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Rumen microbiome: Interacting with host genetics, dietary nutrients metabolism, animal production, and environment

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Rumen microbiome: Interacting with host genetics, dietary nutrients metabolism, animal production, and environment

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Editorial: Rumen microbiome: interacting with host genetics, dietary nutrients metabolism, animal production, and environment

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Editorial on the Research Topic
Rumen microbiome: interacting with host genetics, dietary nutrients metabolism, animal production, and environment

Ruminants play an essential role in global human societies due to their unique ability to convert low-value feeds into high quality protein food (meat and milk) for human consumption using their rumen microbiome. The rumen microbiome is the ruminant's second genome, which facilitates the transformation of feeds into valuable animal products. The rumen microbiome profoundly influences dietary nutrient metabolism, animal production, the quality of animal products, and the environment. Understanding the complex interactions among microbial groups and with the host is essential to optimizing rumen function to meet growing demands for animal products while minimizing the environmental impact of ruminant production.

The aim of this Research Topic is to improve our understanding of the rumen microbiota interactions and fermentation control as the key to developing interventions toward sustainable intensification of ruminant production. The 14 publications gathered in this Research Topic have expanded our knowledge of the role of the rumen microbiome.

Cattle and other ruminants produce large amounts of the potent greenhouse gas methane. Enteric methane is a natural byproduct of microbial fermentation of feed in the rumen or gastrointestinal tract, accounts for 6% of total emissions of CH4 from anthropogenic related sources (Difford et al., 2018). The rumen microbiome, along with the host, influences methane emissions and feed efficiency of dairy cows. The papers by Malik et al. and O'Hara et al. reported the effects of the addition of anti-methanogenic products on the rumen microbiomes of sheep and dairy cows, respectively. Malik et al. reported that the addition of 5% of a tannin-containing supplements to a straw and concentrate mixed diet resulted in significantly reduced enteric methane emissions

from sheep without affecting intake and rumen fermentation. The anti-methanogenic supplementation had no effect on the core microbiome, but significantly reduced rumen protozoa and reduced the activity of key enzymes involved in rumen methanogenesis. O'Hara et al. evaluated the effects of three red seaweeds on rumen prokaryotic communities *in vitro*. The population of rumen methanogens collapsed almost completely after supplementation with *Asparagopsis taxiformis*, and the number of methanogens that recovered during the stabilization phase was small. Similarly, *A. taxiformis* inhibited bacteria involved in acetate and propionate synthesis and fiber degradation, thereby reducing volatile fatty acid synthesis. This product has the potential to decrease the negative environmental impact of livestock production.

The composition of dietary nutrients, dietary components, and feeding systems are important for manipulating rumen microbial composition and fermentation. The study by Rehemujiang et al. found that replacing soybean meal with cottonseed meal or rapeseed meal in the diets of Hu sheep stimulated production of propionate in the rumen, improved the efficiency of energy utilization, and improved production performance of Hu sheep. Zhang et al. explored the effects of two feeding methods, indoor feeding and pasture grazing on the quality of black Tibetan sheep meat through metabolome and microbiome analyses. The results showed that indoor feeding of compound forage diets improved muscle water retention capacity, tenderness, and muscle color of black Tibetan sheep by regulating the metabolism of amino acids, lipids and carbohydrates in muscle tissues. In addition, indoor feeding practices positively affected the fatty acid and amino acid composition of muscle or meat. Hua et al. studied *in vitro* the effects of glucogenic and lipogenic diets on the structure of rumen bacteria and archaea and the *in vitro* rumen metabolome and gas production It was found that the lipogenic diet increased the proportion of cellulolytic bacteria, whereas with the glycogenic diets amylolytic bacteria were relatively more abundant. Bacteria with increased relative abundance in the glycogen diet played a role in the succinate pathway, resulting in higher propionate yield. Jiang et al. showed that supplementing yaks with 0.3% mixed isoacids increased the digestibility of neutral and acid detergent fiber and average daily weight gain, and changed the diversity of rumen bacteria.

New feed additives can promote rumen microbial fermentation and animal productivity. Xu et al. found that adding biochanin A (BCA), an isoflavone phytoestrogen, to the diet of dairy goats improved milk production performance, nitrogen metabolism and feed conversion efficiency. The authors found that BCA supplementation increased antioxidant performance and endocrine hormone levels, and altered composition of rumen microbiota, improved nitrogen metabolism by inhibiting degradation of amino acids, and increased the abundance of cellulolytic bacteria. This finding provides an effective nutritional means to improve the milk production performance of dairy goats. Tondini et al. explored how polyclonal antibodies produced against key rumen cellulolytic bacterial strains inhibited the growth of targeted strains in single culture. Their results show that polyclonal antibodies could be used to inhibit the growth of specific, undesirable rumen bacteria, and improve rumen fermentation patterns.

The host is also a driver for rumen microbiome changes. Wang et al. found that litter size had different effects on rumen microbiota community composition, rumen fermentation, and growth performance of goats. Their results show decreased growth performance and rumen concentration of total VFA, propionate, and butyrate, in triplet goats. Triplet goats have higher levels of *Prevotella* in their rumen and lower abundance in *Rikenellaceae RC9*, which can be related to growth performance of triplet goats.

Rumen development is important for young ruminants. Early consumption of solid feed can promote rumen growth and development in ruminants (Lin et al., 2019) and stimulate the development of rumen microbial communities (Liu et al., 2017). Huang et al. found that increasing the supply of milk replacer from 2 to 4% of lamb body weight increased weight gain, but reduced intake of starter solid feed and slowed down rumen development. High levels of milk replacer feeding increased rumen microbial diversity but reduced the relative abundance of carbohydrate-degrading bacteria. The results of Liu et al. showed that partially replacing alfalfa with oats in lamb diet promoted the production of beneficial fatty acids in *longissimus lumborum* muscle. A review by Du et al. reported the importance of using early intervention methods for the establishment of a healthy calf gastrointestinal microbiota. For example, oral probiotics, and fecal and rumen transplantation of microbiota, can be effective in alleviating calf diarrhea.

Ma et al. analyzed the differences in prokaryotic communities in lamb rumen fed granular TMR and bacterial communities in rumen contents and filtered rumen fluid. The results showed that the microbial DNA yield, bacterial diversity and abundance of fibrolytic bacteria and archaeal *Methanimicrococcus* in lamb rumen fluid were lower than those in mixed rumen content.

Urea is an important non-protein source of nitrogen for ruminants. In the rumen, ureolytic bacteria play a key role in urea nitrogen metabolism, however, the number of ureolytic bacteria isolated from the rumen is scarce. Zhong et al. isolated a novel ureolytic bacterium from cattle rumen and characterized its genome and function. The isolate is a new gram-positive strain of *Enterobacter hormaechei* named *E. hormaechei* Z129 that carries unique genes related to feed conversion, nitrogen dissimilation reduction, and lactose synthesis.

The present Research Topic highlights the interaction of rumen microbes with host and nutrient metabolism and animal production. It also provides innovative ideas for further research in this area informing practical management options to optimize ruminant production. The rumen microbiome not only breaks down ruminant feed, but also controls animal productivity and influences the quality of the final product. Future research should explore the molecular mechanisms by which the rumen microbiome regulates ruminant performance, with the aim of producing high-quality products that improve profitability and reduce environmental impact.

Author contributions

QX: Writing—review and editing. EU: Writing—review and editing, Conceptualization. DM: Writing—review and editing, Conceptualization. SW: Writing—review

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Conflict of interest

SW was employed by Teagasc.

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

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References

Difford, G. F., Plichta, D. R., Lovendahl, P., Lassen, J., Noel, S. J., Hojberg, O., et al. (2018). Host genetics and the rumen microbiome jointly associate with methane emissions in dairy cows. *PLoS Genet.* 14, 7580. doi: 10.1371/journal.pgen.1007580

Lin, L., Xie, F., Sun, D., Liu, J., Zhu, W., and Mao, S. (2019). Ruminal microbiome-host crosstalk stimulates the development of the ruminal epithelium in a lamb model. *Microbiome* 7, 83. doi: 10.1186/s40168-019-0701-y

Liu, J., Bian, G., Sun, D., Zhu, W., and Mao, S. (2017). Starter feeding altered ruminal epithelial bacterial communities and some key immune-related genes' expression before weaning in lambs. *J. Anim. Sci.* 95, 910–921. doi: 10.2527/jas.2016.0985

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Effect of an anti-methanogenic supplement on enteric methane emission, fermentation, and whole rumen metagenome in sheep

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A study was conducted to investigate the impact of an anti-methanogenic product supplementation on enteric methane emissions, whole rumen metagenome and ruminal fermentation in sheep. Twelve adult male sheep were randomly divided into two groups of six animals each. Animals were fed *ad libitum* on a total mixed ration either without (CON) or with an anti-methanogenic supplement (Harit Dhara-HD). The anti-methanogenic supplement contained 22.1% tannic acid in a 3: 1 ratio of condensed and hydrolysable tannins. The supplementation of product revealed a significant reduction in daily enteric methane emission (21.9 vs. 17.2g/d) and methane yield (23.2 vs. 18.2) without affecting the nutrient intake and digestibility. However, the propionate concentration in the HD treatment group was significantly higher than in the CON group. On the contrary, the ammonia nitrogen concentration was lower. The anti-methanogenic supplement significantly decreased the ruminal protozoa in the HD treatment group. Whole rumen metagenome analysis revealed that the core bacterial (*Bacteroidetes* and *Firmicutes*) and archaeal communities (*Methanobrevibacter* and *Methanosarcina*) were comparable between the CON and HD treatment groups. However, the supplementation of anti-methanogenic product led to a considerable reduction in the abundance of *Proteobacteria*, whereas the abundance of *Lentisphaerae* was greater. The supplementation significantly decreased the abundance of *Methanocaldococcus*, *Methanococcoides*, *Methanocella*, and *Methanoregula* methanogens. A total of 36 KO related to methanogenesis were identified in this study. The activities of formate dehydrogenase (EC 1.8.98.6) and tetrahydromethanopterin S-methyltransferase (EC 2.1.1.86) were significantly lowered by the anti-methanogenic product supplementation in sheep. In conclusion, the anti-methanogenic supplement has the potential to decrease enteric methane emission (~22%) at the recommended level (5% of DM) of supplementation. The contribution of minor methanogens vulnerable to supplementation to rumen methanogenesis is not known; hence, the culturing of these archaea should be taken on priority for determining the impact on overall rumen methanogenesis.

KEYWORDS

anti-methanogenic supplement, methane, metagenome, sheep, tannin

Introduction

Globally, livestock are responsible for 15% of anthropogenic greenhouse gas emissions (Gerber et al., 2013). Methane is a significant greenhouse gas (Yusuf et al., 2012), and its atmospheric concentration with an average yearly acceleration of 10 ppb reached 1,890 ppb (Dlugokencky, 2021). Enteric fermentation is one of the primary contributors to atmospheric methane. About 87 to 90 teragrams of methane is generated annually by enteric fermentation (Chang et al., 2019). In addition to contributing to global warming, enteric methane is responsible for a substantial loss of 2%–12% of dietary energy (Johnson and Johnson, 1995). Each liter of enteric methane emission carries 39.5 kJ of the dietary energy away from the animal (Guan et al., 2006). Ionophores have been widely used to improve ruminant productivity while simultaneously reducing enteric methane emissions. However, their use in the diet has been prohibited in many countries (EU directive 1831/2003/EEC). An approach that leads to a significant reduction in enteric methane without affecting ruminal fermentation, the host animal, or residual excretion in products would be one of the most plausible.

Use of plant secondary metabolites containing phyto-sources appears to be one of the promising approaches for enteric methane mitigation. The anti-methanogenic property of tannins has been extensively studied in recent years (Puchala et al., 2005; Grainger et al., 2009; Tan et al., 2011; Jayanegara et al., 2015). Tannins, which are polyphenolic metabolites, can modulate rumen fermentation and mitigate enteric methane emissions (Cieslak et al., 2012; Hassanat and Benchaar, 2013). Based on previous research by our group (Bhatta et al., 2001; Malik et al., 2017a,b; Baruah et al., 2019; Poornachandra et al., 2019; Thirumalaisamy et al., 2022a,b), we have formulated a patent-applied (Indian patent application 201,941,004,992) tanniferous anti-methanogenic supplement "Harit Dhara." The product was formulated primarily using phyto-sources, furnishing the condensed and hydrolysable tannins in a 3:1 ratio in the final product. We hypothesized that the supplementation of anti-methanogenic product in the straw and concentrate-based diet when included at the appropriate level would mitigate the enteric methane emissions and rumen microbial composition without any detrimental implications on the rumen fermentation. This study aimed to (1) investigate the impact of variable levels of supplementation of anti-methanogenic product on *in vitro* methane production and optimize the most promising inclusion level for animal studies; and (2) to ascertain the impact of anti-methanogenic product supplementation on enteric methane emissions, whole rumen metagenome and ruminal fermentation.

Materials and methods

Tannin assays

For the tannin assays, samples of anti-methanogenic supplement in triplicate were ground to a fine powder in a Cyclotec mill (Foss, Denmark). About 20 mg of sample was extracted in 10 ml of aqueous acetone (70%), vortexed for 5 min, and the supernatant (10 µl) was taken for the immediate tannin assay. The tannin assay and the phenol and tannin content in the extract were determined by the Folin–Ciocalteu method (Makkar, 2003) using polyvinylpolypyrrolidone (PVPP). Condensed tannin in the samples was determined following the butanol–HCl–iron method (Makkar, 2003). Total phenols and tannin were expressed as tannic acid equivalents, whereas condensed tannin was leucocyanidin equivalent. The concentration of hydrolysable tannin in the samples was determined by the difference between total and condensed tannin.

Catechin derivatives

About 0.5 g of sample was weighed and placed in a 50 ml centrifuge tube. After that, 25 ml of diluent (70% methanol) was added, vortexed for 10 min, and kept in a water bath at 70°C for 30 min. Subsequently, the solution was brought to room temperature and made up to a final volume of 50 ml with the diluent. The solution was vortexed and filtered through a 0.45 µm syringe filter into the autosampler vial for HPLC analysis. The individual constituents of the tannin were estimated on the HPLC (1,260 Infinity II, Agilent) using a Zorbax Eclipse C18 column (4.6 × 250 mm × 5 µm), UV detection at 270 nm, and gradient elution. Acetonitrile was used in the mobile phase. The standard (1,000 ppm) of individual tannin constituents (Sigma Aldrich, >99% purity) as an internal reference was injected into the HPLC-DAD.

Experiment I-*in vitro*

An *in vitro* study was initially conducted to investigate the impact of the anti-methanogenic supplement named "*Harit Dhara*" (HD) on methane production and to optimize the level of supplementation in the diet. A basal diet consisting of finger millet (*Eleusine coracana*) straw and concentrate mixture in a 1:1 ratio was formulated for the *in vitro* evaluation. The concentrate mixture was formulated with maize grain (320 g/kg), soybean meal (130 g/kg), groundnut cake (120 g/kg), wheat bran (400 g/kg), mineral mixture (20 g/kg), and salt (10 g/kg). The anti-methanogenic supplement was added at 2 (HD_2), 5 (HD_5), and 8% (HD_8) levels of the basal diet, whereas in control (CON), there was no supplementation of the HD.

Chemical composition

The dry matter content of the samples was determined in accordance with AOAC (2012) at 100°C for 12 h, and the dried samples were ground using a Cyclotec mill. For determining total ash, the samples were initially burned in crucibles on a hot plate before being transferred to a muffle furnace at 550°C for 4 h (AOAC, 2012). Organic matter (OM) was calculated by subtracting the total ash from the sample's initial dry weight and

expressing the result as a percentage. Crude protein (CP, N × 6.25) was determined using an automatic nitrogen analyzer (Gerhardt, Germany) in accordance with AOAC (2005). The samples' crude fiber (*CF*) was analyzed with an automatic fiber analyzer (Fibretherm FT12, Gerhardt, Germany) in accordance with AOAC (2005), whereas the fiber fractions were determined according to Van Soest et al. (1991). As per AOAC (2005), the ether extract (EE) was determined using Soxtherm (Gerhardt, Germany).

Total gas and methane

Two adult male fistulated Holstein-Friesian cattle (BW ± SE, 500 ± 15 kg) were used as donors for the ruminal fluid, which was then used as a source of microbial inoculum. The cattle were fed on finger millet straw and concentrate-based diet (1:1). About 200 mg sample was quantitatively transferred to a 100-ml glass syringe (Haeberle, Germany). The buffer medium consisted of macro and micro mineral solutions, buffer, and resazurin was prepared on the previous day of incubation and preserved in a water bath at 39°C with intermittent flushing of carbon dioxide and stirring. Both the solid and liquid fractions (1:2) were ensured while collecting the rumen fluid on the day of incubation The solid and liquid fractions were brought to the laboratory in a thermos flask at 39°C within the 10 min of collection. The rumen content was squeezed through double layers of muslin cloth into a prewarmed thermos flask while slowly flushing the carbon dioxide. About 30 ml of premixed buffer medium consisting of rumen fluid and medium in a 1:2 ratio was dispensed using an automatic dispenser (Eppendorf Varispenser, 50 ml) into the glass syringe through the silicon tube fitted to the nozzle. For each sample, three replicates were taken and the incubation was repeated three times. Thus, a total of nine observations for each sample/treatment were recorded. The incubation was performed in a Hohenheim type water bath at 39°C for 24 h with periodical automatic shaking. In each incubation, three glass syringes containing buffer medium and rumen fluid without feed sample and three Hohenheim hay standard were placed as blanks and positive controls, respectively. After 24 h, the incubation was terminated and the final piston position was recorded. The syringes were placed on the ice in a big plastic tumbler and the gas was transferred to 10 ml pre-evacuated glass vials with the help of airtight gas syringes. The volume of gas produced was calculated by considering the initial and final piston position as well as the blanks. The gas samples collected in glass vials were presented to the gas chromatograph (Agilent 7890B) using airtight Haeberle glass syringes. The analysis of methane using gas chromatograph was performed by uphold the conditions described previously by Malik et al. (2017a, 2019). The dry matter digestibility (DMD), as described by Menke et al. (1979), was used to determine the effect of graded supplementation of HD on the diet fermentation.

Statistical analysis

Data from the *in vitro* study was examined for gaussian normal distribution using the D'Agostino-Pearson normality test at the 0.05 alpha level in GraphPad Prism version 9 (GraphPad Software, San Diego, United States). Total gas, methane, and dry matter digestibility data were analyzed in SPSS version 21.0 (IBM® SPSS, USA) using one-way analysis of variance (ANOVA) with the model.

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

Yij represents an individual observation, represents the mean of the population, Ai represents the effect of the treatment, and ij indicates experimental error. Using Tukey's *post-hoc* procedure, the difference between the means was examined and deemed significant at $p \leq 0.05$.

The linear effect of the variable doses of anti-methanogenic supplement Harit Dhara on *in vitro* total gas, methane, and dry matter digestibility was determined using the orthogonal polynomial contrast coefficient in SPSS version 21.0.

Experiment II-animal study

Animal feeding and management

After obtaining prior approval (NIANP/IAEC/1/2019) from the Institute Animal Ethics Committee (IAEC), a study in sheep was conducted in strict accordance with the IAEC's procedures for animal care and sample collection. Twelve adult male *Mandya* sheep (BW ± SD, 30.7 ± 2.05) were randomly divided into two groups of six animals each. Animals were fed *ad libitum* a total mixed ration (TMR) of finger millet straw and concentrate mixture in equal proportions, either without (CON) or with the anti-methanogenic supplement Harit Dhara (HD treatment). Based on the *in vitro* studies, the level of HD supplementation (5% of DMI) was fixed for the sheep feeding. The sheep were housed in a well-ventilated, concrete shed with individual feeding and watering facilities. Before the commencement of the experiment, the animals were dewormed with an anthelminthic (Fenbendazole @5 mg/kg BW). Animals had access to clean drinking water throughout the day.

Daily methane emission and nutrient digestibility

Following a preliminary feeding of sheep on CON and HD treatment diets for 30 days, the daily enteric methane measurement was performed using sulfur hexafluoride (SF_6) tracer technique (Berndt et al., 2014). The brass permeation tubes, which served as the source of SF_6 gas in the rumen during methane measurement, were charged with 780 ± 52.2 mg pure gas. The charged tubes were calibrated for 70 days at 39°C and the SF_6 release rate was recorded. Ten days prior to the methane measurement, the calibrated tubes were inserted into the sheep rumen. Throughout the duration of the methane measurement (7 days), a PVC canister to collect background gas samples was hung daily on the iron wire mesh fixed to the cement wall on the east side of the shed. The male and

female connectors (Swagelok), capillary tube (Supelco, ID 1/16), teflon tube, and air filter were assembled into the halter and connected to the PVC canister as described by Williams et al. (2014). Individual vacuumed PVC canisters (>90–95 kPa) were tied to each sheep for 24 h to collect breath samples. The timings for the tying and removal of canisters were maintained throughout the duration. After 24 h, the canister was removed from the sheep and the final pressure was measured with a digital pressure meter (Leo 2, Keller). The breath and background samples in the canisters were diluted by two to three times with high-purity N_2 gas, and the dilution pressure was measured to estimate the dilution factor. Using an airtight glass syringe (Hamilton, 1 ml), successive subsamples were collected from canisters containing diluted breath samples. The samples were then analyzed using a gas chromatograph. For the analysis of methane and SF_6, a gas chromatograph (GC 2010 plus, Shimadzu, Japan) equipped with flame ionization and electron capture detectors was used under the same circumstances as previous studies (Malik et al., 2021; Thirumalaisamy et al., 2022b).

Methane (ppm) and SF_6 (ppt) concentrations were calculated using the canister pressures at the beginning, end, and after N_2 dilution (Lassey et al., 2014). The local elevation and atmospheric pressure at the study site (Thirumalaisamy et al., 2022b) were also taken into consideration while calculating the gas concentration.

$$[G_S] = \frac{90 - \tau_f}{\tau_e - \tau_s} \times [G_A]$$

G_S was the concentration of methane (ppm) or SF_6 (ppt), τ_f (kPa) was the final pressure after N_2 dilution, τ_s (kPa) was the post sampling pressure, τ_e was the initial canister pressure, and G_A was the concentration of methane (ppm) or SF_6 (ppt) in the diluted samples.

Daily enteric methane emissions were calculated as per (Moate et al., 2014).

$$R_{CH4} = R_{SF6} \frac{[CH_4]_M - [CH_4]_{BG}}{[SF_6]_M - [SF_6]_{BG}} \times \frac{MW_{CH_4}}{MW_{SF_6}} \times 1000$$

R_{CH4} was CH_4 emission (g/d), R_{SF6} was the SF_6 release rate from permeation tubes (mg/d), $[CH_4]_M$-$[CH_4]_{BG}$ was the methane concentration in the samples and background, $[SF_6]_M$-$[SF_6]_{BG}$ was the SF_6 concentration in the samples and background, and MW_{CH4} and MW_{SF6} represented the molecular mass of the respective gases.

After 30 days of feeding, a seven-day digestibility trial was conducted concurrently with daily methane measurements. The daily total mixed ration (TMR) offered, feed refusals, and fecal output of each individual sheep in the CON and HD treatment groups were recorded. Representative samples of TMR and feed refusals were obtained and dried at 80°C for 24 h. The DMI (g/d) was determined by subtracting the daily feed refusals from the feed offered. Before drawing a representative sample, the total amount of feces voided by each sheep over a period of 24 h was transferred to a large plastic container and thoroughly mixed. One aliquot (1/100th) was taken from the mixed feces for dry matter estimation in a hot air oven at 100°C, while another aliquot (1/1000th) was preserved in 25% sulfuric acid. The chemical constituents in the dried feed, refusals, and feces samples were analyzed using the procedure outlined in the section on chemical analysis. The digestibility coefficient of nutrients was calculated using the following equation.

$$\text{Digestibility coefficient} = \frac{Nutrient\ intake - Excretion\ of\ nutrient}{Intake\ of\ nutrient}$$

Ruminal fluid collection

Rumen fluid samples were collected at the completion of the methane measurement study (38th day of feeding). The samples were collected 3 h post-feeding from the individual sheep with the help of 1 m perforated nylon stomach tube connected to an airtight collection vessel and handheld vacuum pump (Malik et al., 2015; Thirumalaisamy et al., 2022a,b). To avoid saliva contamination, the first 30 ml of ruminal fluid was discarded, and then 45 ml of rumen fluid was collected and strained through a single layer of muslin cloth for the separation of liquid and solid digesta fractions. The strained rumen fluid was separated into three 15 ml subsets for the isolation of gDNA, ammonia-VFA analysis, and protozoa enumeration. Immediately prior to being transported to the lab, tubes holding the ruminal fluid for gDNA and ammonia-VFA were placed on ice, while ruminal fluid subsets for the protozoal count were transported to the laboratory without being placed on ice. The solid digesta fraction retained in the muslin cloth was squeezed and washed with the buffer consisting of 50 mM tris–HCl, 200 mM NaCl, 5 mM sodium EDTA, and 0.05% triton and re-suspended in the liquid fraction preserved for the genomic DNA isolation.

Rumen fermentation

The samples of ruminal fluid were centrifuged at 13,400 rpm for 15 min, and the supernatant was separated into two equal portions. In one portion, 25% metaphosphoric acid was added in a 4:1 (v/v) ratio and stored at-80°C until the VFA analysis. The samples were thawed at room temperature and the VFA concentration was estimated in accordance with Filípek and Dvořák (2009) using a gas chromatograph (Agilent 7890B, Santa Clara, United States). During the analysis, the following GC conditions were upheld (Malik et al., 2021; Thirumalaisamy et al., 2022b): temperature program: 59°C–250°C (20°C/min, 10 min), injector temperature: 230°C, and detector temperature: 280°C.

Individual volatile fatty acids concentration was determined using the following equation:

$$VFA\ con.(mmol) = \frac{Peak\ area\ of\ sample\ x}{\frac{Conc.\ of\ standard\ x\ dilution}{Peak\ area\ of\ standard}}$$

The concentration of ammonia in the second subset of ruminal fluid samples was determined as per Conway (1957). In brief, 1 ml of a boric acid indicator was pipetted into the inner chamber of the disk, while an equivalent volume of saturated sodium carbonate was pipetted into the outer chamber. Approximately 1 ml of ruminal fluid was pipetted directly just outside of the sodium carbonate in the outer chamber. The disk was covered with the lid and left undisturbed at room temperature for 2 h before being titrated with 0.01 N sulfuric acid. Ammonia-N concentration was determined with the following formula:

$$\text{Ammonia} - \text{N}\ (\text{mg}/\text{dl}) = \text{ml of}\ 0.001\text{N}\,H_2SO_4\,\text{x}14$$

Protozoal enumeration

The protozoa in the rumen fluid (third sub-set) transported without putting on the ice were enumerated on the same day following the methodology described by Kamra and Agarwal (2003). Ruminal protozoa enumeration and morphological identification were carried out under a phase-contrast microscope (Nikon Eclipse, Japan) at a 10x objective. Based on the morphology and cilia, the protozoa were categorized into *Entodiniomorphs* and *Holotrichs* in accordance with Hungate (1966). The numbers of protozoa in the rumen fluid from the individual sheep in CON and HD treatment groups were calculated using the following equation:

$$\text{N} = \frac{\text{n} \times \text{A} \times \text{D}}{\text{a} \times \text{v}}$$

Where, *N* was number of protozoa (cells) per ml of rumen fluid, n was the average cell count per microscopic field, A was the area of the slide on which the diluted rumen fluid sample was spread, D was the dilution, a was the area of the microscopic field, and v was the volume of rumen fluid in the cavity. The protozoal numbers were expressed as $\times 10^5$ or $\times 10^4$ cells/ml.

Blood biochemical profile

At the end of the methane measurement trial (38th day), the blood samples (5 ml) were collected from the jugular vein in heparinized tubes. Total protein, albumin, globulin, minerals (Ca, P, Mg) and enzymes (alkaline phosphatase, alanine aminotransferase, glutamyl transferase, and creatine kinase) were analyzed in an automatic blood analyzer (VetScan 2, Abaxis, Germany) following the manufacturer's instructions.

Statistical analysis

Data from the animal traits was examined for gaussian normal distribution using the D'Agostino-Pearson normality test at the 0.05 alpha level in GraphPad Prism version 9 (GraphPad Software, San Diego, CA, United States). Data on the intake, nutrient digestibility, daily enteric methane emissions, fermentation, and protozoal counts between the CON and HD treatment groups were analyzed using the independent sample t test in SPSS version 21.0 (IBM® SPSS, United States). The Leven's test was used to determine the significance of the comparison of the group means at a confidence level of 95%. The principal component analysis (PCA) biplot was constructed by considering methane as a dependent variable and regressing the VFA, protozoa and organic matter intake data for the weightage of the individual parameter on the dependent variable.

DNA isolation

In order to allow feed particles to settle, the ruminal fluid samples were initially spun at 1,000 rpm for 5 min. The supernatant was transferred to a new tube and kept at-80°C until the gDNA was isolated. The samples were initially centrifuged at 13,400 rpm for 10 min at 4°C, and the dense pellet was retained while the supernatant was discarded. The pellet was dissolved using 1 ml of lysis buffer by gentle pipetting. The contents were transferred to a 2 ml sterile screw cap tube with an O-ring (BioSpec, United States) containing pre-sterilized zirconia beads weighing 0.5 g (0.1 mm; BioSpec, United States). The RBB + C technique (Yu and Morrison, 2004) was used to isolate gDNA from samples of ruminal fluid. The quality of the gDNA was determined using 0.8% agarose gel electrophoresis, and the concentration was determined using a Qubit 4.0 (ThermoFisher Scientific, Waltham, United States).

Total methanogens quantification

The relative abundance of total methanogen in the CON and HD treatment group was determined by qPCR (Thermo Fisher Scientific, United States) using SsoFast™ Eva Green® Supermix (Bio-Rad Laboratories Inc., CA, United States). The mcrA forward (TTC GGTGGATCDCARAGRGC3) and reverse (GBARGTC GWAWCCGTAGAATCC) described (Denman et al., 2007) were used for the amplification of total methanogens. In brief, 1 ng of total genomic DNA, 0.25 mM of each forward and reverse primer, and 1X Eva Green Supermix were mixed in a reaction volume of 10 ml. The qPCR conditions were upheld as described previously (Thirumalaisamy et al., 2022a). The relative changes in the abundance of total methanogens were determined by the $2^{-\Delta\Delta CT}$ method. Total methanogens quantification between CON and HD treatment group were compared using *t*-test.

WGS library preparation and paired end sequencing

All gDNA samples were sent to an external sequencing facility (Clevergene Biocorp, Bangalore, India) for Illumina NextSeq500 metagenome sequencing. To generate an average fragment of 350 bp, DNA shearing was performed using a Covaris M220 (Covaris®) with the following settings: duty factor 20%, peak/display power of 50, cycle/burst 200, temperature 20°C, and time 65 s. End Repair

Mix was used to perform end repair on the produced fragments. According to the manufacturer's instructions, the ligated products were size-selected using AMPure XP beads and amplified using the index primers (Illumina True Seq Nano DNA Library Prep Kit). Following the ligation of indexing adaptors to the ends of DNA fragments, PCR-enriched libraries were analyzed on a 4,200 Tape Station System (Agilent Technologies). The libraries were loaded onto NextSeq500 for cluster generation and sequencing, and 150 bp paired-end reads (2 × 150 bp) were obtained.

Structural and functional annotation of rumen metagenome

The MG-RAST Metagenomics Analysis server was used to annotate individual datasets under the project id PRJNA885963. The conventional MG-RAST pipeline was used to perform the quality control and merge of the paired-end reads. The low-quality sequence reads were iterated based on a Phred quality score of 15. The artificially replicated reads were eliminated and the sequences were checked for host contamination using the *B. taurus* UMD v3.0 database. BLAT (Kent, 2002) and the M5nr database (Wilke et al., 2012) were used to taxonomically classify the reads. A minimum *E-value* cut-off of 1×10^{-5} was used, while the minimum percentage identity cut-off was set at 60%. The reads were labeled as "unclassified" if they did not meet the aforementioned criterion at the selected taxonomic level. Sequences that could not be attributed to a taxonomic unit were labeled "No Hits." The FragGeneScan method (Rho et al., 2010) was used to name genes from the metagenomic profile generated by MG-RAST (Wilke et al., 2013), and UCLUST was used to cluster the predicted protein sequences (Edgar, 2010). The longest sequence from each cluster was chosen as the representative sequence for matching with the M5nr reference database (Wilke et al., 2012). The analysis page of MG-RAST was used to export the annotated results and taxonomic affiliations of bacteria and archaea obtained from the RefSeq database. Thereafter, the abundance tables of taxonomic assignments for the bacteria and archaea were uploaded separately into MicrobiomeAnalyst (Chong et al., 2020) for statistical computation and visualization of the metagenome data.

Differential abundance estimation and statistical analysis

The initial filtering of the metagenome data in MicrobiomeAnalyst was performed using the following cut-off values: minimum abundance counts of 4, prevalence in samples of 20%, and a low variance filter of 10%. The data was normalized using rarefication to the minimum library size, and the abundance and evenness of the microbiome composition were evaluated using Shannon's diversity index. Principal coordinate analysis (PCoA) was used to depict the beta diversity based on Bray–Curtis dissimilarity matrices, and Permutational Analysis of Variance (PERMANOVA) was used to calculate the statistical difference. In addition, based on the Bray–Curtis dissimilarity matrices, and Permutational Analysis of Variance (PERMANOVA) in the MicrobiomeAnalyst, the coefficient of determination (R^2) was determine for estimating the proportion of variance. The taxonomic profiles of bacteria and archaea between CON and HD treatment groups at the phylum and genus level were compared using t test. The differential abundance of taxonomical assignment at the genus level was estimated using DeSeq2 (Love et al., 2014) on the raw counts. The fold changes and the corresponding *p* values of the bacterial and archaeal differential abundance were depicted in the volcano plot.

De-novo assembly and annotation

The whole metagenome data set was assembled using Megahit (version 1.2.9) on the KBase portal (Arkin et al., 2018). The contigs were used for predicting prokaryotic genes and translated into amino acids using Find prokaryotic genes (version 2.2) and translated to protein module (version 21.0.2) with translation table 11 containing bacterial, archaeal, and plant plastids, respectively, in the CLC Genomics workbench (version 21.0). The amino acid sequences in the FASTA file were matched to C_Genus_Prokaryotes in Ghost Koala (Kanehisa et al., 2016) and summarized in R (version 4.0.2). The KOs identified in the CON and HD treatment groups were mapped using the KEGG reconstruct pathway tool and visualized the methane metabolism pathway. Further, the KO table was filtered based on the total minimum count of 10 in at least one of the groups, and the differential abundance of the KOs was estimated using DESeq2. The KOs related to the methanogenesis pathways were filtered from the DESeq output.

Results

Tannin-catechin derivatives

Tannin analysis revealed that the formulated product contains 22.1% tannic acid (Cyanidin equivalent). The anti-methanogenic supplement primarily contains condensed tannins (75%), while hydrolysable tannin accounts for 25% of the total tannic acid in the supplement. The condensed to hydrolyzable tannins ratio was 3.12:1 (Table 1). HPLC analysis of catechin derivatives revealed that epigallocatechin (8.23%) was most abundant, followed by epicatechin (0.61%) and gallic acid (0.55%). Other derivatives, such as ellagic acid, catechin and epicatechin, and gallate, accounted for less than 0.2% of the derivatives (Table 1).

In vitro total gas and methane production

The chemical composition of the basal diet and anti-methanogenic supplement is presented in Supplementary Table 1.

The results of the *in vitro* experiments (Table 2) revealed a significant decrease in total gas (TG) production at the highest level (HD_8) of anti-methanogenic agent supplementation. In a similar manner, the dry matter digestibility (DMD) decreased ($p < 0.0001$) when an anti-methanogenic agent was added at a level of 8%. Data on methane production revealed a significant decrease ($p < 0.001$) across all three levels (HD_2, HD_5, and HD_8) of anti-methanogenic supplementation compared to the control ($p < 0.001$). The difference in methane production between the HD treatments was also significant ($p = 0.008$). The addition of anti-methanogenic product at varying concentrations reduced ($p < 0.0001$) the proportion of methane in total gas (Table 2). Overall, there was a tendency of linear reduction in the total gas, methane, DMD and methane as percent of total gas due to the variable doses of the anti-methanogenic supplement.

Methane emission and nutrient digestibility

The chemical composition of the basal diet is presented in Supplementary Table 2. Daily enteric methane measurement (g/d) in sheep revealed a significant reduction ($p = 0.0002$) in the anti-methanogenic supplemental group (17.2 g/d) compared to the CON group (21.9 g/d). Similarly, the anti-methanogenic agent in the HD treatment group also decreased ($p = 0.0008$) methane yield (g/kg DMI) as compared to the CON group. The nutrient intake and digestibility data (Table 3) did not reveal any negative effects of the anti-methanogenic supplement on the intake or nutrient digestibility in sheep.

TABLE 1 Tannin assay and catechin derivatives in anti-methanogenic supplement.

Tannin assay (%)					
Phenol	**Non-tannin phenol**	**Tannic acid**	**CT**	**HT**	**CT: HT**
23.06	0.96	22.09	16.73	5.36	3.12:1
Catechin derivatives (%)					
Catechin	Epicatechin	Gallic acid	Ellagic acid	Epigallocatechin	Epicatechin gallate
0.13	0.61	0.55	0.17	8.23	<0.01

Rumen fermentation and protozoa

Similar to nutrient intake and digestibility, total VFA production (mM) did not differ between the CON and HD treatment groups ($p = 0.062$). In addition, the production of specific VFAs also did not differ between the CON and HD treatment groups. However, the propionate production (mM) in the HD treatment group was higher ($p = 0.007$) than in the CON group. The amount of ammonia nitrogen (mg/dL) was higher in the HD treatment group ($p = 0.026$) than in the control group (CON). Due to the addition of an anti-methanogenic agent, the number of protozoa were significantly reduced in the HD treatment group (Table 4). The inclusion of the anti-methanogenic supplement (HD treatment group) led to a significant reduction in the total number of *Entodinimorphs* and *Holotrichs* protozoa.

The PCA biplot (Figure 1) plotted considering methane yield as the main attribute revealed that the PC1 had relatively more influence (46.51%) than the PC2 (19.92%). *Entodinimorphs* and

TABLE 3 Effect of *Harit Dhara* supplementation on nutrient intake, digestibility, and methane emissions in sheep.

Attribute	**CON**	**HD**	**SEM**	***P***
Body weight (kg)	30.8	30.7	0.593	0.894
Intake (g/d)				
Dry matter	943	941	1.82	0.551
Organic matter	868	877	5.43	0.452
Crude protein	116	117	0.69	0.501
Neutral detergent fiber	385	389	2.40	0.471
Acid detergent fiber	294	297	1.80	0.458
Dig. nutrient intake				
Dry matter	595	611	13.9	0.570
Organic matter	560	575	12.9	0.596
Crude protein	77.8	79.6	1.59	0.591
Neutral detergent fiber	155	166	8.84	0.571
Acid detergent fiber	111	119	7.03	0.586
Methane emissions				
Daily methane (g/d)	21.9	17.2	1.17	0.0002
Methane yield (g/kg DMI)	23.2	18.2	0.489	0.0008

CON, control group (no *Harit Dhara* supplementation); HD, treatment group (*Harit Dhara* supplementation @ 5% basal diet); DMI, dry matter intake; DDMI, digestible dry matter intake; DCP, digestible crude protein intake; DADF, digestible acid detergent fiber; DNDF, digestible neutral detergent fiber; CH_4, methane; SEM, standard error of mean. Mean values bearing different superscripts in a row differ significantly at 5% level.

TABLE 2 Effect of anti-methanogenic supplement on *in vitro* gas production and DMD.

Attributes	**CON**	**HD_2**	**HD_5**	**HD_8**	**SEM**	***P***	**Linear, *P***
Total gas (ml/g)	236^b	234^b	229^b	215^a	2.06	<0.0001	<0.0001
Methane (ml/g)	42.0^c	35.2^b	33.8^b	29.1^a	1.15	<0.0001	<0.0001
Methane (% of TG)	18.3^c	16.2^b	15.7^{ab}	14.7^a	0.321	<0.0001	<0.0001
DMD (%)	63.8^b	62.9^b	62.3^b	60.7^a	0.274	<0.0001	<0.0001
Methane reduction* (ml/g)	-	15.9^a	19.3^a	30.6^b	2.26	0.008	<0.0001

CON, basal diet + 0 *Harit Dhara*; HD_2, basal diet + 2% *Harit Dhara*; HD_5, basal diet + 5% *Harit Dhara*; HD_8, basal diet + 8% *Harit Dhara*. DMD, dry matter digestibility; dDM, digestible dry matter; TG, total gas. Mean values bearing different superscripts in a row differ significantly at 5% level. *Reduction is in percent.

TABLE 4 Effect of *anti-methanogenic* supplement on rumen fermentation and protozoa in sheep.

Attributes	CON	HD	SEM	*P*
TVFA (mmol)	67.9	75.9	2.18	0.062
Ammonia N (mg/dl)	10.8	9.57	0.304	0.026
VFA (mmol)				
Acetate	47.8	51.2	1.52	0.285
Propionate	10.9	13.0	0.432	0.007
Butyrate	7.07	9.07	0.526	0.051
Isobutyrate	0.69	0.80	0.046	0.227
Valerate	0.51	0.61	0.033	0.124
Isovalerate	0.88	1.24	0.102	0.068
A/P	4.19	4.24	0.115	0.835
Protozoa				
Total ($\times 10^5$ cells/ml)	5.71	0.79	0.811	<0.0001
Entodiniomorphs ($\times 10^5$ cells/ml)	5.52	0.79	0.788	<0.0001
Holotrichs ($\times 10^4$ cells/ml)	1.98	nd	0.389	0.004

CON, control group (no *Harit Dhara* supplementation); HD, treatment group (*Harit Dhara* supplementation @ 5% basal diet); SEM, standard error of mean. Mean values bearing different superscripts in a row differ significantly at 5% level.

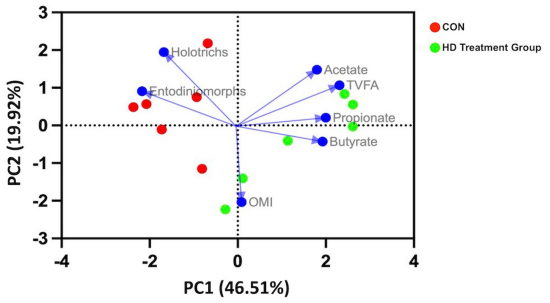

FIGURE 1
PCA biplot of CON and HD treatment group samples. The samples (points) were distinguished by VFA, protozoa, and organic matter intake (arrows labeled). The first principal component is dominated by VFA, whereas the second principal component is dominated by organic matter intake.

Holotrichs' protozoa had a significant impact on methane production. The close angle of *Entodinimorphs* on PC loadings revealed that they were closely correlated with methane production as compared to the *Holotrichs*. The propionic acid had more influence on the PC1, whereas acetate had an impact on PC2 and was found to be positively correlated with the methane yield.

Blood biochemical profile

Blood analysis revealed comparable enzymatic activity, mineral content, and protein (albumin, globulin, total protein, and blood urea nitrogen) between the CON and HD treatment groups (Supplementary Table 3). Results indicated that the anti-methanogenic supplement did not alter the blood biochemical profile.

Total methanogens

Results from the study revealed a significant ($p = 0.016$) reduction (−1.46 log fold change) in the total rumen methanogens quantified by qPCR in the HD treatment group as compared to CON (Supplementary Figure 1).

Rumen whole metagenome

A total of 182,783,020 reads and 18,278,302 ± 583,571 sequences per sample were generated by metagenomic shotgun sequencing of ruminal fluid samples from sheep in CON and HD treatment groups. Each sample yielded an average of 5.85 GB of paired-end read data (range 31.9–44.8 million). A total of 84.69% of the submitted sequences passed the MG-RAST pipeline's quality control. For taxonomic analysis, each sample's reads mapped to the RefSeq database were rarefied to 15,452,419 for bacteria and 178,972 for archaea.

The shotgun dataset contains 28 bacterial phyla, 110 orders, and 571 genera. *Bacteroidetes* (57.9%), *Firmicutes* (26.1%), *Proteobacteria* (6.21%), *Actinobacteria* (2.31%) and *Fibrobacteres* (2.23%) were the most abundant bacterial phyla, aggregately constituting ~95% of the ruminal bacteria in sheep. The Bacterial phyla dataset revealed similar *Bacteroidetes* ($p = 0.726$), *Firmicutes* ($p = 0.170$), *Fibrobacteres* ($p = 0.314$) and *Actinobacteria* ($p = 0.181$) abundance between CON and HD treatment groups (Supplementary File 1). However, the supplementation of anti-methanogenic agent in the HD treatment group led to a reduction ($p = 0.013$) in the abundance of *Proteobacteria*. Similarly, the abundance of *Tenericutes* ($p = 0.010$) and *Elucimicrobia* ($p = 0.019$) was also adversely affected by the supplement. On the contrary, the abundance of *Lentisphaerae* in the HD treatment (0.67%) was greater ($p = 0.032$) than the CON group (0.47%, Figure 2A). The average *Firmicutes* to *Bacteroidetes* ratio was comparable between the groups. At the order level, *Bacteroidales* and *Clostridiales*, with a corresponding average abundance of 53.5 and 18.8%, were two prominent orders of the bacteria. The abundances of *Bacteroidales* ($p = 0.689$) and *Clostridiales* ($p = 0.086$) between the CON and HD treatment groups were not significantly different (Figure 2B). However, the minor bacterial orders such as *Cytophagales* ($p = 0.027$), *Sphingobacteriales* ($p = 0.032$), *Alteromonadales* ($p = 0.030$) and *Enterobacteriales* ($p = 0.037$) were differentially distributed between the CON and HD treatment groups. In the present study, *Prevotella* (35.1%), others (26.2%), and *Bacteroides* (14.9%) were three prominent bacterial genera (Figure 2C).

On the other hand, 4 phyla, 17 orders, and 54 genera of archaea were identified in the present study in sheep. The *Euryarchaeota* was the most abundant phylum, accounting for 98.4% of the total archaea, while the *Crenarchaeaota* accounted for 1.30% of the total archaea (Figure 3A). The archaea assigned to the *Korarchaeaota* and *Thaumarchaeota* were also identified; however, at an extremely low frequency (0.2%). *Methanobacteriales* (73.9%) and *Methanosarcinales* (7.10%) were the two most abundant archaeal orders, but their

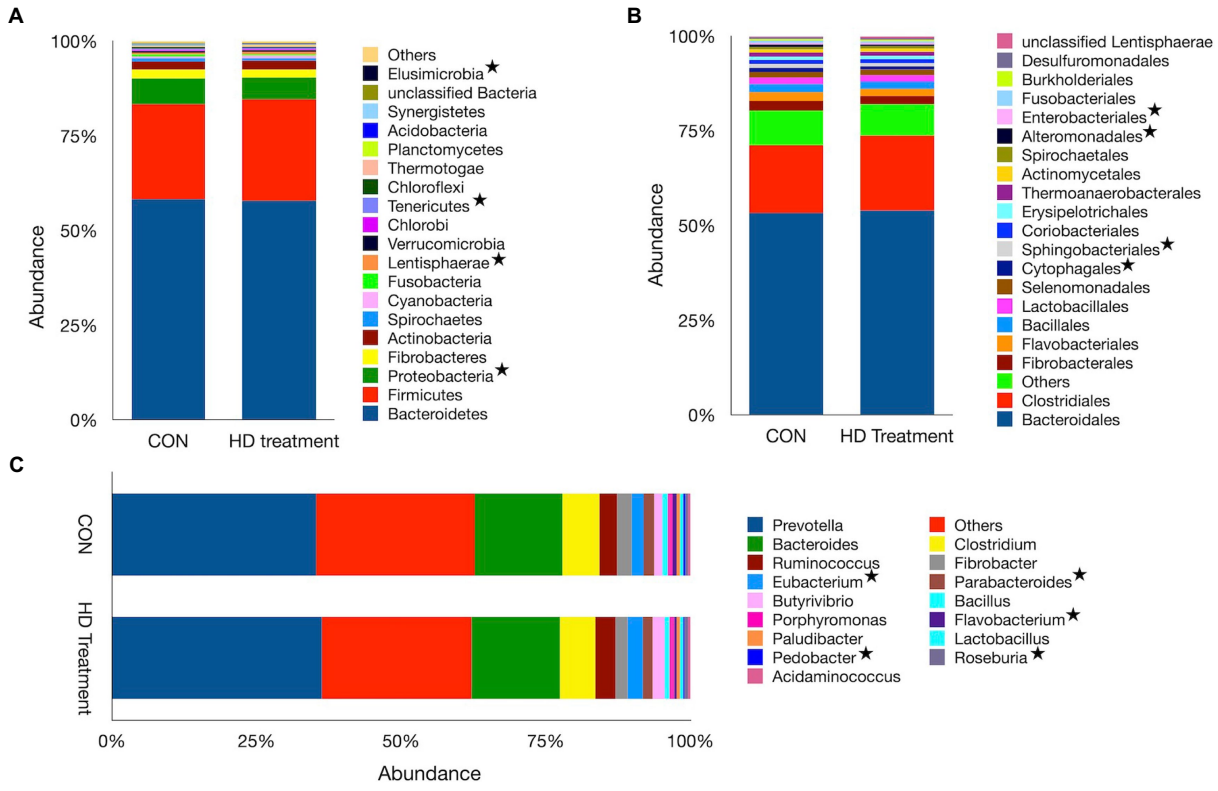

FIGURE 2
Bacterial profiles at different phylogenetic levels observed in the CON and HD treatment groups. The relative abundances of bacterial taxa at the phylum **(A)**, order **(B)**, and genus **(C)** levels are represented by bar plots. The category "Others" represents taxa with a relative abundance of <1%. Asterisks denote taxa that significantly differ in abundance between the CON and the HD treatment groups.

distribution did not vary between the groups (Figure 3B). The *Methanococcales* and *Methanomicrobiales* were another two important archaeal orders, each of which was distributed with an individual frequency of ~5% (Supplementary File 1). The abundance of minor archaeal orders such as *Desulfurococcales* ($p = 0.029$), *Sulfolobales* ($p = 0.043$), and *Methanocellales* ($p = 0.043$) differed significantly between the CON and HD treatment groups.

The *Methanobrevibacter* genus, with a similar distribution ($p = 0.07$) between the two groups, constituted the largest fraction (avg. 61.5%) of the total archaea in sheep (Figure 3C). Similarly, the abundance of *Methanosarcina*, with a mean distribution of 5.19%, was not significantly different between groups ($p = 0.065$). The anti-methanogenic supplementation in the HD treatment group significantly decreased the abundances of *Methanocaldococcus* ($p = 0.011$), *Methanococcoides* ($p = 0.042$), *Methanocella* ($p = 0.043$), and *Methanoregula* ($p = 0.040$). In contrast, the abundance of the second largest genus, *Methanothermobacter,* was significantly greater in the HD treatment group ($p = 0.029$).

Volcano plot revealed that the abundances of six (*Candidatus Reigiella*, *Elusimicrobium*, *Tolumonas*, *Moritella*, *Aeromonas*, and unclassified *Vibrionales*) among the 571 identified genera were decreased by >2-fold in the HD treatment group (Figure 4). However, no genus was found to be upregulated in the HD treatment group. The volcano plot revealed a two-fold downregulation in *Methanohalobium* and *Aciduliprofoundum* archaeal genera.

Alpha and beta diversity

The diversity and richness of bacterial communities in the CON and HD treatment groups, as measured by the Shannon index (Figure 5A), were not significantly different ($p = 0.42$). Similarly, there was no difference in the diversity and richness of archaeal communities between the two groups ($p = 0.15$). The Bray–Curtis dissimilarity visualized using PCoA plots revealed comparable bacterial community composition between the two groups ($p = 0.099$, Figure 5B). Similar patterns were observed in the archaeal community structure between the CON and HD treatment groups (Figures 5C,D; $p = 0.096$). The coefficient of determination (R^2) was non-significant between the CON and HD treatment groups for bacteria ($r = 0.22$) and archaea ($r = 0.30$).

KEGG analysis

KEGG analysis revealed that all three methanogenesis pathways (hydrogenotrophic, methylotrophic, and aceticlastic)

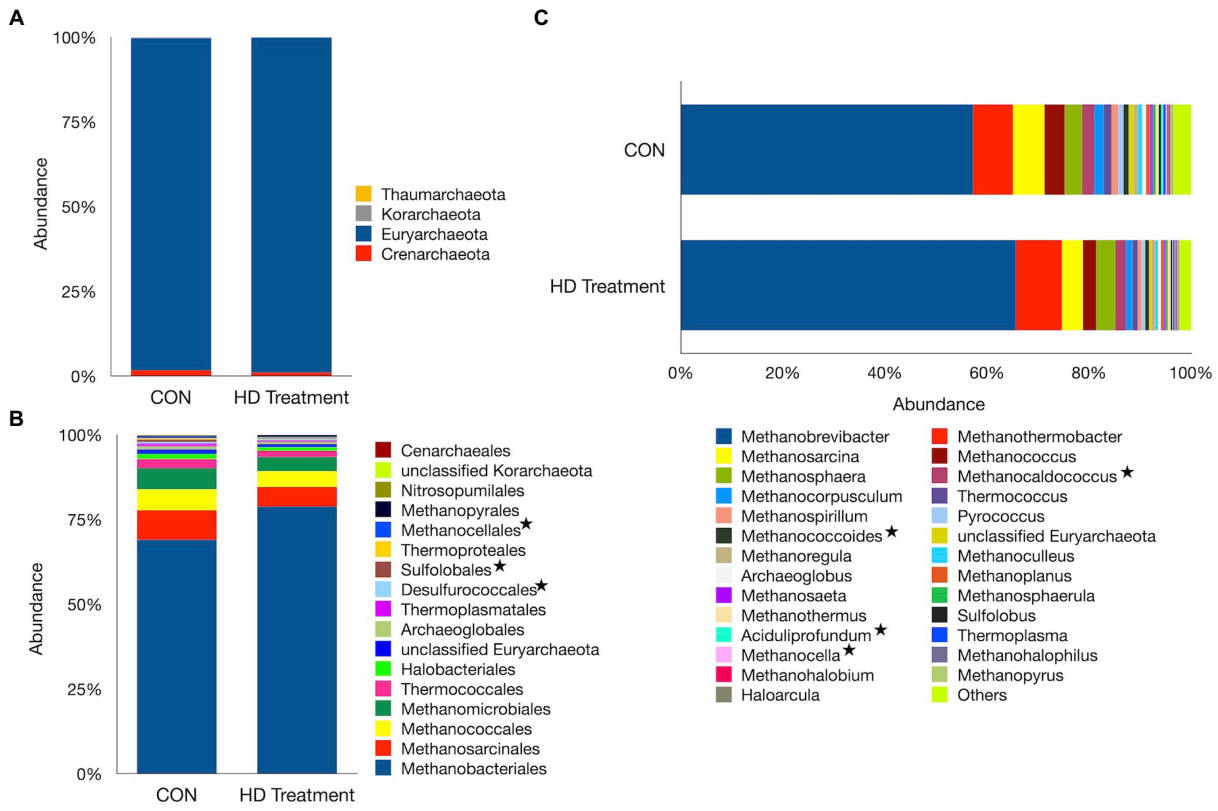

FIGURE 3
Archaeal profiles at different phylogenetic levels. Bar graphs illustrate the relative abundances of archaeal taxa at the phylum **(A)**, order **(B)**, and genus **(C)** levels. In the plots, the category "Others" represents taxa with relative abundances of <1%. Asterisks denote taxa that differ in abundance between the CON and the HD treatment groups.

were active in both CON and HD treatment groups (Figure 6). A total of 36 KO related to methanogenesis were identified in this study (Supplementary File 1). The EC 1.8.98.6 (formate dehydrogenase) assigned to a total of 6 KO was less abundant in the HD treatment group. Out of six KO, the abundance of K22516 ($p = 0.029$) and K00125 ($p = 0.044$), representing alpha and beta units respectively, were significantly lower in the anti-methanogenic supplemented group. However, the abundance of K03388, K03389, K03390, and K14127 was not different between the groups. The KEGG enzyme 2.1.1.86 (tetrahydromethanopterin S-methyltransferase), composed of eight different subunits, was also significantly different between the two groups. More specifically, the abundance of K00581 (subunit E, $p = 0.027$) and K00584 (subunit H, $p = 0.037$) was lower in the HD treatment group. The KEGG analysis did not find any difference in the number of other subunits (A-D, F-G).

K00125 ($p = 0.044$) and K22516 ($p = 0.029$) assigned to the KEGG enzyme 1.17.98.3 (formate dehydrogenase) were significantly lower in the HD treatment group. Results from the study indicated that K22516 and K00125 were assigned to two KEGG enzymes, EC1.8.98.6 and EC 1.17.98.3. On the contrary, the supplementation of anti-methanogenic product in the HD treatment group increased the abundance of ACDS (acetyl CoA decarbonylase, EC 2.3.1.169). A total of three KO (K00193, K00194, and K00197) enzymes were assigned to the ACDS and, among these, only K00197 was significantly different ($p = 0.042$) between the two groups.

Discussion

A significant reduction (~22%) in the daily enteric methane emission in sheep demonstrated that the anti-methanogenic supplement *Harit Dhara* has tremendous potential for the mitigation of enteric methane up to the desirable limit where ruminal fermentation is not compromised. Ruminal microorganisms reside in the ecosystem in a syntrophic manner to perform a specific functional task. The relationship between ruminal protozoa and methanogens is an excellent illustration of syntrophy. Methane mitigation by the supplementation of tanniferous sources is achieved in both indirect and direct manners (Bhatta et al., 2009; Gemeda and Hassen, 2015). The indirect inhibition is mediated by halting the interspecies H_2 transfer because of the decrease in rumen protozoa (Malik et al., 2017a,b; Baruah et al., 2019). A significant reduction in the numbers of protozoa in the HD treatment group, associated with a decrease in methane emissions, revealed that protozoa were one of

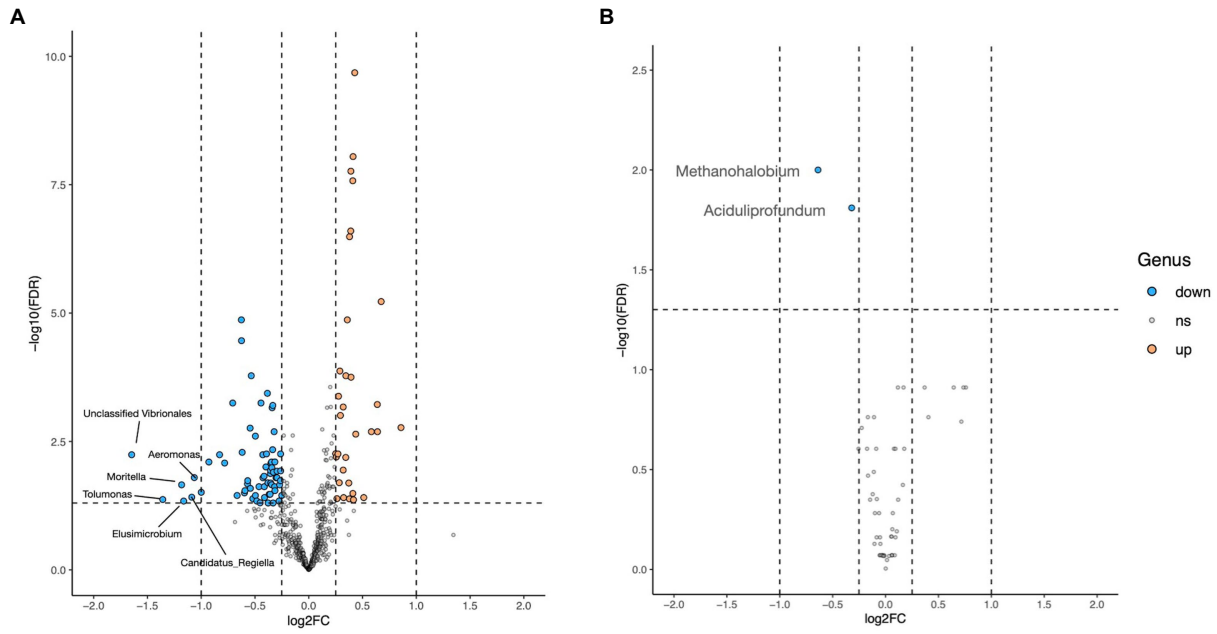

FIGURE 4
Volcano plot showing the difference in bacterial **(A)** and archaeal **(B)** abundance at the genus level between the CON and HD treatment groups. The blue dot indicates downregulation, the orange dot indicates upregulation, while gray dots depict no change. The log2 fold change is represented on the x-axis represents, while the y-axis represents the -log10 of FDR-corrected *p*-values.

the most significant factors contributing to rumen methanogenesis. Previous studies on sheep (Malik et al., 2017a,b; Baruah et al., 2019; Poornachandra et al., 2019) support our findings that a considerable reduction in enteric methane emissions can be attributed to the reduction in ruminal protozoa due to tanniferous phyto-sources. Further, the comparison of tannin-driven partial defaunation in this paper with the previous studies (Qin et al., 2012; Guyader et al., 2014; Nguyen et al., 2016) reported partial defaunation with other agents other than tanniferous sources also revealed a similar reduction in the enteric methane emission.

Protozoa are abundant but unwanted inhabitants of the rumen (Morgavi et al., 2012). The digestion of fiber could be negatively impacted by the defaunation (Ushida et al., 1990), as they appear to have better carboxymethylcellulase activity than bacteria (Coleman, 1985). On the other hand, some studies revealed no difference (Jouany, 1996; Han et al., 1999), or even increased fibrolytic activity due to the defaunation (Soetanto et al., 1985; Romulo et al., 1986). Despite their significant representation in the rumen microbiota, the role of protozoa in fiber digestibility is still controversial and debatable (Williams and Coleman, 1992). The increased propionate production in the HD treatment group can be due to the partial defaunation that promotes the development of succinate-producing bacteria (Kurihara et al., 1978; Li et al., 2020).Major succinate-producing bacteria in the rumen are *Ruminicoccus flavefaciens*, *Bacteroides succinogenes* and *Selenomonas ruminantium* (Scheifinger and Wolin, 1973). The relative abundance comparison of the major succinate producing bacteria in our study revealed the marginally higher distribution of *Ruminicoccus* (CON 2.9 vs. HD 3.3%), and *Selenomonas* (CON 0.43 vs. 0.47%) at the genus level (Supplementary File 1). The anti-methanogenic supplement offered to the HD treatment group lowered protein breakdown, as indicated by the significantly lower rumen ammonia concentration. It is a well-known fact that when tannin sources are added, nitrogen breakdown moves from the rumen to the intestine (Orlandi et al., 2015). Additionally, defaunation may be one of the causes of the reduced ammonia concentration in the rumen (Newbold et al., 2015).

The extent of volatile fatty acids production is primarily dependent on organic fermentation (Dijkstra, 1994) and remains the same until the diet composition in terms of the proportion of structural and soluble carbohydrates does not vary. The basal diet in the CON and HD treatment groups was similar in respect of ingredients and their proportions, and therefore the organic matter intake and digestibility in the present study also did not vary between the two groups. The similar diet composition, intake and digestibility, particularly of organic matter, can be accountable for the similar volatile fatty acids production. However, the sheep in the HD treatment group additionally received the anti-methanogenic supplement, primarily consisting of condensed tannins. The adverse impact of the tannins on the fermentation has been reported, particularly when supplemented at such high levels (Kumar and Vaithiyanathan, 1990). In a recent study, Guerreiro et al. (2021) reported that condensed tannin adversely impacted volatile fatty acid production; however, the adverse impact was more pronounced at higher doses (75–100 g/kg DM). The concentration of tannins (10.4 g/kg DM) in the HD treatment

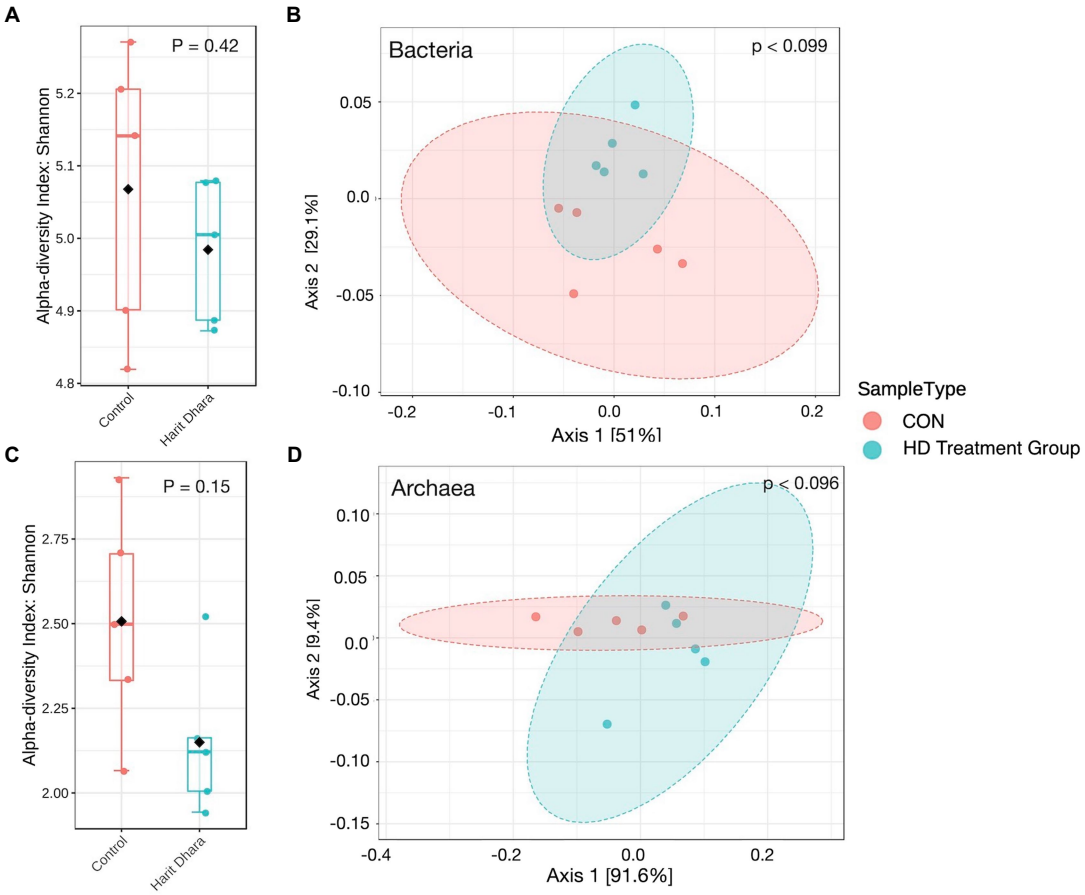

FIGURE 5
Alpha and beta diversity plots of bacteria **(A,B)** and archaea **(C,D)**.

group study was not adequate to induce an adverse impact on the fermentation of organic matter and the subsequent volatile fatty acids production. These findings are consistent with the findings of Bhatta et al. (2015), who reported that tannin supplementation has no effect on volatile fatty acid production at low levels.

The similar blood profile between CON and HD treatment groups shows that ingesting tannin-rich anti-methanogenic supplement at the appropriate level had no effect on the blood's enzymes, proteins, or minerals (Supplementary Table 3). Our results are supported by earlier research (McSweeney et al., 1988; Makkar, 2003), which concluded that the condensed tannin is not absorbed into the bloodstream and can damage an organ if it enters the bloodstream in the event of intestinal damage. On the other hand, Zhao et al. (2019) reported that Hu sheep fed on maize straw and a concentrate-base diet supplemented with 0.1% tannin had a significantly higher level of globulin and alkaline phosphatase as compared to control.

Other ways in which tanniferous phyto-sources limit intestinal methanogenesis include a reduction in fiber digestion and ruminal fermentation (McSweeney et al., 2001; Tavendale et al., 2005; Mueller-Harvey, 2006). However, similar feed intake, digestibility, and volatile fatty acid concentrations across the CON and HD treatment groups did not support that the decrease in methane in the present study was due to reduced fiber digestion. The reduction in methane emissions resulting from poor fiber digestion is dependent on the concentration (Chiquettei et al., 1988; Tan et al., 2011), molecular size (McLeod, 1974), and type of tannins (Getachew et al., 2008; Bhatta et al., 2009). Therefore, the optimal concentrations of tannins in the diet led to a reduction in methane emissions without affecting fiber digestion or ruminal fermentation (Carulla et al., 2005; Tavendale et al., 2005). The concentration of tannins in the HD treatment group at the given intake was 10.4 g/kg DM, which is significantly below the concentration of 20 g/kg DM (Waghorn et al., 1987), at which tannins exert the most detrimental effect on fermentation. The reduction in methane output without impairing intake and fermentation in our investigation corroborated previous findings in sheep supplemented with a nearly same concentration of tannins (Malik et al., 2017a,b; Baruah et al., 2019; Poornachandra et al., 2019).

Further, the comparable core bacterial communities, such as *Bacteroidetes* and *Firmicutes* between the groups also demonstrated that the anti-methanogenic supplement did not affect the fiber digestibility in the sheep rumen. The composition of the bacterial metagenome in this study was consistent with previous reports

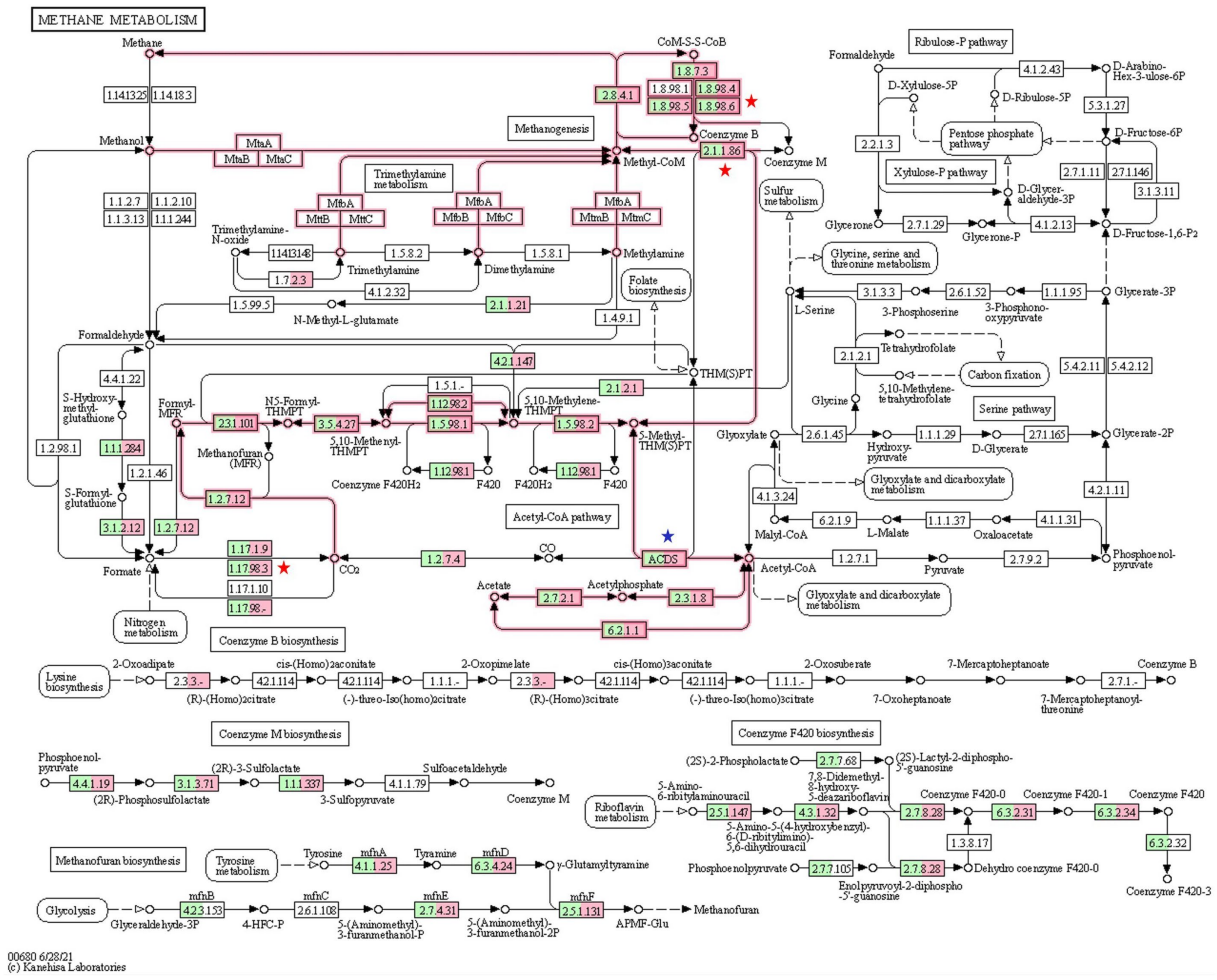

FIGURE 6
KEGG analysis for methanogenesis pathways. The numbers in the boxes represent the EC numbers of enzymes involved in methanogenesis pathways. The green color inside the box represents relative genes from the CON group microbiome, while the box in pink refers to the relative genes from the HD treatment group. At several steps in the pathway, genes related to methane metabolism has been significantly reduced in the HD treatment group. KO numbers and gene annotation of the enzymes are shown in Supplementary File.

(Khafipour et al., 2009; Jami and Mizrahi, 2012; McLoughlin et al., 2020; Malik et al., 2022). However, the anti-methanogenic supplement in the HD treatment group specifically decreased the abundance of *Proteobacteria*, which can be ascribed to the tannins antibacterial properties. Our findings are in good agreement with the previous studies (Taguri et al., 2004; Yao et al., 2006; Min et al., 2007), which reported the adverse impact of tannins on *Proteobacteria*. Tannins act mainly on the microbial cell membrane (Liu et al., 2013). However, this action is microbe species-specific and closely linked to the structure (Huang et al., 2018). The antibacterial effect of condensed tannins against *Proteobacteria*, such as *Escherichia* and *Shigella*, is well documented (Funatogawa et al., 2004; Banso and Adeyemo, 2007; Liu et al., 2013).

Conversely, despite the lower abundances of *Lentisphaerae* (0.47–0.67%), we observed significant differences in abundances between the two groups. The low abundance of *Lentisphaerae* in the rumen of sheep is consistent with previous findings (Myer et al., 2015; Lourenco et al., 2020). It has been reported that bacteria belonging to the phylum *Lentisphaerae* are linked to feed efficiency (Mao et al., 2013; Guan et al., 2017). In a study, Ahmad et al. (2020) demonstrated a linear relationship between the abundance of *Lentisphaerae* and the energy levels in yak. The greater production of volatile fatty acids, an indicator of energy, also revealed a greater *Lentisphaerae* abundance in the HD treatment group. Nevertheless, further confirmation is necessary to establish the mechanism and role of *Lentisphaerae* in the overall feed conversion efficiency.

The supplementation of tanniferous sources directly inhibited methanogens, resulting in a substantial decrease in rumen methanogenesis. A few well-described mechanisms of tannins action on rumen archaea that result in methane reduction include compositional change in cell membranes, enzymatic inhibition, and lack of substrate and metallic ions (McSweeney et al., 2001). Tannin exerts a detrimental effect on methanogens by tanning

proteins at accessible sites inside or on methanogens (Field and Lettinga, 1987; McSweeney et al., 2001). The tanniferous anti-methanogenic supplement significantly decreased the abundance of ruminal archaea such as *Methanocaldococcus*, *Methanococcoides*, *Methanocella*, and *Methanoregula* in the HD treatment group as compared to that of CON (3.3 vs. 4.4%). Archaea from the domain *Euryarchaeota* constitute 3%–5% of the rumen microbiota (Yanagita et al., 2000; Henderson et al., 2015; Malik et al., 2021). The dominance of *Methanobacteriales* as dominant archaea in the sheep rumen is in good agreement with the previous reports (Malik et al., 2022; Thirumalaisamy et al., 2022a). Similarly, our results on the dominance and abundance of *Methanobrevibacter* is in line with our previous research (Baruah et al., 2018; Malik et al., 2022; Thirumalaisamy et al., 2022a, 2022b).

A significant decrease in the abundances of *Methanocaldococcus*, *Methanococcoides*, *Methanocella*, and *Methanoregula* in the HD treatment group warrants further research to determine how tanniferous material affects their abundance. A recent report (Kibegwa et al., 2022) revealed that the distribution of *Methanococcus* in the rumen is influenced by the diet composition and its distribution tends to be greater on high roughage diets than on concentrate diets. However, the contribution of *Methanocaldococcus*, *Methanococcoides*, *Methanocella*, and *Methanoregula* to ruminal methanogenesis must be determined before concluding that their decreased abundance contributed to the lowering of methane emissions.

The *Crenarchaeota*-affiliated methanogens identified in this study have been reported previously in the rumen (Janssen and Kirs, 2008; Zhao et al., 2020; Malik et al., 2021). However, information regarding their participation in and contribution to rumen methanogenesis is scanty. *Korarchaeaota* is the third and least-characterized archaeal phylum (Barns et al., 1994, 1996). This phylum is typically found in terrestrial hot springs (Spear et al., 2005; Takai and Sako, 2006; Elkins et al., 2008) and marine hydrothermal vents (Marteinsson et al., 2001; Rogers et al., 2005). *Korarchaeaotes* are effective peptide degraders (Elkins et al., 2008), and their inability to synthesize cofactors, vitamins, and purines renders them either a symbiotic microbe or an effective scavenger (Nealson, 2008). This is, to the best of our knowledge, the first study to report the presence of *Korarchaeaota* in the rumen.

Thaumarchaeota was previously classified as a subgroup of *Crenarchaeota*; however, *Cenarchaeum symbiosum* genome sequencing in 2008 resulted in its classification as a separate archaeal phylum. The analysis of conserved signature indels (CSIs) and signature proteins provided insights into the distinction between *Thaumarchaeota* and *Crenarchaeota* (Gupta and Shami, 2011). *Thaumarchaeotes* are the dominant ammonia-oxidizing archaea in soil systems (Schleper and Nicol, 2010) and in the deep sea (Zhong et al., 2020). In a previous study (Deusch et al., 2017), based on tryptic peptide analysis (meta-proteome), the *Thaumarchaeota* phylum was identified in the rumen of Jersey cows fed corn or grass silage and/or grass hay in a rotational manner. The occurrence of *Thaumarchaeota* in our study at an extremely low frequency is in congruence with a previous report (Deusch et al., 2017). On the contrary, Wang et al. (2016) reported that *Thaumarchaeota* was one of the dominant phyla and constituted 15% of the total archaea community in the goat rumen. They conclude that *Thaumarchaeotes* participate in the oxidation of ammonia to nitrite and, due to their acid tolerance ability, thrive better on a high-concentrate diet. Nevertheless, this could not be substantiated by comparing the findings of Wang et al. (2016) with those of Deusch et al. (2017), despite the fact that the latter study contained more protein than the former. Due to the limited number of studies, the correlation between *Thaumarchaeota* distribution frequency and diet cannot be confirmed and requires further investigation.

The abundance of KEGG orthologs K00581 and K00582 representing tetrahydromethanopterin methyltransferase (EC 2.1.1.86) was significantly lower in the HD treatment group. This enzyme is accountable for the methyl transfer to coenzyme M (Gottschalk and Thauer, 2001). More specifically, the lower abundance of E and F subunits of the enzyme might lead to a sodium ion translocation-driven reduction in methanogenesis. Similarly, the KEGG orthologues K22516 and K00125, accountable for the activity of formate dehydrogenase (EC 1.8.98.6 & EC 1.17.98.3), were less abundant in the HD treatment group. This enzyme facilitates electron transfer to the HdrA subunit (Thauer, 2012), which might have reduced the reduction of ferredoxin and thereby methanogenesis. In contrast, the higher abundance of K00197, accountable for the acetyl-CoA decarbonylase/synthase complex (EC 2.3.1.169) in the HD treatment group, might have increased the transfers of methyl group (Li et al., 2013). However, in this study, we have not checked the activity of the above enzymes *per se* and are not sure if the lower abundance was transcribed.

KEGG analysis results revealed that the activity of the enzymes such as formate dehydrogenase and tetrahydromethanopterin methyltransferase involved in the formation of methane from CO_2/formate through a hydrogenotrophic pathway was comparatively less in the HD treatment group. This likely contributed to a reduction in methanogenesis as a whole. *Methanocella* and *Methanoregula* are hydrogenotrophic methanogens that utilize H_2/CO_2 for methane production (Sakai et al., 2008; Yashiro et al., 2011); consequently, the partial inhibition of hydrogenotrophic methanogenesis in the present study can be attributed to overall less methanogens population as revealed by qPCR and the low abundances of the above two methanogens in the HD treatment group. Similarly, the lower distribution of obligatory methylotrophic *Methanococcoides* (Franzmann et al., 1992) in the HD treatment group should account for the reduced methane emission in sheep fed on an anti-methanogenic supplement-based diet. Due to the absence of pure cultures and the fact that the total abundance of these methanogens in the rumen is 5%, it is extremely difficult to determine the contribution of a particular genus to the overall rumen methanogenesis. To the greatest extent possible, ruminal archaea must be cultured and the roles of *Methanococcoides* and hydrogenotrophic genera (*Methanocella* and *Methanoregula*) in rumen methanogenesis must be investigated.

Since this product was formulated with readily available, inexpensive agricultural phyto-sources, has little food-feed

competition and could therefore be one of the promising products for the small and marginal farmers to reduce the negative environmental consequences of livestock production and productivity enhancement.

Conclusion

From the study, it can be inferred that the anti-methanogenic supplement (HD treatment) at the 5% level in the straw and concentrate-based diet led to a significant decrease (~22%) in the enteric methane emission without compromising the intake and rumen fermentation. The supplement has the ability to significantly reduce ruminal protozoa and concurrently lower methane emissions. The comparable makeup of bacterial and archaeal metagenomes between groups revealed that the supplement had no effect on the core microbiome and the modest alterations in prokaryotic communities were due to minor species. Despite the vulnerability of minor methanogens in the archaeal community, the anti-methanogenic supplement greatly decreased the activity of key enzymes such as formate dehydrogenase and tetrahydromethanopterin S-methyltransferase involved in rumen methanogenesis. The culturing of minor rumen methanogens, such as *Methanocaldococcus*, *Methanococcoides*, *Methanocella*, and *Methanoregula*, should be taken up as a priority, and we should look into the impact of these archaea on the overall rumen methanogenesis. This product is formulated with readily available, inexpensive agricultural waste phyto-sources, has little food-feed competition, and could be one of the promising tools for small and marginal farmers to reduce the negative environmental impact of livestock production while improving the productivity.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/bioproject/, PRJNA885963.

Ethics statement

After obtaining prior approval (NIANP/IAEC/1/2019) from the Institute Animal Ethics Committee (IAEC), a study in sheep was conducted in strict accordance with the IAEC's procedures for animal care and sample collection.

Author contributions

PM, RB, and AK conceived and designed the study. PM, ST, and AM performed the *in vitro* and *in vivo* studies and executed the chemical composition and ruminal fluid analysis. AK and ST performed the molecular and bioinformatics analysis as well as visualization of the data. RB and HR acquired the required funding from the ICAR and ILRI. PM and RB wrote the draft and final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1048288/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Effect of anti-methanogenic supplement on the total methanogens (log2).

SUPPLEMENTARY FILE 1
Bacterial abundance, Archaeal abundance, KO Abundance, Statistical comparison results of Bacterial, Archaeal genera and KOs.

References

Ahmad, A. A., Yang, C., Zhang, J., Kalwar, Q., Liang, Z., Li, C., et al. (2020). Effects of dietary energy levels on rumen fermentation, microbial diversity, and feed efficiency of yaks (Bos grunniens). *Front. Microbiol.* 11:625. doi: 10.3389/fmicb.2020.00625

AOAC. (2005). *Official method of analysis*. Association of Officiating Analytical Chemists, Washington DC, USA.

AOAC. (2012). *Official method of analysis*. Association of Officiating Analytical Chemists, Washington DC, USA.

Arkin, A. P., Cottingham, R. W., Henry, C. S., Harris, N. L., Stevens, R. L., Maslov, S., et al. (2018). KBase: the United States department of energy systems biology knowledgebase. *Nat. Biotechnol.* 36, 566–569. doi: 10.1038/nbt.4163

Banso, A., and Adeyemo, J. (2007). Evaluation of antibacterial properties of tannins isolated from Dichrostachys cinerea. *Afr. J. Biotechnol.* 6, 1785–1787. doi: 10.5897/AJB2007.000-2262

Barns, S. M., Delwichet, C. F., Palmert, J. D., and Pace, N. R. (1996). Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences. *Proc. Natl. Acad. Sci.* 93, 9188–9193. doi: 10.1073/pnas.93.17.9188

Barns, S. M., Fundyga, R. E., Jeffries, M. W., and Pace, N. R. (1994). Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. *Proc. Natl. Acad. Sci.* 91, 1609–1613. doi: 10.1073/pnas.91.5.1609

Baruah, L., Malik, P. K., Kolte, A. P., Dhali, A., and Bhatta, R. (2018). Methane mitigation potential of phyto-sources from Northeast India and their effect on rumen fermentation characteristics and protozoa in vitro. *Vet. World* 11, 809–818. doi: 10.14202/vetworld.2018.809-818

Baruah, L., Malik, P. K., Kolte, A. P., Goyal, P., Dhali, A., and Bhatta, R. (2019). Rumen methane amelioration in sheep using two selected tanniferous phyto-leaves. *Carbon Manag* 10, 299–308. doi: 10.1080/17583004.2019.1605480

Berndt, A., Boland, T. M., Deighton, M. H., Gere, J. I., Grainger, C., Hegarty, R. S., et al. (2014). in *Guidelines for use of Sulphur hexafluoride (SF6) tracer technique to measure enteric methane emissions from ruminants*. ed. M. Lambert (New Zealand: New Zealand agricultural greenhouse gas research Centre).

Bhatta, R., Krishnamoorthy, U., and Mohammed, F. (2001). Effect of tamarind (Tamarindus indica) seed husk tannins on in vitro rumen fermentation. *Anim. Feed Sci. Technol.* 90, 143–152. doi: 10.1016/S0377-8401(01)00204-8

Bhatta, R., Saravanan, M., Baruah, L., and Prasad, C. S. (2015). Effects of graded levels of tannin-containing tropical tree leaves on in vitro rumen fermentation, total protozoa and methane production. *J. Appl. Microbiol.* 118, 557–564. doi: 10.1111/jam.12723

Bhatta, R., Uyeno, Y., Tajima, K., Takenaka, A., Yabumoto, Y., Nonaka, I., et al. (2009). Difference in the nature of tannins on in vitro ruminal methane and volatile fatty acid production and on methanogenic archaea and protozoal populations. *J. Dairy Sci.* 92, 5512–5522. doi: 10.3168/jds.2008-1441

Carulla, J. E., Kreuzer, M., Machmüller, A., and Hess, H. D. (2005). Supplementation of Acacia mearnsii tannins decreases methanogenesis and urinary nitrogen in forage-fed sheep. *Aust. J. Agr. Res.* 56, 961–970. doi: 10.1071/AR05022

Chang, J., Peng, S., Ciais, P., Saunois, M., Dangal, S. R. S., Herrero, M., et al. (2019). Revisiting enteric methane emissions from domestic ruminants and their δ13CCH4 source signature. *Nat. Commun.* 10, 3420–3414. doi: 10.1038/s41467-019-11066-3

Chiquettei, J., Cheng, K.-J., Costerton, J. W., and Milligan, L. P. (1988). Effect of tannins on the digestibility of two isosynthetic strains of birdsfoot trefoil (Lotus corniculatus L.) using in vitro and in sacco techniques. *Can. J. Anim. Sci.* 68, 751–760. doi: 10.4141/cjas88-084

Chong, J., Liu, P., Zhou, G., and Xia, J. (2020). Using microbiome analyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nat. Protoc.* 15, 799–821. doi: 10.1038/s41596-019-0264-1

Cieslak, A., Zmora, P., Pers-Kamczyc, E., and Szumacher-Strabel, M. (2012). Effects of tannins source (Vaccinium vitis idaea L.) on rumen microbial fermentation in vivo. *Anim. Feed Sci. Technol.* 176, 102–106. doi: 10.1016/j.anifeedsci.2012.07.012

Coleman, G. S. (1985). The cellulase content of 15 species of entodiniomorphid protozoa, mixed bacteria and plant debris isolated from the ovine rumen. *J. Agric. Sci.* 104, 349–360. doi: 10.1017/S0021859600044038

Conway, E. J. (1957). *Microdiffusion analysis and volumetric error. 4th Edn*. London: Crosby Lockwood and Son Ltd.

Denman, S. E., Tomkins, N. W., and McSweeney, C. S. (2007). Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. *FEMS Microbiol. Ecol.* 62, 313–322. doi: 10.1111/j.1574-6941.2007.00394.x

Deusch, S., Camarinha-Silva, A., Conrad, J., Beifuss, U., Rodehutscord, M., and Seifert, J. (2017). A structural and functional elucidation of the rumen microbiome influenced by various diets and microenvironments. *Front. Microbiol.* 8:1605. doi: 10.3389/fmicb.2017.01605

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

Dlugokencky, E. (2021). NOAA/GML. Available at: https://gml.noaa.gov/ccgg/trends_ch4/.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Elkins, J. G., Podar, M., Graham, D. E., Makarova, K. S., Wolf, Y., Randau, L., et al. (2008). A korarchaeal genome reveals insights into the evolution of the Archaea. *Proc. Natl. Acad. Sci.* 105, 8102–8107. doi: 10.1073/pnas.0801980105

Field, J. A., and Lettinga, G. (1987). The methanogenic toxicity and anaerobic degradability of a hydrolyzable tannin. *Water Res.* 21, 367–374. doi: 10.1016/0043-1354(87)90217-X

Filípek, J., and Dvořák, R. (2009). Determination of the volatile fatty acid content in the rumen liquid: comparison of gas chromatography and capillary isotachophoresis. *Acta Vet. Brno* 78, 627–633. doi: 10.2754/avb200978040627

Franzmann, P. D., Springer, N., Ludwig, W., Conway De Macario, E., and Rohde, M. (1992). A Methanogenic Archaeon from ace Lake, Antarctica: Methanococcoides burtonii sp. nov. *Syst. Appl. Microbiol.* 15, 573–581. doi: 10.1016/S0723-2020(11)80117-7

Funatogawa, K., Hayashi, S., Shimomura, H., Yoshida, T., Hatano, T., Ito, H., et al. (2004). Antibacterial activity of hydrolyzable tannins derived from medicinal plants against helicobacter pylori. *Microbiol. Immunol.* 48, 251–261. doi: 10.1111/j.1348-0421.2004.tb03521.x

Gemeda, B. S., and Hassen, A. (2015). Effect of tannin and species variation on in vitro digestibility, gas, and methane production of tropical browse plants. *Asian Australas. J. Anim. Sci.* 28, 188–199. doi: 10.5713/ajas.14.0325

Gerber, P. J., Hristov, A. N., Henderson, B., Makkar, H., Oh, J., Lee, C., et al. (2013). Technical options for the mitigation of direct methane and nitrous oxide emissions from livestock: a review. *Animal* 7, 220–234. doi: 10.1017/S1751731113000876

Getachew, G., Pittroff, W., Putnam, D. H., Dandekar, A., Goyal, S., and DePeters, E. J. (2008). The influence of addition of gallic acid, tannic acid, or quebracho tannins to alfalfa hay on in vitro rumen fermentation and microbial protein synthesis. *Anim. Feed Sci. Technol.* 140, 444–461. doi: 10.1016/j.anifeedsci.2007.03.011

Gottschalk, G., and Thauer, R. K. (2001). The Na-translocating methyltransferase complex from methanogenic archaea. *Biochim. Biophys. Acta* 1505, 28–36. doi: 10.1016/S0005-2728(00)00274-7

Grainger, C., Clarke, T., Auldist, M. J., Beauchemin, K. A., Mcginn, S. M., Waghorn, G. C., et al. (2009). Potential use of Acacia mearnsii condensed tannins to reduce methane emissions and nitrogen excretion from grazing dairy cows. *Can. J. Anim. Sci.* 89, 241–251. doi: 10.4141/CJAS08110

Guan, H., Wittenberg, K. M., Ominski, K. H., and Krause, D. O. (2006). Efficacy of ionophores in cattle diets for mitigation of enteric methane. *J. Anim. Sci.* 84, 1896–1906. doi: 10.2527/jas.2005-652

Guan, Y., Yang, H., Han, S., Feng, L., Wang, T., and Ge, J. (2017). Comparison of the gut microbiota composition between wild and captive sika deer (Cervus nippon hortulorum) from feces by high-throughput sequencing. *AMB Express* 7:212. doi: 10.1186/s13568-017-0517-8

Guerreiro, O., Alves, S. P., Costa, M., Duarte, M. F., Jerónimo, E., and Bessa, R. J. B. (2021). Effects of increasing doses of condensed tannins extract from cistus ladanifer l. on in vitro ruminal fermentation and biohydrogenation. *Animals* 11, 1–13. doi: 10.3390/ani11030761

Gupta, R. S., and Shami, A. (2011). Molecular signatures for the Crenarchaeota and the Thaumarchaeota. *Antonie Van Leeuwenhoek* 99, 133–157. doi: 10.1007/s10482-010-9488-3

Guyader, J., Eugène, M., Nozière, P., Morgavi, D. P., Doreau, M., and Martin, C. (2014). Influence of rumen protozoa on methane emission in ruminants: a meta-analysis approach. *Animal* 8, 1816–1825. doi: 10.1017/S1751731114001852

Han, C. Y., Lu, D. X., Hu, M., and Tan, Z. L. (1999). Influence of controlling protozoa on the degradation and utilization of dietary fibre and protein in the rumen and nitrogenous flow entering the duodenum of sheep. *Asian Austral. J. Anim. Sci.* 12, 1241–1245. doi: 10.5713/ajas.1999.1241

Hassanat, F., and Benchaar, C. (2013). Assessment of the effect of condensed (acacia and quebracho) and hydrolysable (chestnut and valonea) tannins on rumen fermentation and methane production in vitro. *J. Sci. Food Agric.* 93, 332–339. doi: 10.1002/jsfa.5763

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., and Janssen, P. H. (2015). Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Sci. Rep.* 5:14567. doi: 10.1038/srep14567

Huang, Q., Liu, X., Zhao, G., Hu, T., and Wang, Y. (2018). Potential and challenges of tannins as an alternative to in-feed antibiotics for farm animal production. *Anim. Nutr.* 4, 137–150. doi: 10.1016/j.aninu.2017.09.004

Hungate, R. E. (1966). *The rumen and its microbes*. Academic Press Inc, New York, USA.

Jami, E., and Mizrahi, I. (2012). Composition and similarity of bovine rumen microbiota across individual animals. *PLoS One* 7:e33306. doi: 10.1371/journal.pone.0033306

Janssen, P. H., and Kirs, M. (2008). Structure of the archaeal community of the rumen. *Appl. Environ. Microbiol.* 74, 3619–3625. doi: 10.1128/AEM.02812-07

Jayanegara, A., Goel, G., Makkar, H. P. S., and Becker, K. (2015). Divergence between purified hydrolysable and condensed tannin effects on methane emission, rumen fermentation and microbial population in vitro. *Anim. Feed Sci. Technol.* 209, 60–68. doi: 10.1016/j.anifeedsci.2015.08.002

Johnson, K. A., and Johnson, D. E. (1995). Methane emissions from cattle. *J. Anim. Sci.* 73, 2483–2492. doi: 10.2527/1995.7382483x

Jouany, J. P. (1996). Effect of rumen protozoa on nitrogen utilization by ruminants. *J. Nutr.* 126, 1335S–1346S. doi: 10.1093/jn/126.suppl_4.1335S

Kamra, D. N., and Agarwal, N. (2003). *Techniques in rumen microbiology*. Izatnagar, Bareilly, India: Indian Veterinary Research Institute.

Kanehisa, M., Sato, Y., and Morishima, K. (2016). BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. *J. Mol. Biol.* 428, 726–731. doi: 10.1016/j.jmb.2015.11.006

Kent, W. J. (2002). BLAT-the BLAST-like alignment tool. *Genome Res.* 12, 656–664. doi: 10.1101/gr.229202

Khafipour, E., Li, S., Plaizier, J. C., and Krause, D. O. (2009). Rumen microbiome composition determined using two nutritional models of subacute ruminal acidosis. *Appl. Environ. Microbiol.* 75, 7115–7124. doi: 10.1128/AEM.00739-09

Kibegwa, F. M., Bett, R. C., Charles, K., Gachuiri, C. K., Machuka, E., Stomeo, F., et al. (2022). Diversity and functional analysis of rumen and fecal microbial communities associated with dietary changes in crossbreed dairy cattle. bioRxiv [Preprint].

Kumar, R., and Vaithiyanathan, S. (1990). Occurrence, nutritional significance and effect on animal productivity of tannins in tree leaves. *Anim. Feed Sci. Technol.* 30, 21–38. doi: 10.1016/0377-8401(90)90049-E

Kurihara, Y., Takechi, T., and Shibata, F. (1978). Relationship between bacteria and ciliate protozoa in the rumen of sheep fed on a purified diet. *J. Agric. Sci.* 90, 373–381. doi: 10.1017/S0021859600055489

Lassey, K. R., Martin, R. J., Williams, S. R. O., Berndt, A., Iwaasa, A. D., Hegarty, R. S., et al. (2014). "Analysis of breath samples" in *Guidelines for use of sulphur hexafluoride (SF6) tracer technique to measure enteric methane emissions from ruminants*. ed. M. G. Lambert (New Zealand: New Zealand Agricultural Greenhouse Gas Research Centre), 89–112.

Li, A., Chu, Y. N., Wang, X., Ren, L., Yu, J., Liu, X., et al. (2013). A pyrosequencing-based metagenomic study of methane-producing microbial community in solid-state biogas reactor. *Biotechnol. Biofuels* 6, 3–17. doi: 10.1186/1754-6834-6-3

Li, Z., Wang, X., Alberdi, A., Deng, J., Zhong, Z., Si, H., et al. (2020). Comparative microbiome analysis reveals the ecological relationships between rumen methanogens, acetogens, and their hosts ecology of methanogens and acetogens in the rumen. *Front. Microbiol.* 11:1311. doi: 10.3389/fmicb.2020.01311

Liu, X. L., Hao, Y. Q., Jin, L., Xu, Z. J., McAllister, T. A., and Wang, Y. (2013). Anti-Escherichia coli O157:H7 properties of purple prairie clover and sainfoin condensed tannins. *Molecules* 18, 2183–2199. doi: 10.3390/molecules18022183

Lourenco, J. M., Kieran, T. J., Seidel, D. S., Glenn, T. C., da Silveira, M. F., Callaway, T. R., et al. (2020). Comparison of the ruminal and fecal microbiotas in beef calves supplemented or not with concentrate. *PLoS One* 15:e0231533. doi: 10.1371/journal.pone.0231533

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15:550. doi: 10.1186/s13059-014-0550-8

Makkar, H. P. S. (2003). *Quantification of tannins in tree and shrub foliage*. Dordrecht, Netherlands: Springer Science & Business Media.

Malik, P. K., Kolte, A. P., Bakshi, B., Baruah, L., Dhali, A., and Bhatta, R. (2017a). Effect of tamarind seed husk supplementation on ruminal methanogenesis, methanogen diversity and fermentation characteristics. *Carbon Manag.* 8, 319–329. doi: 10.1080/17583004.2017.1357403

Malik, P. K., Kolte, A. P., Baruah, L., Saravanan, M., Bakshi, B., and Bhatta, R. (2017b). Enteric methane mitigation in sheep through leaves of selected tanniniferous tropical tree species. *Livest. Sci.* 200, 29–34. doi: 10.1016/j.livsci.2017.04.001

Malik, P. K., Soren, N. M., Thulasi, A., and Prasad, C. S. (2015). Simple method for rumen content collection from 2 days old lambs. *Indian Vet. J.* 92, 46–48.

Malik, P. K., Trivedi, S., Kolte, A. P., Sejian, V., Bhatta, R., and Rahman, H. (2022). Diversity of rumen microbiota using metagenome sequencing and methane yield in Indian sheep fed on straw and concentrate diet. *Saudi J. Biol. Sci.* 29:103345. doi: 10.1016/j.sjbs.2022.103345

Malik, P. K., Trivedi, S., Mohapatra, A., Kolte, A. P., Sejian, V., Bhatta, R., et al. (2021). Comparison of enteric methane yield and diversity of ruminal methanogens in cattle and buffaloes fed on the same diet. *PLoS One* 16:e0256048. doi: 10.1371/journal.pone.0256048

Malik, P. K., Uyeno, Y., Kolte, A. P., Kumar, R., Trivedi, S., and Bhatta, R. (2019). Screening of phyto-sources from foothill of Himalayan mountain for livestock methane reduction. *SN Appl. Sci.* 1:232. doi: 10.1007/s42452-019-0229-6

Mao, S. Y., Zhang, R. Y., Wang, D. S., and Zhu, W. Y. (2013). Impact of subacute ruminal acidosis (SARA) adaptation on rumen microbiota in dairy cattle using pyrosequencing. *Anaerobe* 24, 12–19. doi: 10.1016/j.anaerobe.2013.08.003

Marteinsson, V. T., Kristjánsson, J. K., Kristmannsdóttir, H., Dahlkvist, M., Sæmundsson, K., Hannington, M., et al. (2001). Discovery and description of giant submarine smectite cones on the seafloor in Eyjafjordur, northern Iceland, and a novel thermal microbial habitat. *Appl. Environ. Microbiol.* 67, 827–833. doi: 10.1128/AEM.67.2.827-833.2001

McLeod, M. N. (1974). Plant tannins-their role in forage quality. *Nutr. Abstr. Rev.* 44, 803–815. doi: 10.11209/jim.25.1

McLoughlin, S., Spillane, C., Claffey, N., Smith, P. E., O'Rourke, T., Diskin, M. G., et al. (2020). Rumen microbiome composition is altered in sheep divergent in feed efficiency. *Front. Microbiol.* 11:1981. doi: 10.3389/fmicb.2020.01981

McSweeney, C. S., Kennedy, P. M., and John, A. A. (1988). Effect of ingestion of hydrolysable tannins in Terminalia oblongata on digestion in sheep fed Stylosanthes hamata. *Aust. J. Agr. Res.* 39, 235–279. doi: 10.1071/AR9880235

McSweeney, C. S., Palmer, B., McNeill, D. M., and Krause, D. O. (2001). Microbial interactions with tannins: nutritional consequences for ruminants. *Anim. Feed Sci. Technol.* 91, 83–93. doi: 10.1016/S0377-8401(01)00232-2

Menke, K. H., Raab, L., Salewski, A., Steingass, H., Fritz, D., and Schneider, W. (1979). The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor in vitro. *J. Agric. Sci.* 93, 217–222. doi: 10.1017/S0021859600086305

Min, B. R., Pinchak, W. E., Anderson, R. C., and Callaway, T. R. (2007). Effect of tannins on the in vitro growth of Escherichia coli O157:H7 and in vivo growth of generic Escherichia coli excreted from steers. *J. Food Prot.* 70, 543–550. doi: 10.4315/0362-028X-70.3.543

Moate, P. J., Richard, S., Williams, O., Deighton, M. H., Pryce, J. E., Hayes, B. J., et al. (2014). Mitigation of enteric methane emissions from the Australian dairy industry. In *Proceedings of the 5th Australasian Dairy Science Symposium*, 121–140. Doi:10.13140/2.1.2172.4489.

Morgavi, D. P., Martin, C., Jouany, J. P., and Ranilla, M. J. (2012). Rumen protozoa and methanogenesis: not a simple cause-effect relationship. *Br. J. Nutr.* 107, 388–397. doi: 10.1017/S0007114511002935

Mueller-Harvey, I. (2006). Unravelling the conundrum of tannins in animal nutrition and health. *J. Sci. Food Agric.* 86, 2010–2037. doi: 10.1002/jsfa.2577

Myer, P. R., Smith, T. P. L., Wells, J. E., Kuehn, L. A., and Freetly, H. C. (2015). Rumen microbiome from steers differing in feed efficiency. *PLoS One* 10:e0129174. doi: 10.1371/journal.pone.0129174

Nealson, K. (2008). A Korarchaeote yields to genome sequencing. *Proc. Natl. Acad. Sci.* 105, 8805–8806. doi: 10.1073/pnas.0804670105

Newbold, C. J., de la Fuente, G., Belanche, A., Ramos-Morales, E., and McEwan, N. R. (2015). The role of ciliate protozoa in the rumen. *Front. Microbiol.* 6:1313. doi: 10.3389/fmicb.2015.01313

Nguyen, S. H., Barnett, M. C., and Hegarty, R. S. (2016). Use of dietary nitrate to increase productivity and reduce methane production of defaunated and faunated lambs consuming protein-deficient chaff. *Anim. Product. Sci.* 56, 290–297. doi: 10.1071/AN15525

Orlandi, T., Kozloski, G., Alves, T. P., Mesquita, F. R., and Ávila, S. C. (2015). Digestibility, ruminal fermentation and duodenal flux of amino acids in steers fed grass forage plus concentrate containing increasing levels of Acacia mearnsii tannin extract. *Anim. Feed Sci. Technol.* 210, 37–45. doi: 10.1016/j.anifeedsci.2015.09.012

Poornachandra, K. T., Malik, P. K., Dhali, A., Kolte, A. P., and Bhatta, R. (2019). Effect of combined supplementation of tamarind seed husk and soapnut on enteric methane emission in crossbred cattle. *Carbon Manag.* 10, 465–475. doi: 10.1080/17583004.2019.1640136

Puchala, R., Min, B. R., Goetsch, A. L., and Sahlu, T. (2005). The effect of a condensed tannin-containing forage on methane emission by goats. *J. Anim. Sci.* 83, 182–186. doi: 10.2527/2005.831182x

Qin, W. Z., Li, C. Y., Kim, J. K., Ju, J. G., and Song, M. K. (2012). Effects of defaunation on fermentation characteristics and methane production by rumen microbes in vitro when incubated with starchy feed sources. *Asian-Australas J. Anim. Sci.* 25, 1381–1388. doi: 10.5713/ajas.2012.12240

Rho, M., Tang, H., and Ye, Y. (2010). FragGeneScan: predicting genes in short and error-prone reads. *Nucleic Acids Res.* 38:e191. doi: 10.1093/nar/gkq747

Rogers, K. L., Amend, J. P., and Rogers, K. L. (2005). Archaeal diversity and geochemical energy yields in a geothermal well on Vulcano Island, Italy. *Geobiology* 3, 319–332. doi: 10.1111/j.1472-4669.2006.00064.x

Romulo, B. H., Bird, S. H., and Leng, R. A. (1986). The effects of defaunation on digestibility and rumen fungi counts in sheep fed high-fibre diets. In *Proceedings of the Australian Society of Animal Production* (Australia).

Sakai, S., Imachi, H., Hanada, S., Ohashi, A., Harada, H., and Kamagata, Y. (2008). Methanocella paludicola gen. Nov., sp. nov., a methane-producing archaeon, the first isolate of the lineage "Rice cluster I", and proposal of the new archaeal order Methanocellales Ord. Nov. *Int. J. Syst. Evol. Microbiol.* 58, 929–936. doi: 10.1099/ijs.0.65571-0

Scheifinger, C. C., and Wolin, M. J. (1973). Propionate formation from cellulose and soluble sugars by combined cultures of Bacteroides succinogenes and Selenomonas ruminantium. *Appl. Microbiol.* 26, 789–795. doi: 10.1128/am.26.5.789-795.1973

Schleper, C., and Nicol, G. W. (2010). Ammonia-oxidising archaea - physiology, ecology and evolution. *Adv. Microb. Physiol.* 57, 1–41. doi: 10.1016/B978-0-12-381045-8.00001-1

Soetanto, H., Gordon, G. L., Hume, I. D., and Leng, R. A. (1985). The role of protozoa and fungi in fibre digestion in the rumen of sheep. in *Proceedings of the 3rd AAAP Animal Science Congress, Efficient Animal Production for Asian Welfare*, 805–807.

Spear, J. R., Walker, J. J., McCollom, T. M., and Pace, N. R. (2005). Hydrogen and bioenergetics in the Yellowstone geothermal ecosystem. *Proc. Natl. Acad. Sci.* 102, 2555–2560. doi: 10.1073/pnas.0409574102

Taguri, T., Tanaka, T., and Kouno, I. (2004). Antimicrobial activity of 10 different plant polyphenols against bacteria causing food-borne disease. *Biol. Pharm. Bull.* 27, 1965–1969. doi: 10.1248/bpb.27.1965

Takai, K., and Sako, Y. (2006). A molecular view of archaeal diversity in marine and terrestrial hot water environments. *FEMS Microbiol. Ecol.* 28, 177–188. doi: 10.1111/j.1574-6941.1999.tb00573.x

Tan, H. Y., Sieo, C. C., Abdullah, N., Liang, J. B., Huang, X. D., and Ho, Y. W. (2011). Effects of condensed tannins from Leucaena on methane production, rumen fermentation and populations of methanogens and protozoa in vitro. *Anim. Feed Sci. Technol.* 169, 185–193. doi: 10.1016/j.anifeedsci.2011.07.004

Tavendale, M. H., Meagher, L. P., Pacheco, D., Walker, N., Attwood, G. T., and Sivakumaran, S. (2005). Methane production from in vitro rumen incubations with Lotus pedunculatus and Medicago sativa, and effects of extractable condensed tannin fractions on methanogenesis. *Anim. Feed Sci. Technol.* 123-124, 403–419. doi: 10.1016/j.anifeedsci.2005.04.037

Thauer, R. K. (2012). The Wolfe cycle comes full circle. *Proc. Natl. Acad. Sci. U. S. A.* 109, 15084–15085. doi: 10.1073/pnas.1213193109

Thirumalaisamy, G., Malik, P. K., Kolte, A. P., Trivedi, S., Dhali, A., and Bhatta, R. (2022a). Effect of silkworm (Bombyx mori) pupae oil supplementation on enteric methane emission and methanogens diversity in sheep. *Anim. Biotechnol.* 33, 128–140. doi: 10.1080/10495398.2020.1781147

Thirumalaisamy, G., Malik, P. K., Trivedi, S., Kolte, A. P., and Bhatta, R. (2022b). Effect of long-term supplementation with silkworm pupae oil on the methane yield, ruminal protozoa, and archaea community in sheep. *Front. Microbiol.* 13:780073. doi: 10.3389/fmicb.2022.780073

Ushida, K., Kayouli, C., de Smet, S., and Jouany, J. P. (1990). Effect of defaunation on protein and fibre digestion in sheep fed on ammonia-treated straw-based diets with or without maize. *Br. J. Nutr.* 64, 765–775. doi: 10.1079/bjn19900078

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

Waghorn, G. C., Ulyatt, M. J., John, A., and Fisher, M. T. (1987). The effect of condensed tannins on the site of digestion of amino acids and other nutrients in sheep fed on Lotus corniculatus L. *Br. J. Nutr.* 57, 115–126. doi: 10.1079/bjn19870015

Wang, J., Xue, C., Song, Y., Wang, L., Huang, Q., and Shen, Q. (2016). Wheat and rice growth stages and fertilization regimes alter soil bacterial community structure, but not diversity. *Front. Microbiol.* 7:1207. doi: 10.3389/fmicb.2016.01207

Wilke, A., Glass, E., Bischof, J., Braithwaite, D., Souza, M., and Gerlach, W. (2013). *MG-RAST technical report and manual for version 3.3. 6–Rev 1*, Lemont, IL: Argonne National Laboratory.

Wilke, A., Harrison, T., Wilkening, J., Field, D., Glass, E. M., Kyrpides, N., et al. (2012). The M5nr: a novel non-redundant database containing protein sequences and annotations from multiple sources and associated tools. *BMC Bioinformatics* 13, 1–5. doi: 10.1186/1471-2105-13-141

Williams, A. G., and Coleman, G. S. (1992). "Role of protozoa in the rumen," in *In the rumen protozoa*. eds. A. G. Williams and G. S. Coleman (New York: Springer), 317–347.

Williams, S. R. O., Moate, P. J., and Deighton, M. H. (2014). "Sampling background air" in *Guidelines for Use of Sulphur hexaflouride (SF6) Tracer Technique to Measure Enteric Methane Emissions from Ruminants*. ed. M. G. Lambert (New Zealand: New Zealand agricultural greenhouse gas research Centre), 81–88.

Yanagita, K., Kamagata, Y., Kawaharasaki, M., Suzuki, T., Nakamura, Y., and Minato, H. (2000). Phylogenetic analysis of methanogens in sheep rumen ecosystem and detection of Methanomicrobium mobile by fluorescence in situ hybridization. *Biosci. Biotechnol. Biochem.* 64, 1737–1742. doi: 10.1271/bbb.64.1737

Yao, K., He, Q., Ying Jia, D., and Shi, B. (2006). The potential of wattle tannin extracts for fine use. *Nat. Prod. Res.* 20, 271–278. doi: 10.1080/14786410500074754

Yashiro, Y., Sakai, S., Ehara, M., Miyazaki, M., Yamaguchi, T., and Imachi, H. (2011). Methanoregula formicica sp. nov., a methane-producing archaeon isolated from methanogenic sludge. *Int. J. Syst. Evol. Microbiol.* 61, 53–59. doi: 10.1099/ijs.0.014811-0

Yu, Z., and Morrison, M. (2004). Improved extraction of PCR-quality community DNA from digesta and fecal samples. *Biotechniques* 36, 808–812. doi: 10.2144/04365st04

Yusuf, R. O., Noor, Z. Z., Abba, A. H., Hassan, M. A. A., and Din, M. F. M. (2012). Methane emission by sectors: a comprehensive review of emission sources and mitigation methods. *Renew. Sustain. Energy Rev.* 16, 5059–5070. doi: 10.1016/j.rser.2012.04.008

Zhao, M. D., Di, L. F., Tang, Z. Y., Jiang, W., and Li, C. Y. (2019). Effect of tannins and cellulase on growth performance, nutrients digestibility, blood profiles, intestinal morphology and carcass characteristics in Hu sheep. *Asian-Australas J. Anim. Sci.* 32, 1540–1547. doi: 10.5713/ajas.18.0901

Zhao, Y., Xue, F., Hua, D., Wang, Y., Pan, X., Nan, X., et al. (2020). Metagenomic insights into effects of thiamine supplementation on carbohydrate-active enzymes' profile in dairy cows fed high-concentrate diets. *Animals* 10:304. doi: 10.3390/ani10020304

Zhong, H., Zhong, H., Lehtovirta-Morley, L., Liu, J., Liu, J., Zheng, Y., et al. (2020). Novel insights into the Thaumarchaeota in the deepest oceans: their metabolism and potential adaptation mechanisms. *Microbiome* 8:78. doi: 10.1186/s40168-020-00849-2

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Glucogenic and lipogenic diets affect *in vitro* ruminal microbiota and metabolites differently

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This study was conducted to evaluate the effects of two glucogenic diets (C: ground corn and corn silage; S: steam-flaked corn and corn silage) and a lipogenic diet (L: sugar beet pulp and alfalfa silage) on the ruminal bacterial and archaeal structures, the metabolomic products, and gas production after 48h *in vitro* fermentation with rumen fluid of dairy cows. Compared to the C and S diets, the L dietary treatment leaded to a lower dry matter digestibility (DMD), lower propionate production and ammonia-nitrogen concentration. The two glucogenic diets performed worse in controlling methane and lactic acid production compared to the L diet. The S diet produced the greatest cumulative gas volume at any time points during incubation compared to the C and L diet. The metabolomics analysis revealed that the lipid digestion especially the fatty acid metabolism was improved, but the amino acid digestion was weakened in the L treatment than in other treatments. Differences in rumen fermentation characteristics were associated with (or resulting from) changes in the relative abundance of bacterial and archaeal genera. The rumen fluid fermented with L diet had a significantly higher number of cellulolytic bacteria, including the genera of *Ruminococcus*, *Butyrivibrio*, *Eubacterium*, *Lachnospira*, unclassified *Lachnospiraceae*, and unclassified *Ruminococcaceae*. The relative abundances of amylolytic bacteria genera including *Selenomonas_1*, *Ruminobacter*, and *Succinivibrionaceae_UCG-002* were higher in samples for diets C and S. The results indicated that the two glucogenic diets leaded to a higher relative abundance of bacteria which functions in succinate pathway resulting in a higher propionate production. The steam-flaked corn diet had a higher gas production and lower level of metabolites in fatty acids and amino acids. Most highly abundant bacteria were observed to be not sensitive to dietary alterations of starch and fiber, except for several amylolytic bacteria and cellulolytic bacteria. These finding offered new insights on the digesting preference of ruminal bacteria, which can assist to improve the rumen functioning.

KEYWORDS

glucogenic/lipogenic diet, rumen fermentation, microbiota, gas production, metabolomics, PICRUSt

Introduction

Dietary carbohydrates, such as starch and fiber, provide substrates for rumen microbes. Changes in carbohydrate composition and content in ruminant rations lead to the changes in microbial community and subsequently to changes in fermentation end-products, including the volatile fatty acids (VFAs), carbon dioxide (CO_2), methane (CH_4), and hydrogen (H_2; Carberry et al., 2012). The major ruminal VFAs include acetate, propionate, and butyrate. Acetate is the primary precursor of milk fatty acids and is termed a lipogenic nutrient, while propionate being the primary precursor of milk lactose is a glucogenic nutrient (van Knegsel et al., 2005). For ruminant animals, lipogenic nutrients in metabolism are supplied by acetate and butyrate from ruminal degradation of fiber or dietary fat, if not derived from the mobilization of body fat reserves (van Knegsel et al., 2005). In contrast, glucogenic nutrients in metabolism can originate from the ruminal fermentation of dietary starch to propionate, rumen bypass of dietary starch which is then digested in the small intestine and absorbed as glucose, or gluconeogenesis (Van Knegsel et al., 2007). Previous research showed that glucogenic nutrients increased plasma glucose and insulin concentrations, whereas lipogenic nutrients did not (van Knegsel et al., 2005). However, changes in microbial communities and their metabolic activities under lipogenic and glucogenic diets are also essential to investigate to unravel the production pathways of the affected metabolites and better understand how rumen functioning is regulated.

Ground corn is a major dietary energy source because of its high amount of readily fermentable starch. Steam-flaking can disintegrate the crystalline structure of cereal starch by gelatinisation (Ding et al., 2007), and subsequently, this can increase the accessibility to the starch granules of ruminal amylases and amylolytic microorganisms (Huntington, 1997). Previous studies showed that steam-flaked corn improved the ruminal degradability of starch, resulting in high production of ruminal propionate, and increased efficiency in microbial protein synthesis (Zhou et al., 2015). However, the alteration in rumen microbial communities underneath these improvements is still not clear, which is essential for manipulate the starch degradation in the rumen.

The effects of dietary treatments on the ruminal microbes and microbial metabolism when incubated *in vitro* are rarely reported although the *in vitro* gas production technique is routinely used to evaluate dry matter (DM) degradation rate, amount and proportion of VFAs production, and gas composition of various feeds and ingredients. This technique also yields valuable information on the effects of feedstuff on rumen microbial activity and predicts the kinetics of fermentation (Pellikaan et al., 2011a). With the 16S rRNA sequencing technology, a fast and cost-effective way of microbial analysis and their correlations with environmental factors coupled with liquid chromatography-mass spectrometry (LC–MS), an effective technique for metabolomics analysis, more knowledge on changes in ruminal microbiota metabolism can be generated (Zhang J. et al., 2017).

Although studies on differences in rumen fermentation between glucogenic and lipogenic diets have been conducted, the changes in rumen bacterial community and functions are not yet fully understood. We hypothesized that glucogenic and lipogenic diets when evaluated using the *in vitro* gas production technique should lead to clear differences in bacterial communities and functions which affect intermediary metabolites besides the well-known differences in fermentation end-products and CH_4 production.

Materials and methods

Experimental design

Animal care followed the Chinese guidelines for animal welfare, and all protocols were approved by the Animal Care and Use Committee of the Chinese Academy of Agricultural Sciences (IAS2019-6).

Three total mixed rations (TMR) were designed: two glucogenic diets including a ground corn diet (C, which used ground corn and corn silage as the primary energy sources), a steam-flaked corn diet (S, which used steam-flaked corn and corn silage as the primary energy sources), and a lipogenic diet (L) mainly containing sugar beet pulp and alfalfa silage as the energy sources. In addition, other ingredients, including soybean meal, oat and alfalfa hay, and calcium hydrogen phosphate were used to balance the ration to meet the nutritional requirements of dairy cattle (Table 1). Diets were isocaloric and were equal in the digestible crude protein.

In vitro incubation

Six lactating dairy cows (Holstein) were selected as rumen fluid donors for all three runs of this *in vitro* study, with different two cows for each run. The cows received a diet containing (% DM basis) a concentrated mixture (45%), alfalfa and oat hay (20%), corn silage (20%), and alfalfa silage (15%). The cows were fed three time daily at 7:00, 13:00, and 19:00, and they had free access to water and feed.

The fermentation substrates were the ground DM of each experimental diets. 0.5 g of substrates were firstly weighed into 150-ml serum bottles, with three replicate bottles for each dietary treatment within one fermentation run. A phosphate-bicarbonate buffer medium was anaerobically prepared as described by Menke and Steingass (1988).

Equal volumes of fresh ruminal fluid were collected through a stomach tube from two cows in each fermentation run, 2 h after the first feeding (09:00 h), then poured into a sterilized and pre-warmed thermos flask (2,000 ml) leaving no headspace in the flask. After transportation to the laboratory, the rumen fluid was

TABLE 1 Ingredient and nutritional composition of two glucogenic (C, S) and a lipogenic (L) diet.

Item	Experimental diet		
	C	L	S
Ingredient, % of dry matter			
Ground corn	28	0	0
Sugar beet pulp	0	28	0
Steam flaked corn	0	0	28
Soybean meal	18.5	12	18.5
Oat hay	5	19	5
Alfalfa hay	10	10	10
Corn silage	38	0	38
Alfalfa silage	0	30	0
Dicalcium phosphate	0.5	1	0.5
Composition, g/kg of dry matter			
Crude protein	174.4	174.6	172.1
Ether extract	24.3	20.4	31.7
Starch	192.9	39.7	163.8
Neutral detergent fiber	326	562.2	320.2
Acid detergent fiber	197.9	348.9	199.1
Ash	47.6	98.7	47
Calcium	9.3	12.8	11.1
Phosphorus	10.4	4.9	11.9
NE_L, MJ/kg of dry matter	7.3	7.9	7.4

Nutrient composition of the experimental diets was calculated according to National Research Council (NRC) (2001). Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; and S, corn and steam-flaked corn diet. NE_L, net energy for lactation and calculated according to National Research Council (NRC) (2001).

strained through four layers of cheesecloth and transferred into a flask placed in a water bath of 39°C maintaining anaerobic conditions. The strained rumen fluid inoculum (25 ml) and anaerobic buffer (50 ml) were successively combined with substrates into each bottle, with the CO_2 continuously flushing in the headspace of bottles. After sealing with butyl rubber stoppers, the serum bottles were connected to the gas inlets of an automated gas production recording system (AGRS), as reported by Zhang and Yang (2011). Each fermentation run lasted for 48 h and was repeated for three runs within 2 weeks (Supplementary Figure S1).

Sampling and chemical analysis

Calibrated gas volumes were automatically recorded and cumulative gas production was expressed against the time of incubation. At 48 h of incubation, 20 μl of gas was collected through a 20 μl gastight syringe from each bottle to test the CH_4 concentration using gas chromatography (GC, 7890B, Agilent Technologies, United States). The GC was equipped with a capillary column (USF727432H, 30 m × 0.25 mm × 0.25 μm, Agilent, California, United States) and a flame ionization detector (FID). Nitrogen (N_2, 99.99%) was used as the carrier gas, with column settings as follows: the inlet pressure 18.85 psi, the total flow 30.2 ml/min, the column flow 1.7 ml/min, the linear speed 39.8 cm/s, the split ratio 15, the sweeping flow 3 ml/min, and the cycling flow 8 ml/min. The hydrogen and airflow were 40 ml/min and 400 ml/min, respectively. Temperatures were set to 100°C for the injection point, 80°C for the column oven, and 120°C for the detector.

At 48 h, all bottles were transferred into an ice-water mixture to terminate the incubation. The pH value of the fermented substrates was determined using a portable pH meter (PHB-4, INESA, Shanghai, China). Then the substrates were filtered through a nylon bag (12 cm × 8 cm i.d. and 50 μm of pore size) and the residue left in the bag was used to analyze apparent dry matter digestibility (DMD) gravimetrically. Two replicates of 3 ml fluid samples were quickly collected into two cryogenic vials and immediately frozen in liquid nitrogen until being stored at −80°C, which were used for 16S rRNA sequencing and metabolomics analysis separately. A sample of 1 ml fluid was mixed with 0.25 ml of 25% meta-phosphoric acid to evaluate the VFA contents *via* the GC (7890B, Agilent Technologies, United States; Mao et al., 2008). Also, 1 ml of fluid was collected to analyze the ammonia nitrogen (NH_3-N) according to the indophenol way (Broderick and Kang, 1980) and another 1 ml fluid was used to measure the lactic acid concentration by a commercial kit (A019-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, China; Pan et al., 2016). Two replicated 3 ml fluid samples were stored under −80°C for later microbial and metabolomics analysis, separately.

DNA extraction and amplification

The DNA of microbes was extracted from supernatant samples using the QIAamp DNA Stool Mini Kit (M5635-02, OMEGA, United States). The concentration of DNA was evaluated with the Nano Drop spectrophotometer (Thermo Scientific, United States), and then the agarose gel (1% w/v) electrophoresis was used to check the DNA quality.

The 16S rRNA gene of bacteria and archaea were separately amplified with the general primers (Supplementary material) based on the hypervariable region (V3–V4). The PCR was performed (Supplementary material) and the products were firstly extracted from an agarose gel (2% w/v), then purified with the commercial Extraction Kit (Axygen Biosciences, United States). The DNA products were finally quantified with QuantiFluor™-ST (Promega, United States).

Illumina miSeq sequencing and analysis

Purified amplicons were mixed in an equimolar ratio and paired-end sequenced (2 × 300 bp) through the MiSeq platform (Illumina, San Diego, United States) according to the

manufacturer's standard (Majorbio Bio-Pharm Technology Co. Ltd., Shanghai, China).

The raw fastq was quality-filtered with FLASH following the protocol previously reported by Pan et al. (2017). With a 97% sequence similarity cut-off, the operational taxonomic units (OTUs) were clustered through UPARSE. The taxonomy was calculated with the RDP Classifier against the SILVA (SSU123) 16S rRNA database with a confidence threshold of 70%. The principal coordinates analysis (PCoA) was analyzed with the method of unweighted UniFrac distance to compare the interrelationships of bacterial communities between diets using the R software (3.4.4). The community richness and diversity were analyzed by the alpha diversity indexes including the OTU, Chao 1, ACE, Shannon, and Simpson (Hua et al., 2021).

Inferred metagenomics analysis

The metagenome functions of ruminal bacteria were predicted using the analysis of Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt). Firstly, the closed OTU table was normalized by the 16S rRNA copy number whereafter the results were exported into the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The PCoA was conducted to calculate the similarities of the predicted functions among groups by the R software (3.4.4). The top 10 abundant functions were further analyzed to determine significant differences among diets using Welch's *t*-test in R software (3.4.4).

Metabolomics processing

The method was modified from the procedure described by Wang et al. (2021). The rumen fluid samples were firstly thawed under room temperature whereafter 200 μl supernatant of each sample was collected into a 1.5 ml centrifuge tube and mixed with 800 μl extracting solution [methanol: acetonitrile = 1:1 (v/v)]. Each sample was then vortexed for 30 s and extracted ultrasonically (40 kHz) at 5°C for 30 min before being treated under −20°C for 30 min. All samples were centrifuged (13,000 × *g*, 4°C, 15 min) and the supernatant transferred to a new tube, mixed with 100 μl acetonitrile solution (acetonitrile: water = 1:1), vortexed for 30 s, extracted ultrasonically (40 kHz) at 5°C for 5 min, and centrifuged (13,000 × *g*, 4°C, 10 min) where after 200 μl of the supernatant was carefully transferred to sample vials for LC–MS/MS analysis. At the same time, 20 μl of supernatant was collected from each sample and mixed as the quality control sample (QC) which were injected at regular intervals throughout the analytical runs in order to obtain system repeatability.

Chromatographic separation of the metabolites was performed on the ultra-performance liquid chromatography (UPLC) coupled with a triple time-of-flight (TOF) system (UPLC-Triple TOF, AB Sciex, United States). The system was equipped with the ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm id, 1.8 μm; Waters, Milford, United States). Mobile phase A consisted of 5% acetonitrile water and 0.1% formic acid, the mobile phase B contained 95% acetonitrile-isopropanol (1:1, v/v) and 0.1% formic acid. The injection volume was 10 μl, the flow rate was 0.4 ml/min, and the column temperature was 40°C. The elution gradient of the mobile phases was shown in the Supplementary material. After being treated with an electrospray ionization (ESI) source, the signals of mass spectra were scanned in both positive mode and negative mode. The optimal conditions for mass spectra were shown in the Supplementary material.

Metabolomics data analysis

After UPLC-TOF/MS analyses, the raw data were imported into Progenesis QI 2.3 (Waters Corporation, Milford, United States) for a series of pre-processing, including filtration of the baseline, identification and integration of the peak, correction of the retention time, and alignment of the peak. After the pre-processing, a data matrix was generated consisting of the retention time (RT), mass-to-charge ratio (m/z) values, and peak intensity. The MS and MS/MS information was searched in the Human metabolome database (HMDB; http://www.hmdb.ca/) and Metlin database.[1] Results were shown in the form of a data matrix.

After being pre-processed, the data matrix was analyzed on the Majorbio Cloud Platform.[2] Using the R package of ROPLS (version1.6.2), PCA was applied to obtain an overview of the metabolic data, general clustering, trends, or outliers among groups whereafter orthogonal partial least squares discriminate analysis (OPLS-DA) was performed to observe the global difference of the metabolites between comparable groups. The variable importance in the projection (VIP) was calculated in the OPLS-DA model, and the *p*-value was estimated with paired Student's *t*-test. Statistically affected metabolites among groups were selected with VIP > 1 and $p \leq 0.05$. The affected metabolites between every two groups were summarized into different metabolic groups and mapped into their biochemical pathways through the KEGG database. The metabolic pathway enrichment analysis of the metabolic groups was conducted with the Fisher's exact test using the Python package of Scipy. stats (version1.0.0, SciPy.org).

Correlation between bacterial community and rumen metabolites

Correlation between the affected bacterial genera with a relative abundance >0.5% and the rumen fermentation

1 https://metlin.scripps.edu/

2 https://cloud.majorbio.com

parameters, as well as the correlation between these affected bacterial genera and the differential metabolites (VIP > 1.5, fold change >2 or < 0.5, $p \leq 0.05$), was separately assessed by Pearson's correlation analysis in R (version 3.4.4). These correlations were visualized using the R package of Pheatmap.

Curve fitting and calculations

Data of the cumulative gas production curve were in accordance with the monophasic model using a non-linear least squares regression procedure NLIN in SAS 9.3 (SAS Institute Inc., Cary, NC, United States; Pellikaan et al., 2011b):

$$GP = \frac{A}{1 + \left(\frac{C}{t}\right)^{B}}$$

in which GP is the total gas produced (ml/g OM), A is the asymptotic gas production (ml/g OM), B equals the switching characteristic of the curve, and C is the time at which half of the asymptote has been reached and t is the time (h). The maximum rate of gas production (R_{max}, ml/g OM/h) and the time when R_{max} appears (TR_{max}, h) were separately calculated using the equations below:

$$R_{max} = \frac{A \times C^{B} \times B \times TR_{max}^{(-B-1)}}{\left(1 + \left(C^{B} \times TR_{max}^{(-B)}\right)\right)^{2}}$$

$$TR_{max} = C \times \left(\frac{B-1}{B+1}\right)^{\frac{1}{B}}$$

Statistical analysis

All fermentation end-products and gas kinetics data were analyzed using PROC MIXED of SAS 9.3 (SAS Institute Inc., Cary, NC, United States). The statistical model was

$$Y_{ij} = \mu + D_i + B_j + e_{ij}$$

where Y_{ij} is the dependent variable, μ is the overall mean, D_i is the fixed effect of diet ($i = 1–3$), B_j is the random effect of run ($j = 1–3$), e_{ij} is the random residual error. The Student–Newman–Keuls (SNK) multiple comparison procedure in the LSMEANS statement was used to test differences among treatments. Significance was considered at $p \leq 0.05$, and a trend was declared at $0.05 < p \leq 0.10$.

Results

Effect of treatments on gas production

The cumulative gas productions at 6, 12, 24, and 48 h of *in vitro* incubation showed the same direction of effects (Table 2), where the diet S treatment had the highest gas production compared to the other two treatments, while the diet L treatment gave the lowest gas production ($p < 0.001$). The CH_4 production for diet S was higher than that for diet L ($p = 0.043$), but both diets did not differ from the diet C. The *in vitro* DMD for diets C and S was greater ($p < 0.001$) than that for diet L.

The cumulative gas production curve derived from the monophasic model is shown in Supplementary Figure S2. As for the curve fit parameter estimates (Table 2), the S treatment had the highest asymptotic gas production (A) compared to the other treatments ($p < 0.001$), the switching characteristic (B) of diet L treatment was lower, while diet S treatment had lower halftime (C), compared to the other two diets ($p < 0.001$). The S treatment had the highest maximum gas production rate (R_{max}) followed by treatment L and treatment C ($p < 0.001$).

Effect of treatments on fermentation end-products

The values of fermentation end-products and pH at 48 h are shown in Table 3. Compared with the L treatment, both C and S

TABLE 2 Comparison of cumulative gas production at 6, 12, 24, and 48h, curve fit parameters, head space methane concentration, and dry matter digestibility at 48h among two glucogenic (C, S) and a lipogenic (L) diet under *in vitro* fermentation with rumen fluid of dairy cows.

Item	Experimental diet			SEM	*p* value
	C	L	S		
Gas production (ml/g OM)					
6 h	94.37^{b}	73.06^{c}	106.08^{a}	3.783	<0.001
12 h	118.14^{b}	100.08^{c}	132.45^{a}	3.671	<0.001
24 h	125.67^{b}	108.68^{c}	139.29^{a}	3.561	<0.001
48 h	128.24^{b}	110.95^{c}	141.40^{a}	3.464	<0.001
Curve fit parameters					
A (ml/g OM)	139.77^{b}	124.60^{c}	155.84^{a}	3.438	<0.001
B	1.41^{a}	1.18^{c}	1.36^{b}	0.04	0.001
C (h)	3.91^{b}	4.22^{a}	3.57^{c}	0.129	<0.001
Rmax (ml/h/g OM)	23.48^{c}	24.49^{b}	27.83^{a}	0.936	<0.001
TRmax (h)	1.03^{a}	0.51^{c}	0.88^{b}	0.1	<0.001
Methane (%, 48 h)	11.64^{ab}	9.23^{b}	13.45^{a}	0.844	0.043
DMD (%, 48 h)	87.72^{a}	75.82^{b}	87.64^{a}	0.979	<0.001

A, asymptotic gas production; B, switching characteristic of the curve; C, time at which half of the asymptote has been reached; Rmax, maximum rate of gas production; TRmax, time at which Rmax occurs. DMD, dry matter digestibility. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; and S, steam-flaked corn and corn silage diet. OM, organic matter. SEM, standard error of the mean. ^{a,b,c}means within a row with different superscripts differ significantly ($p \leq 0.05$).

TABLE 3 Effect of two glucogenic (C, S) and a lipogenic (L) diet on the ruminal pH and end-products after 48h *in vitro* fermentation with rumen fluid.

Item	Experimental diet			SEM	*p* value
	C	L	S		
pH	6.61[b]	6.74[a]	6.62[b]	0.011	<0.001
NH_3-N (mg/dl)	70.14[a]	52.98[b]	65.70[a]	1.817	0.001
Volatile fatty acids (mmol/l)					
Acetate	68.9	71.87	71.29	1.129	0.65
Propionate	27.58[a]	24.42[b]	29.34[a]	0.582	0.004
Acetate:Propionate	2.50[b]	2.94[a]	2.43[b]	0.037	<0.001
Butyrate	11.86[a]	10.42[b]	12.31[a]	0.258	0.015
Valerate	0.68	0.6	0.71	0.025	0.356
Isobutyrate	5.66	5.31	5.81	0.111	0.306
Isovalerate	7.34	6.66	7.39	0.165	0.208
Total VFA	124.5	122.2	129.3	2.1	0.492
Lactic acid (mmol/l)	0.51[a]	0.40[b]	0.50[a]	0.016	0.011

Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. SEM, standard error of the mean. Total VFA, total volatile fatty acid (acetate + propionate + butyrate + valerate + isobutyrate + isovalerate). [a,b] means within a row with different superscripts differ significantly ($p \leq 0.05$).

TABLE 4 Effect of two glucogenic (C, S) and a lipogenic (L) diet on the alpha diversity indices of ruminal bacteria and archaea communities after 48h *in vitro* fermentation with rumen fluid of dairy cows.

Item	Experimental diet			SEM	*p* value
	C	L	S		
Bacteria					
OTU	1,403[b]	1,493[a]	1,408[b]	9.377	0.031
Chao 1	1,652	1,717	1,655	6.734	0.241
ACE	1,652	1,717	1,655	6.734	0.136
Shannon	5.58	5.78	5.56	0.026	0.339
Simpson	0.021	0.012	0.025	0.001	0.295
Archaea					
OTU	152[c]	182[a]	173[b]	4.114	0.028
Chao 1	306	352	364	8.095	0.056
ACE	518	533	590	10.201	0.427
Shannon	1.10[b]	1.57[a]	1.17[b]	0.068	0.018
Simpson	0.584[a]	0.357[b]	0.539[a]	0.032	0.024

Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. ACE, abundance-based coverage estimator. OTU, operational taxonomic units. SEM, standard error of the mean. [a,b,c] means within a row with different superscripts differ significantly ($p \leq 0.05$).

treatments resulted in higher lactic acid concentration ($p = 0.011$) but lower pH value ($p < 0.001$). The fermentation end-products for diet L possessed a significantly lower NH_3-N concentration ($p = 0.001$) and a lower lactic acid level ($p = 0.011$). Both C and S diets leaded to greater propionate ($p = 0.004$) and butyrate ($p = 0.015$) concentrations and lower acetate to propionate ratio ($p < 0.001$) in the fermentation end-products compared to the L diet.

Effect of treatments on ruminal bacteria and archaea

The alpha diversity parameters of both the ruminal bacteria and archaea as influenced by the three dietary treatments are shown in Table 4. A total of 1,070,928 quality sequence reads across all samples were acquired with an average read length of 421 bp. The total number of reads from each sample varied from 28,949 to 70,861, with an average reads number of 38,919. The entire sequences were assigned to 2,042 OTUs using a cut-off of 97% sequence similarity. The richness and diversity estimators (Table 4) showed the total number of observed OTUs out of the rumen fluid in the L treatment was higher than in the other treatments ($p = 0.031$). No differences in other diversity estimators (Chao 1, ACE, Shannon, and Simpson indices) were observed among the three groups. The alpha diversity estimates of archaea (Table 4) showed that the total number of observed OTUs for the C diet was lower compared to the S and L diets ($p = 0.028$). Both the C and S diets leaded to a significantly lower Shannon diversity index and a higher Simpson diversity index for archaea in comparison with the L diet ($p = 0.024$).

To visualize the impact of the diets on overall rumen bacteria and archaea communities, a PCoA was performed (Figures 1A,B). The rumen bacterial community showed a clear separation between the S and L dietary treatment along PC1, explaining >39% of the total variation, and the L diet was separated from the C diet along PC2, explaining >20% of the total variation (Figure 1A). The ruminal archaea communities in the rumen fluid samples from the L diet were clearly distinguished from those in the C and S diets, with approximately 76% of the variance explained along PC1 (Figure 1B).

A total of 21 bacterial phyla were identified from all samples among dietary treatments, with Bacteroidetes, Firmicutes, and Proteobacteria being the top three predominant phyla, representing 45.0–49.4, 36.1–41.6, and 3.9–6.5% of all sequences, respectively (Supplementary Table S1). The rumen fluid samples from L treatment showed a higher relative abundance of Tenericutes than samples from the other two treatments ($p = 0.042$).

At the genus level, a total of 176 bacterial genera were identified which together accounted for 96% of all sequences. 89 of these identified genera with a relative abundance of ≥0.1% in at least one sample were further analyzed. Among all genera, 26 genera were affected by dietary treatments (Supplementary Table S2), with the top 20 of these genera listed in Table 5. 12 of these affected genera had higher relative abundances in the rumen fluid for L diet compared to the other two diets, including *SP3-e08* ($p = 0.011$), *Christensenellaceae_R-7_group* ($p = 0.029$), *Ruminococcaceae_UCG-014* ($p = 0.026$), *Family_XIII-AD3011_group* ($p = 0.004$), unclassified_*o_Clostridiales* ($p = 0.010$), *Selenomonas_1* ($p = 0.005$), *Lachnospiraceae_ND3007_group* ($p = 0.025$), *[Eubacterium]_coprostanoligenes_group* ($p < 0.001$), unclassified_*f_Lachnospiraceae* ($p = 0.014$), unclassified_*f_Ruminococcaceae* ($p = 0.001$), *Ruminococcaceae_UCG_013* ($p = 0.006$), *Ruminococcus_1* ($p = 0.022$),

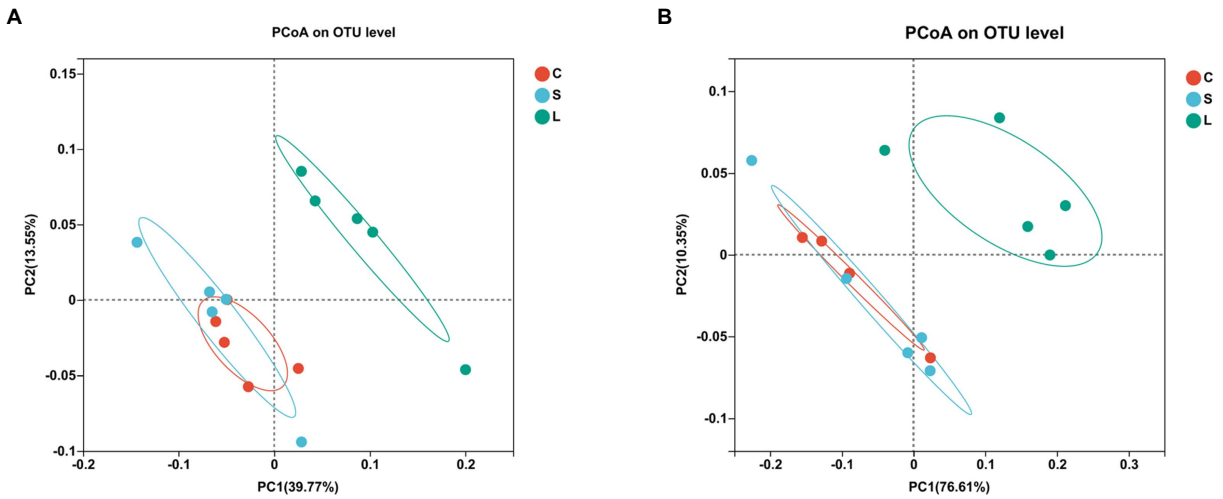

FIGURE 1
Principal coordinate analysis (PCoA) of the ruminal bacteria **(A)** and archaea **(B)** communities on OTU level among two glucogenic (C, S) and a lipogenic (L) diet after 48h *in vitro* fermentation with rumen fluid of dairy cows. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; and S, steam-flaked corn and corn silage diet.

TABLE 5 Effect of two glucogenic (C, S) and a lipogenic (L) diet on the relative abundance (%) of the top 20 affected bacteria and the top three differential archaea at the genus level after 48h *in vitro* fermentation with rumen fluid of dairy cows.

Domain/ Phylum	Genus/others	Experimental diet			SEM	*p* value
		C	L	S		
Bacteria						
Bacteroidetes	*SP3-e08*	0.11^{b}	0.17^{a}	0.10^{b}	0.009	0.011
Firmicutes	*Christensenellaceae_R-7_group*	1.28^{b}	1.70^{a}	1.06^{b}	0.077	0.029
	Ruminococcus_2	0.87^{a}	0.55^{b}	1.0^{a}	0.055	0.018
	Ruminococcus_1	0.18^{b}	0.31^{a}	0.18^{b}	0.068	0.022
	Ruminococcaceae_UCG-014	0.74^{b}	1.05^{a}	0.70^{b}	0.045	0.026
	Ruminococcaceae_UCG-013	0.18^{b}	0.35^{a}	0.15^{b}	0.026	0.006
	Unclassified_f_Ruminococcaceae	0.19^{b}	0.35^{a}	0.20^{b}	0.021	0.001
	Butyrivibrio_2	0.24^{b}	0.62^{a}	0.29^{b}	0.076	0.037
	Selenomonas_1	0.38^{b}	0.26^{b}	0.88^{a}	0.077	0.005
	Family_XIII_AD3011_group	0.72^{b}	1.25^{a}	0.50^{c}	0.091	0.004
	Family_XIII_UCG-002	0.12^{a}	0.13^{a}	0.08^{b}	0.007	0.026
	Unclassified_o_Clostridiales	0.70^{b}	1.07^{a}	0.60^{b}	0.058	0.01
	[Eubacterium]_coprostanoligenes_group	0.63^{b}	0.91^{a}	0.50^{b}	0.05	<0.001
	[Eubacterium]_nodatum_group	0.19^{ab}	0.24^{a}	0.14^{b}	0.011	0.021
	[Eubacterium]_oxidoreducens_group	0.11^{b}	0.19^{a}	0.11^{b}	0.011	0.044
	Lachnospiraceae_ND3007_group	0.56^{b}	1.12^{a}	0.68^{b}	0.07	0.025
	Unclassified_f_Lachnospiraceae	0.36^{b}	0.78^{a}	0.36^{b}	0.057	0.014
Proteobacteria	*Ruminobacter*	1.90^{a}	0.16^{b}	1.14^{a}	0.206	<0.001
Saccharibacteria	*Candidatus_Saccharimonas*	0.75^{b}	1.16^{a}	1.01^{ab}	0.048	0.037
Archaea						
Euryarchaeota	*Methanobrevibacter*	78.3^{a}	57.6^{b}	74.0^{a}	3.382	0.014
	Candidatus_Methanomethylophilus	3.68^{b}	11.42^{a}	4.40^{b}	1.067	0.001
	Halostagnicola	0.04	0.02	0.04	0.003	0.076

Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. SEM, standard error of the mean. SEM, standard error of the mean. ^{a,b,c} means within a row with different superscripts differ significantly ($p \leq 0.05$).

Butyrivibrio_2 ($p = 0.037$), *[Eubacterium]_oxidoreducens_group* ($p = 0.044$), and *Family_XIII_UCG-002* ($p = 0.026$). However, the relative abundances of *Ruminococcus_2* ($p = 0.018$), *Ruminobacter* ($p < 0.001$), and *Succinivibrionaceae_UCG-002* ($p = 0.004$) were lower for the L dietary treatment. The S dietary treatment resulted in a greater relative abundance of *Selenomonas_1* ($p = 0.005$), while lower relative abundances of *Family_XIII_UCG-002* ($p = 0.026$) and *Family_XIII_ AD3011_group* ($p = 0.004$) compared to the other two diets. Compared with the S dietary treatment, the rumen fluid for the C treatment had a higher relative abundance of the *Family_XIII_ AD3011_group* ($p = 0.004$), *Family_XIII_UCG-002* ($p = 0.026$), and *Succinivibrionaceae_UCG-002* ($p = 0.004$).

In terms of the archaea community, the Euryarchaeota was the most predominant phyla. At the genus level, five archaeal genera were identified from all samples (Supplementary Table S3), and the affected ones with $p \leq 0.1$ by treatments are shown in Table 5. Compared to the L diet, both C and S diets leaded to a significantly higher relative abundance of *Methanobrevibacter* ($p = 0.014$) but a lower relative abundance of *Candidatus_Methanomethylophilus* ($p = 0.001$) and tended to lead to a higher relative abundance of *Halostagnicola* ($p = 0.076$).

Predicted metagenomic functions of the ruminal bacteria

The functional prediction was conducted with PICRUSt in order to further understand the functioning of ruminal bacteria. Forty functional pathways (level 2) were predicted out of all samples (Supplementary Table S4), with amino acid metabolism, carbohydrate metabolism, and membrane transport being the top three functions. The PCoA analysis showed that samples from the L treatment clustered differently from those in the C and S diets (Figure 2A). The differences of the top 15 most abundant functions among treatments are presented in Figure 2. Compared to the C diet, the S dietary treatment resulted in a higher ($p = 0.044$) relative abundance in energy metabolism (Figure 2B), while the L diet leaded to a higher ($p = 0.045$) relative abundance of membrane transport functions but lower relative abundances in amino acid metabolism ($p = 0.027$), replication and repair ($p = 0.01$), translation ($p = 0.015$), metabolisms of cofactors and vitamins ($p = 0.025$), nucleotide metabolism ($p = 0.034$), and cellular processes and signaling ($p = 0.003$; Figure 2C). Compared to diet L, the S diet leaded to higher relative abundances in amino acid metabolism ($p = 0.022$), translation ($p = 0.018$), replication and repair ($p = 0.01$), and cellular processes and signaling ($p = 0.003$; Figure 2D).

Rumen metabolomics profiling

The total ion chromatogram of the QC samples in the positive and negative ion modes indicates the highly reliable repeatability and precision of the data obtained in this analysis (Supplementary Figure S3). Metabolomic data were first examined by PCA in both positive and negative ion mode to obtain an overview of the differences among treatments (Figures 3A,B). The results showed that the metabolites in the rumen fluid fermented with diet L could be separated from those with the other diets. The OPLS-DA score plots indicated a clear separation and discrimination of metabolites between treatments under both positive and negative ion modes (Supplementary Figures S4A,C,E, S5A,C,E). Next, the response permutation test was to assess the OPLS-DA model in distinguishing the metabolite data between two treatments, in which the cumulative values of R^2Y in the positive (0.9880, 0.8027, and 0.8598 for C vs. S, C vs. L, and L vs. S, respectively, Supplementary Figures S4B,D,F) and negative (0.9856, 0.8697, and 0.8361 for C vs. S, C vs. L, and L vs. S, respectively, Supplementary Figures S5B,D,F) ion models were all above 0.80 indicating the stability and reliability of the model.

A total of 801 metabolites (460 in positive ion mode and 341 in negative ion mode) were identified from all rumen fluid samples fermented with three diets, containing 50.3% of the lipids and lipid-like molecules, 13.9% of the organoheterocyclic compounds, 10.9% of the organic acids and derivatives, 9.7% of organic oxygen compounds, 6.4% of both the benzenoids and the phenylpropanoids, and polyketides in the superclass level of the HMDB classification (Supplementary Figure S6).

Supplementary Table S5 shows that based on VIP > 1 and $p \leq 0.05$, a total of 272 significantly affected metabolites (168 positively and 104 negatively ionized metabolites) were obtained from the comparison of L vs. C. Among these metabolites, 20 were classified as the fatty acids and conjugates, 20 as the amino acids, peptides, and analogues, 19 as the triterpenoids, and 14 as the carbohydrates and carbohydrate conjugates. From the comparison of L vs. S, 260 significantly affected metabolites (157 positively and 103 negatively ionized metabolites) were identified (Supplementary Table S6), among which 20 metabolites were classified as the fatty acids and conjugates, 20 as the amino acids, peptides, and analogues, 18 as the triterpenoids, and 14 as the carbohydrates and carbohydrate conjugates. As for the comparison of C vs. S (Supplementary Table S7), 89 significantly affected metabolites (63 positively and 26 negatively ionized metabolites) were detected, of which 12 metabolites were classified as the amino acids, peptides, and analogues, six as the fatty acids and conjugates, and 6 as the carbohydrates and carbohydrate conjugates. The significantly enriched metabolic pathways containing these affected metabolites are shown in Figure 4.

Correlation between bacteria and the fermentation parameters

The Pearson correlation analysis was conducted to assess the correlation between the affected bacteria and the rumen fermentation parameters. As shown in Figure 5, the DMD was negatively correlated with the *Christensenellaceae_R-7_group*, *[Eubacterium]_coprostanoligenes_group*, *Ruminococcaceae_ UCG-014*, *Family_XIII-AD3011_group* and *Lachnospiraceae_ ND3007_group* but positively correlated with the *Selenomonas_1*, *Ruminobacter*, *Succinivibrionaceae_UCG-002*, and

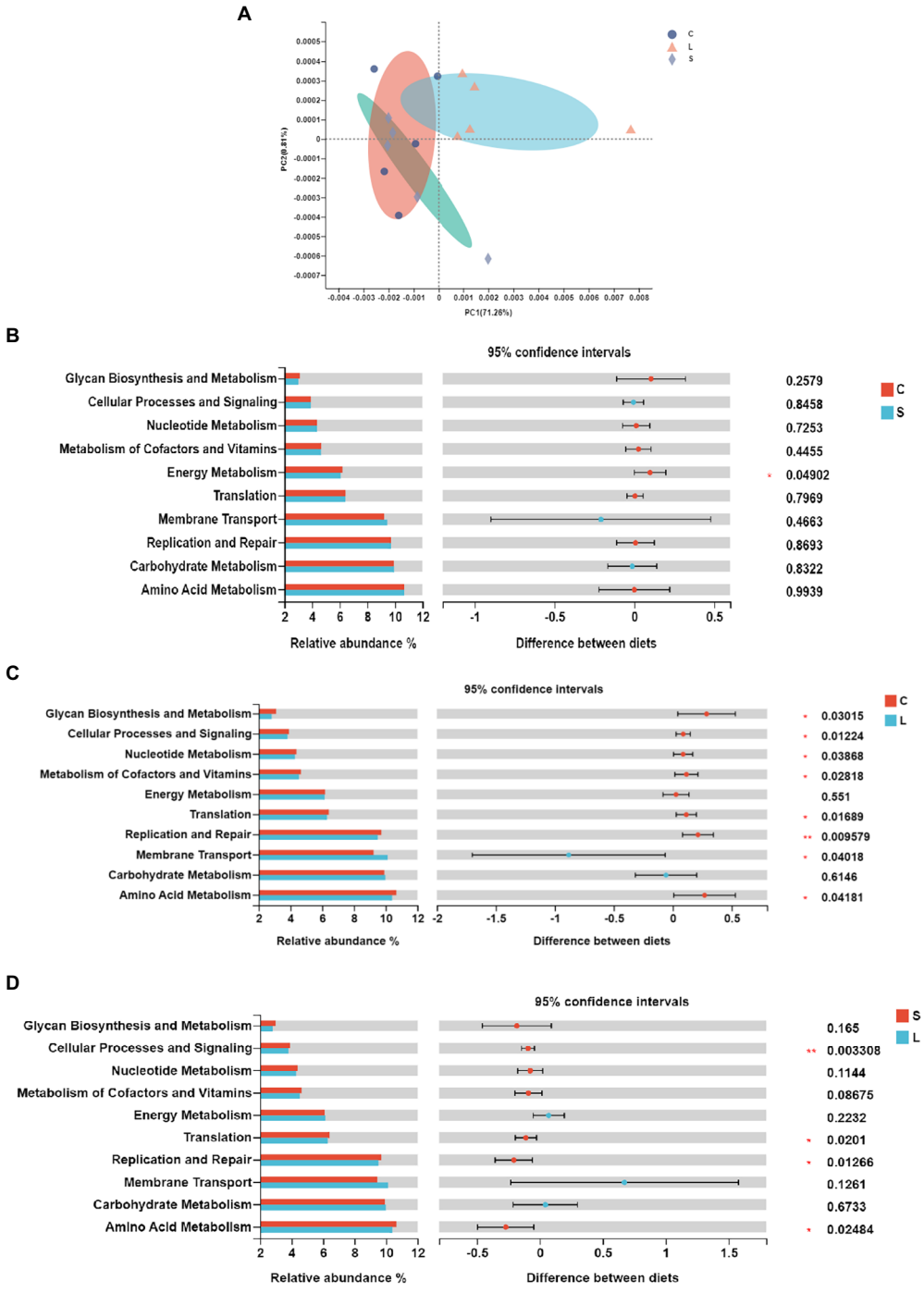

FIGURE 2
Principal coordinate analysis **(A)** and pairwise comparison **(B–D)** of the KEGG pathways of the bacteria in the rumen fluid of dairy cows after 48h *in vitro* fermentation with two glucogenic (C, S) and a lipogenic (L) diet. **(B)** C vs. S; **(C)** C vs. L; and **(D)** S vs. L. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; and S, steam-flaked corn and corn silage diet.

Ruminococcus_2. The NH_3-N concentration was positively correlated with the *Succinivibrionaceae_UCG-002* and *Ruminococcus_2*. The acetate concentration was negatively correlated with the *Succinivibrionaceae_UCG-002* and *Ruminobacter*. In addition, the propionate concentration was positively correlated with the *Selenomonas_1*.

Correlation between affected bacteria and the affected metabolites

As is shown in Figure 6, differently abundant bacterial genera were closely correlated with the affected metabolites in the

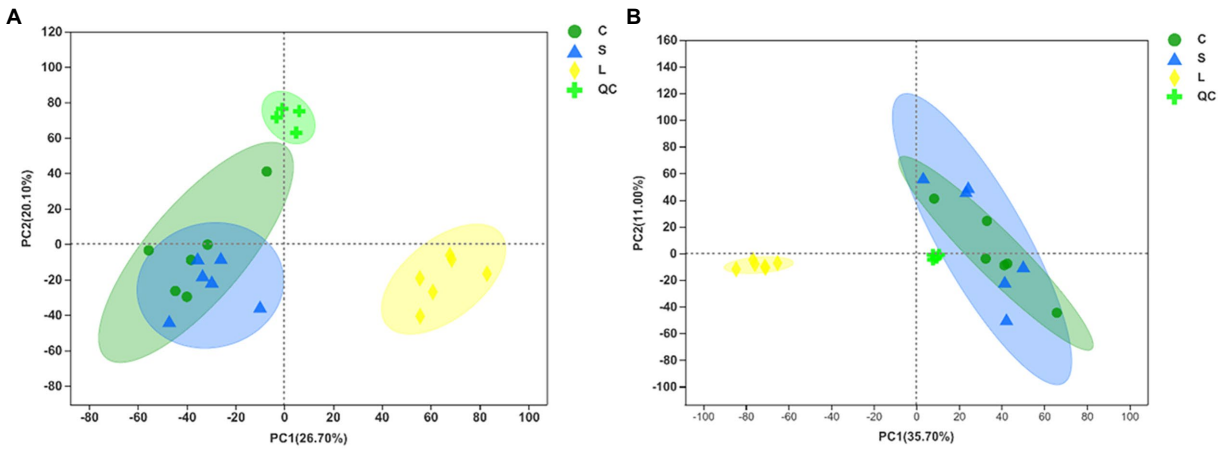

FIGURE 3
Principal component analysis (PCA) of metabolites following positive **(A)** and negative **(B)** mode ionization based on metabolomics analysis in the ruminal fluid samples of dairy cows after 48h *in vitro* fermentation with two glucogenic and a lipogenic diet. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. QC, quality control samples.

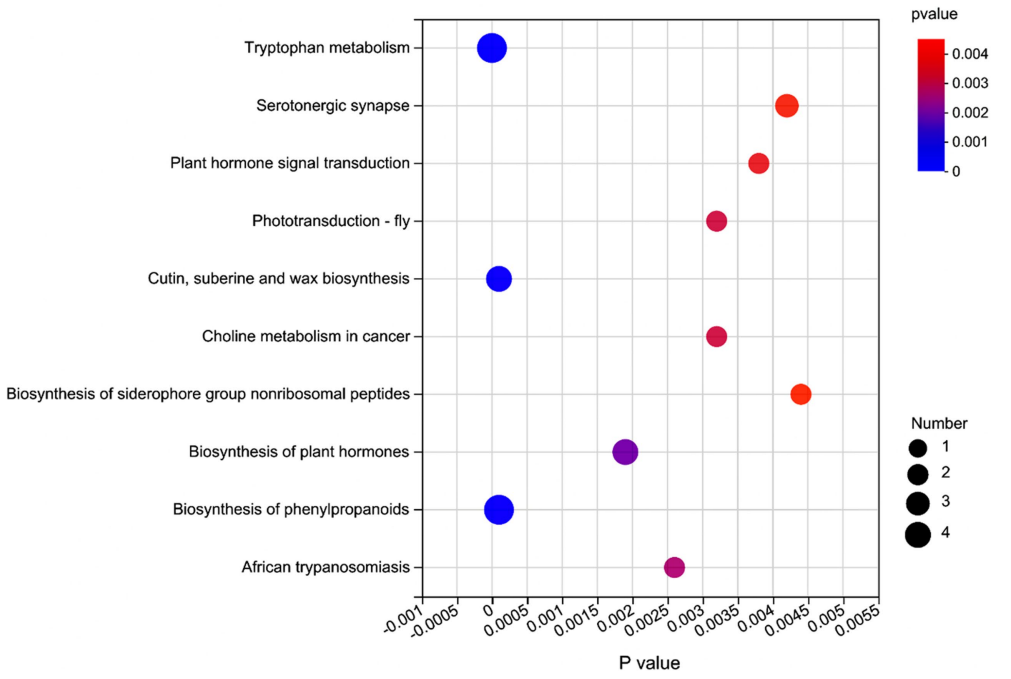

FIGURE 4
Metabolic pathway enrichment analysis of significant differential metabolites in the rumen fluid of dairy cows after 48h *in vitro* fermentation with two glucogenic and a lipogenic diet. The color is to distinguish the enrichment significance (*p* value), the darker the color, the more significantly the metabolic pathway is enriched. The *y* axis indicates the name of the KEGG metabolic pathway (top 10). The *x* axis indicates the *p* value. A larger size dot indicates a higher pathway enrichment.

fermentation fluid. Specifically, the *Family_XIII-AD3011_group*, *[Eubacterium]_coprostanoligenes_group*, and *Christensenellaceae_R-7_group* and *Ruminococcaceae_UCG-010* were positively correlated to the dihydrocumambrin A, norpropoxyphene, D-rrobilin, stearoyllactic acid, 15(R)-15-methyl prostaglandin A2, ganoderic acid A, calenduloside E, and 2-octenedioic acid, but negatively correlated with the noreleagnine, captopril-cysteine disulfide, 2-hepteneoylglycine, 3-propyl-1,2-cyclopentanedione, phenyl vinyl sulfide, N6-acetyl-5S-hydroxy-L-lysine, and indoleacetic acid. Similarly, the *Lachnospiraceae_ND3007_group* was positively correlated to the dihydrocumambrin A, norpropoxyphene, stearoyllactic acid,

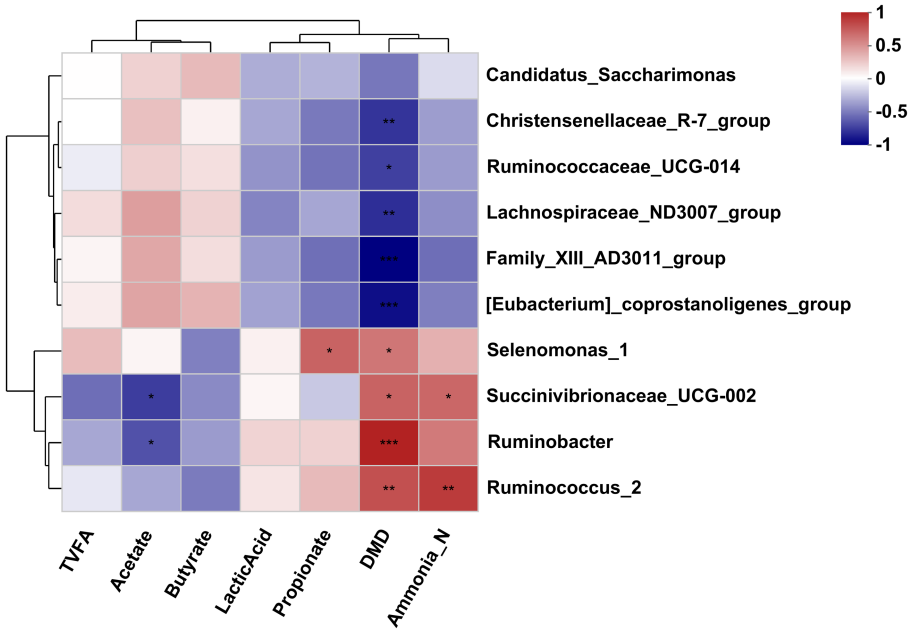

FIGURE 5
Correlation analysis between differential bacteria genus and differential fermentation parameters in the rumen fluid of dairy cows after 48h *in vitro* fermentation with two glucogenic and a lipogenic diet. Each row represents a bacteria genus, only the genera with a relative abundance >0.5% are selected; each column represents a fermentation parameter. The color blue means negative correlation, and the color red means positive correlation. *0.01<$p \leq 0.05$, **0.001<$p \leq 0.01$, ***$p \leq 0.001$.

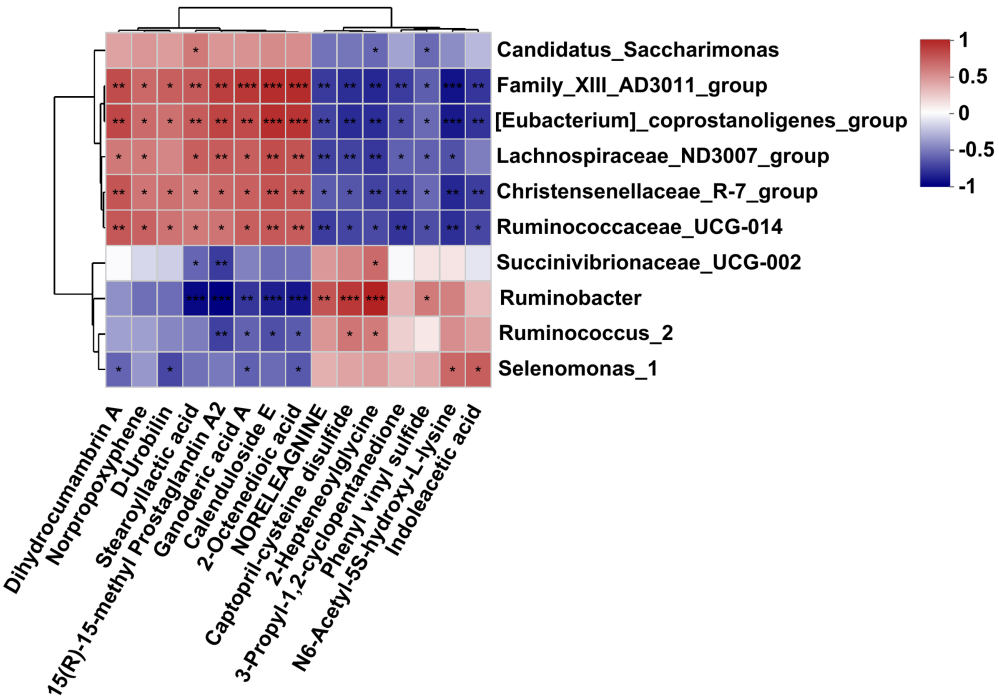

FIGURE 6
Correlation analysis of differential bacteria genus and differential metabolites in the rumen fluid of dairy cows after 48h *in vitro* fermentation with two glucogenic and a lipogenic diet. Each row represents a bacteria genus, only the genera with relative abundance >0.5% are selected; each column represents a metabolite, only the affected metabolites with VIP>1.5, FC<0.5 and>2 are considered. The color blue means negative correlation, the color red means positive correlation. *0.01<$p \leq 0.05$, **0.001<$p \leq 0.01$, ***$p \leq 0.001$.

15(R)-15-methyl prostaglandin A2, ganoderic acid A, calenduloside E, and 2-octenedioic acid, but negatively correlated with the noreleagnine, captopril-cysteine disulfide, 2-hepteneoylglycine, 3-propyl-1,2-cyclopentanedione, phenyl vinyl sulfide, and N6-acetyl-5S-hydroxy-L-lysine. In addition, *Ruminobacter* was negatively correlated with stearoyllactic acid, 15(R)-15-methyl prostaglandin A2, ganoderic acid A, calenduloside E, and 2-octenedioic acid, but positively correlated with noreleagnine, Captopril-cysteine disulfide, 2-hepteneoylglycine, and phenyl vinyl sulfide. The *Succinivibrionaceae_UCG-002* was negatively correlated with the stearoyllactic acid and 15(R)-15-methyl prostaglandin A2, but positively correlated with 2-hepteneoylglycine. *Ruminococcus_2* was negatively correlated with the 15(R)-15-methyl prostaglandin A2, ganoderic acid A, calenduloside E, and 2-octenedioic acid, but positively correlated with the captopril-cysteine disulfide and 2-hepteneoylglycine. The *Selenomonas_1* was negatively correlated with the dihydrocumambrin A, D-urobilin, ganoderic acid A, and 2-octenedioic acid, but positively correlated with the N6-acetyl-5S-hydroxy-L-lysine and indoleacetic acid. Moreover, *Candidatus_saccharimonas* was positively correlated with the stearoyllactic acid but negatively correlated with the 2-hepteneoylglycine and phenyl vinyl sulfide.

Discussion

The present research reports the influence of two glucogenic diets and a lipogenic diet on ruminal fermentation end-products using an *in vitro* incubation system and provides the unknown information on metabolites formed and the bacterial communities in response to the glucogenic and lipogenic diets.

Influence on feed digestion and gas production

The L diet had a lower DMD than the other two diets, which is consistent with previous studies (Ruppert et al., 2003). The ruminal bacteria can be assigned to different functional groups, such as cellulolytic, amylolytic, and proteolytic, based on their preferential use of energy. Starch digestion in the rumen is affected by dietary starch source, grain processing, and adhering capacity of ruminal microbiota to the diet particles (Huntington, 1997). The main amylolytic bacteria included *Streptococcus bovis*, *Bacteroides amylophilus*, *Prevotella* spp., *Succinimonas amylolytica*, *Selenomonas ruminantium*, and *Butyrivibrio* spp. (Xia et al., 2015). For the present study, the relative abundance of amylolytic bacteria genera, including *Selenomonas_1*, *Ruminobacter*, and *Succinivibrionaceae_UCG-002*, were higher in diet C and S compared to diet L and also were significantly positively correlated with DMD. These increased genera may likely have contributed to the higher DMD in diets C and S.

The fiber degradation in the rumen is mainly attributed to the ruminal cellulolytic bacteria (Jeyanathan et al., 2014). *Fibrobacter succinogenes* (belong to the genus *Fibrobacter*), *Ruminococcus flavefaciens*, and *Ruminococcus albus* (belong to the genus *Ruminococcus*) were considered the dominant cellulolytic bacterial species due to their high capacity for cellulose digesting (Krause et al., 2003). In addition, the genera of *Butyrivibrio* and *Eubacterium* were also reported to be cellulolytic (Thoetkiattikul et al., 2013). Moreover, some unclassified taxa, including those assigned to *Ruminococcaceae*, *Lachnospiraceae*, *Christensenellaceae*, *Rikenellaceae*, *Prevotellaceae*, *Clostridium*, and *Bacteroidales* were proven to have the capacity of adhering tightly to forages in the rumen, which indicates that these new taxa might play a significant role in forage digestion (Liu et al., 2016). In this study, there were significantly higher relative abundances of the cellulolytic bacterial genera in the rumen fluid fermented with diet L, including *Ruminococcus*, *Eubacterium*, *Butyrivibrio_2*, and *Lachnospira*, and the potential cellulolytic taxa, including unclassified_*Lachnospiraceae*, unclassified_*Ruminococcaceae*, and unclassified_*o_Clostridiales*. A previous study indicated that the digestibility of cellulose was lower when high corn silage was fed to dairy cows compared to high alfalfa silage (Ruppert et al., 2003). This could explain the higher cellulolytic bacteria in diet L. These bacteria were proved to be more sensitive to lipogenic nutrients in diet.

In the present study, the gas production of the ruminant feeds was highly correlated with their digestibility and available energetic contents, the higher DMD of two glucogenic diets was the reason for their higher gas production than diet L, which agree with the early work of Menke and Steingass (1988). The steam-flaked corn, compared to ground corn, increased the gas production of the total mixed rations (TMR) incubated with buffered rumen liquor *in vitro* and increased the gas production rate, which agrees with the data of Qiao et al. (2015). The processing conditions (increased moisture content, pressure, and temperature) involved in producing steam-flaking corn have been shown to improve the enzymatic hydrolysis of starch *in vitro*, thereby improving the digestibility of corn (de Peters et al., 2003). Starch digestibility was shown to be positively related to the percentage of starch that was gelatinized *in vitro* (Huntington, 1997). The gelatinization of the starch in the steam-flaking corn was highly likely the reason for their higher gas production.

Methanogenesis is an ancient metabolism of the methanogens belonging to the phylum *Euryarcheota*, domain archaea (Hook et al., 2010). All methanogens belong to seven euryarchaeal orders, including *Methanococcales*, *Methanopyrales*, *Methanobacteriales*, *Methanosarcinales*, *Methanomicrobiales*, *Methanocellales*, and *Thermoplasmatales* (Hook et al., 2010). Three classical CH_4-producing pathways were reported previously, including the hydrogenotrophic methanogenesis mainly using CO_2/H_2 or formate as substrate, acetoclastic methanogenesis with acetate as substrate and methylotrophic methanogenesis with methylated C1 compounds as substrate (Hedderich and Whitman, 2006). Methanogens are known to grow better syntrophically *in*

vitro (Sakai et al., 2009). For ruminants, *Methanobrevibacter* was recognized as the dominant genus producing CH_4 (Leahy et al., 2013), mainly through the CO_2/H_2 pathway using CO_2 or formate as the elector acceptor and H_2 as the electron donor (Liu and Whitman, 2008). Our results are in line with the aforementioned observations and illustrate that the relative abundance of the dominant genus *Methanobrevibacter* followed the same trend as gas production and CH_4 proportion. The higher gas production of the S relative to the L diet might supply more substrates (CO_2/H_2) for the methanogenesis of *Methanobrevibacter*, which may lead to higher CH_4 production. The genus of *Candidatus_Methanomethylophilus* is also known as a CH_4-producing methanogen, which mainly depends on methanol as substrate *via* the methylotrophic methanogenesis pathway (Lino et al., 2013). In the present study, the L diet increased the genus of *Candidatus_Methanomethylophilus* significantly. Since this genus was newly defined, its methanogenesis pathway and its relationship with dietary ingredients deserve further research.

Influence on lactic acid and NH_3-N

High starch concentration would decrease rumen pH (Nocek et al., 2002). The present study found that lactic acid concentration was negatively related to the pH value. The low pH value of the C and S diet is likely mainly attributed to their increase in lactic acid production. In addition, no difference between the S and C diets existed in the pH value, which is in line with the previous study (Cooper et al., 2002).

The NH_3-N concentration in the *in vitro* rumen fluid cultures is determined by the balance of the formation and utilization rate of NH_3-N by microorganisms (Shen et al., 2017). Due to the same level of crude protein among all treatments, the supplemented amount of nitrogen available for the microbiota can be considered equal. The efficiency of ruminal NH_3-N utilization is determined by the capacities of microbes to metabolize NH_3-N. Cellulolytic bacteria which degrade structural carbohydrates (e.g., NDF) grow slowly and mainly use NH_3-N as an N source, whereas amylolytic bacteria which degrade non-structural carbohydrates (e.g., starch) grow rapidly with higher requirements and use ammonia, peptides, and amino acid as N sources (Fox et al., 1992). In our study, the NH_3-N concentration in diet L was significantly lower than in the other two diets. The L diet showed higher relative abundances of cellulolytic bacteria, among which the genera *Succinivibrionaceae_UCG-002* and *Ruminococcus_2* was proved to positively correlate with the NH_3-N concentration. These bacteria might use NH_3-N as the main N source leading to a lower NH_3-N concentration.

Influence on VFA

Ruminal fermentation of carbohydrates leads to the formation of VFA. The primary ruminal VFAs are acetate, propionate, and butyrate, the molar proportions of which are mainly determined by the diet. Propionate and acetate are the main precursors of milk glucose and fatty acids, respectively. Cellulose ferments to acetate to a greater extent than propionate, whereas readily degradable starch is fermented less to acetate and more to propionate. Consistent with this, our data showed that both the C and S diets had a higher concentration of propionate and a lower acetate to propionate ratio compared to the L diet. The ruminal propionate is formed *via* two main pathways, the succinate pathway and the acrylate pathway (Jeyanathan et al., 2014). The former is known as the major pathway for propionate-production in the rumen and involves a large number of bacterial species, such as bacteria related to succinate production and utilization (*Fibrobacter succinogenes* and *Selenomonas ruminantium*), and lactate production and utilization (e.g., *Streptococcus Bovis* and *Selenomonas ruminantium*; Jeyanathan et al., 2014). The genus *Selenomonas_1* had a positive correlation with the propionate concentration, and the relative abundance of *Selenomonas_1* and *Succinivibrionaceae_UCG-002* in the L diet was lower than that in the other diets. The *Selenomonas_1* and *Succinivibrionaceae_UCG-002* may contribute to the higher propionate production in diets C and S by enhancing the succinate pathway according to current knowledge. These roles need to be confirmed by further research.

Influence on bacterial function

To further study the differences in the functional roles of rumen bacteria among dietary treatments, PICRUSt was used to estimate the potential functions of the bacteria. Compared with the C and S diets, the predicted pathway of amino acid metabolism had a lower level in the L dietary treatment (Figures 2C,D). The increased amino acid metabolism in diets S and C may lead to higher amino acid production with excessive amounts of amino acids contributing to the higher NH_3-N concentration *via* deamination (Petri et al., 2019). Also, the *Ruminobacter amylophilus* is known for its proteolytic activity (Amlan and Ye, 2014), which could explain that the diets C and S with a higher number of the genus *Ruminobacter* had enhanced function of amino acids metabolism. Moreover, the L diet reduced the relative abundance of the translation, replication and repair, as well as cellular processes and signaling, which is probably attributed to the rapid turnover rate of bacteria (Zhang et al., 2017a). As predicted by PICRUSt, the bacteria in the C diet had an enriched function for energy metabolism compared to the S diet, suggesting that the bacterial capacity of energy intake may be improved by the ground corn compared to the steam-flaked corn.

Influence on rumen metabolites

The metabolomics analysis provides direct evidence for changes in microbial activities among diets. The metabolomics

data showed that the dietary treatments altered most metabolites related to lipid and protein digestion. The enriched metabolic pathways that were predicted by PICRUSt, such as amino acid metabolism and cellular processes and signaling, were similar to the enriched metabolic pathways through the metabolome functions analysis, such as the tryptophan metabolism and sphingolipid signaling pathways.

Most affected metabolites in the lipids and lipid-like molecules were higher in the diet L compared to the other two diets, indicating that the L diet could promote lipid utilization to some degree. Most metabolites belonging to fatty acids and conjugates were also higher in the diet L. Previous *in vitro* bacterial culturing experiments have shown that fatty acids had a negative effect on bacterial growth (Henderson, 1973). The bacterial communities were modified differently by the fatty acid supplements, where cellulolytic strains of bacteria showed to be more sensitive to fatty acids than the amylolytic ones (Doreau and Ferlay, 1995). The present contribution also observed a strong correlation between the cellulolytic bacteria and metabolites associated with fatty acid. The initial step of lipid metabolism in the rumen is the hydrolysis of the ester linkages, which is predominantly controlled by rumen bacteria (Bauman et al., 2003). The strains of *Butyrivibrio fibrisolvens* have been reported to play an important role in the degradation of polyunsaturated fatty acids in the rumen (Latham et al., 1972), including hydrolysing phospholipids and glycolipids (Harfoot and Hazlewood, 1997). Besides, some strains of *Borrelia* (Yokoyama and Davis, 1971), a strain in each of *Ruminocccus* and *Eubacterium* (Kemp et al., 1975) and two strains of cellulolytic *Clostridium* spp. (Viviani et al., 1968) have also been reported to participate in biohydrogenation. The higher abundance of genera *Butyrivibrio_2*, *Ruminococcus_1*, *Ruminococcaceae_UCG-013*, *Ruminococcaceae_UCG-014*, and *Unclassified_o_Clostridiales* in diet L is in line with the higher level of the metabolites related to fatty acids and conjugates. Correlation analysis proved that the different fatty acid metabolites had significant relations with several cellulolytic bacteria, including the *Ruminococcaceae_UCG-014* and *[Eubacterium]_coprostanoligenes_group*. These cellulolytic bacteria might contribute to the higher fatty acid production in the L diet.

Most metabolites associated with the amino acids, peptides, and analogues were decreased in the L compared to the C and S diets, which was also in line with the PICRUSt result. The ruminal amino acids mainly arise from the dietary protein degradation and protein produced by microbiota. A large number of microbial species contribute to the ruminal proteolysis, with starch-degrading bacteria contributing more to the protein degradation in the rumen than the cellulolytic bacteria (Zhang et al., 2017a). This could explain the high level of amino acids, peptides, and analogues in the C and S diets. Besides, in the *de novo* synthesis of ruminal amino acids, acetate and propionate can be used as carbon sources and the compounds like ammonia as the nitrogen sources by the microbes (Zhang et al., 2017b). The high concentrations of propionate, butyrate and NH_3-N in diet C and S also agrees with their higher levels of amino acids.

In conclusion, the glucogenic diet had greater effects than the lipogenic diet in terms of improving the dry matter digestibility, increasing propionate concentration and promoting amino acid metabolism. The improvement in propionate production may be attributed to the increased number of bacterial spp. functioning in the succinate pathway. Compared to ground corn, steam-flaked corn did not show more differences in fermentation end-products except for increasing gas production and lower production of some fatty acids and amino acids. Several amylolytic and cellulolytic bacteria were observed to be sensitive to the dietary changes, while most highly abundant bacteria were stable or minorly affected. The affected bacteria showed to have high associations with certain metabolites. This study has offered a deeper understanding of ruminal microbial functions which could assist the improvement of rumen functions and thereby in the ruminant production. Moreover, these findings provide essential references for future *in vivo* studies.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number PRJNA661353.

Ethics statement

The animal study was reviewed and approved by the Animal Care and Use Committee of the Chinese Academy of Agricultural Sciences.

Author contributions

DH, WH, WP, and BX designed the research. DH, YZ, FX, and YW performed the research. WH, WP, and BX supervised the research. LJ supported the equipment for all the testing. DH analyzed the data and wrote the manuscript. WH and WP supervised the manuscript writing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1039217/full#supplementary-material

References

Amlan, K. P., and Yu, Z. (2014). Effects of vanillin, quillaja saponin, and essential oils on in vitro fermentation and protein-degrading microorganisms of the rumen. *Appl. Microbiol. Biotechnol.* 98, 897–905. doi: 10.1007/s00253-013-4930-x

Bauman, D. E., Perfield, J. W. II, Veth, De M. J., and Lock, A. L. (2003). New perspectives on lipid digestion and metabolism in ruminants in Cornell Nutrition Conference Proceedings Papers. 175–189.

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

Carberry, C. A., Kenny, D. A., Han, S., McCabe, M. S., and Waters, S. M. (2012). Effect of phenotypic residual feed intake and dietary forage content on the rumen microbial community of beef cattle. *Appl. Environ. Microbiol.* 78, 4949–4958. doi: 10.1128/AEM.07759-11

Cooper, R. J., Milton, C. T., Klopfenstein, T. J., Scott, T. L., Wilson, C. B., and Mass, R. A. (2002). Effect of corn processing on starch digestion and bacterial crude protein flow in finishing cattle. *J. Anim. Sci.* 80, 797–804. doi: 10.1051/gse:2002008

de Peters, E. J., Getachew, G., Fadel, J. G., Zinn, R. A., Taylor, S. J., Pareas, J. W., et al. (2003). *In vitro* gas production as a method to compare fermentation characteristics of steam-flaked corn. *Anim. Feed Sci. Technol.* 105, 109–122. doi: 10.1016/S0377-8401(03)00042-7

Ding, J., Zhang, L., Wan, R., Ren, L. P., and Meng, Q. X. (2007). Disintegration of starch crystal structure by steam flaking may be responsible for the improvement of *in vitro* ruminal fermentation of steam flaked sorghum grains. *J. Anim. Feed Sci.* 16, 448–453. doi: 10.1002/anie.201107669

Doreau, M., and Ferlay, A. (1995). Effect of dietary lipids on nitrogen metabolism in the rumen: a review. *Livest. Prod. Sci.* 43, 97–110. doi: 10.1016/0301-6226(95)00041-I

Fox, D. G., Sniffen, C. J., O'Connor, J. D., Russell, J. B., and van Soest, P. J. (1992). A net carbohydrate and protein system for evaluating cattle diets: III. Cattle requirements and diet adequacy. *J. Anim. Sci.* 70, 3578–3596. doi: 10.2527/1992.70113578x

Harfoot, C. G., and Hazlewood, G. P. (1997). "Lipid metabolism in the rumen," in *The Rumennology*. eds. P. N. Hobson and C. S. Stewart (Switzerland: Springer International publishing), 103–126.

Hedderich, R., and Whitman, W. (2006). "Physiology and biochemistry of the methane-producing archaea," in *Prokaryotes: A Handbook on the Biology of Bacteria*. eds. S. Falkow, E. Rosenberg, K. Schleifer, E. Stackebrandt and M. Dworkin (New York: Springer), 1050–1079.

Henderson, C. (1973). The effects of fatty acids on pure cultures of rumen bacteria. *J. Agric. Sci.* 81, 107–112. doi: 10.1017/S0021859600058378

Hook, S. E., Wright, A. D. G., and McBride, B. W. (2010). Methanogens: methane producers of the rumen and mitigation strategies. *Archaea* 2010, 1–11. doi: 10.1155/2010/945785

Hua, D., Zhao, Y., Nan, X., Xue, F., Wang, Y., Jiang, L., et al. (2021). Effect of different glucogenic to lipogenic nutrient ratios on rumen fermentation and bacterial community in vitro. *J. Appl. Microbiol.* 130, 1868–1882. doi: 10.1111/jam.14873

Huntington, G. B. (1997). Starch utilization by ruminants: from basics to the bunk. *J. Anim. Sci.* 75, 852–867. doi: 10.2527/1997.753852x

Kemp, P., White, R. W., and Lander, D. J. (1975). The hydrogenation of unsaturated fatty acids by five bacterial isolates from the sheep rumen, including a new species. *J. Gen. Microbiol.* 90, 100–114. doi: 10.1099/00221287-90-1-100

Jeyanathan, J., Martin, C., and Morgavi, D. P. (2014). The use of direct-fed microbials for mitigation of ruminant methane emissions: a review. *Animal* 8, 250–261. doi: 10.1017/S1751731113002085

Krause, D. O., Denman, S. E., Mackie, R. I., Morrison, M., Rae, A. L., Attwood, G. T., et al. (2003). Opportunities to improve fiber degradation in the rumen: microbiology, ecology, and genomics. *FEMS Microbiol. Rev.* 27, 663–693. doi: 10.1016/S0168-6445(03)00072-X

Latham, M. J., Storry, J. E., and Sharpe, M. E. (1972). Effect of low-roughage diets on the microflora and lipid metabolism in the rumen. *Appl. Microbiol.* 24, 871–877. doi: 10.1128/am.24.6.871-877.1972

Leahy, S. C., Kelly, W. J., Ronimus, R. S., Wedlock, N., Altermann, E., and Attwood, G. T. (2013). Genome sequencing of rumen bacteria and archaea and its application to methane mitigation strategies. *Animal* 7, 235–243. doi: 10.1017/S1751731113000700

Lino, T., Tamaki, H., Tamazawa, S., Ueno, Y., Ohkuma, M., Suzuki, K. I., et al. (2013). Candidatus methanogranum caenicola: a novel methanogen from the anaerobic digested sludge, and proposal of *Methanomassiliicoccaceae fam. Nov.* and *Methanomassiliicoccales Ord. Nov.*, for a methanogenic lineage of the class Thermoplasmata. *Microbes Environ.* 28, 244–250. doi: 10.1264/jsme2.ME12189

Liu, Y., and Whitman, W. B. (2008). Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. *Ann. N. Y. Acad. Sci.* 1125, 171–189. doi: 10.1196/annals.1419.019

Liu, J., Zhang, M., Xue, C., Zhu, W., and Mao, S. (2016). Characterization and comparison of the temporal dynamics of ruminal bacterial microbiota colonizing rice straw and alfalfa hay within ruminants. *J. Dairy Sci.* 99, 9668–9681. doi: 10.3168/jds.2016-11398

Mao, S. Y., Zhang, G., and Zhu, W. Y. (2008). Effect of disodium fumarate on ruminal metabolism and rumen bacterial communities as revealed by denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA. *Anim. Feed Sci. Technol.* 140, 293–306. doi: 10.1016/j.anifeedsci.2007.04.001

Menke, K. H., and Steingass, H. (1988). Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. *Anim. Res. Dev.* 28, 7–55.

National Research Council (NRC) (2001). *Nutrient Requirements of Dairy Cattle 7th Edn.* Washington, DC, USA: National Academy Press.

Nocek, J. E., Allman, J. G., and Kautz, W. P. (2002). Evaluation of an indwelling ruminal probe methodology and effect of grain level on diurnal pH variation in dairy cattle. *J. Dairy Sci.* 85, 422–428. doi: 10.3168/jds.S0022-0302(02)74090-3

Pan, X., Xue, F., Nan, X., Tang, Z., Wang, K., Beckers, Y., et al. (2017). Illumina sequencing approach to characterize thiamine metabolism related bacteria and the impacts of thiamine supplementation on ruminal microbiota in dairy cows fed high-grain diets. *Front. Microbiol.* 8:1818. doi: 10.3389/fmicb.2017.01818

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

Pellikaan, W. F., Hendriks, W. H., Uwimana, G., Bongers, L. J. G. M., Becker, P. M., and Cone, J. W. (2011a). A novel method to determine simultaneously methane production during *in vitro* gas production using fully automated equipment. *Anim. Feed Sci. Technol.* 168, 196–205. doi: 10.1016/j.anifeedsci.2011.04.096

Pellikaan, W. F., Stringano, E., Leenaars, J., Bongers, D. J. G. M., Schuppen, S., Van, L., et al. (2011b). Evaluating effects of tannins on extent and rate of *in vitro* gas and CH_4 production using an automated pressure evaluation system (APES). *Anim. Feed Sci. Technol.* 166-167, 377–390. doi: 10.1016/j.anifeedsci.2011.04.072

Petri, R. M., Mickdam, E., Klevenhusen, F., Beyer, B., and Zebeli, Q. (2019). Effects of the supplementation of plant-based formulations on microbial fermentation and predicted metabolic function *in vitro*. *Anaerobe* 57, 19–27. doi: 10.1016/j.anaerobe.2019.03.001

Qiao, F. Q., Wang, F., Ren, L. P., Zhou, Z. M., Meng, Q. X., and Bao, Y. H. (2015). Effect of steam-flaking on chemical compositions, starch gelatinization, *in vitro* fermentability, and energetic values of maize, wheat and rice. *J. Integr. Agric.* 14, 949–955. doi: 10.1016/S2095-3119(14)60913-8

Ruppert, L. D., Drackley, J. K., Bremmer, D. R., and Clark, J. H. (2003). Effects of tallow in diets based on corn silage or alfalfa silage on digestion and nutrient use by lactating dairy cows. *J. Dairy Sci.* 86, 593–609. doi: 10.3168/jds.S0022-0302(03)73638-8

Sakai, S., Imachi, H., Sekiguchi, Y., Tseng, I. C., Ohashi, A., Harada, H., et al. (2009). Cultivation of methanogens under low-hydrogen conditions by using the coculture method. *Appl. Environ. Microbiol.* 75, 4892–4896. doi: 10.1128/AEM.02835-08

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

Thoetkiattikul, H., Mhuantong, W., Laothanachareon, T., Tangphatsornruang, S., Pattarajinda, V., Eurwilaichitr, L., et al. (2013). Comparative analysis of microbial profiles in cow rumen fed with different dietary fiber by tagged 16S rRNA gene pyrosequencing. *Curr. Microbiol.* 67, 130–137. doi: 10.1007/s00284-013-0336-3

van Knegsel, A. T. M., van den Brand, H., Dijkstra, J., Tamminga, S., and Kemp, B. (2005). Effect of dietary energy source on energy balance, production, metabolic disorders and reproduction in lactating dairy cattle. *Reprod. Nutr. Dev.* 45, 665–688. doi: 10.1051/RND:2005059

Van Knegsel, A. T. M., Van Den Brand, H., Dijkstra, J., Van Straalen, W. M., Heetkamp, M. J. W., Tamminga, S., et al. (2007). Dietary energy source in dairy cows in early lactation: energy partitioning and milk composition. *J. Dairy Sci.* 90, 1467–1476. doi: 10.3168/jds.S0022-0302(07)71632-6

Viviani, R., Borgatti, A. R., and Matteuzzi, D. (1968). Isolation of typical rumen bacteria active in unsaturated fatty acid biohydrogenation. *Boll. Soc. Ital. Biol. Sper.* 44, 2185–2189.

Wang, Y., Nan, X., Zhao, Y., Wang, Y., Jiang, L., Xiong, B., et al. (2021). Ruminal degradation of rumen-protected glucose influences the ruminal microbiota and metabolites in early-lactation dairy cows. *Appl. Environ. Microbiol.* 87, e1908–e1920. doi: 10.1128/AEM

Xia, Y., Kong, Y., Seviour, R., Yang, H. E., Forster, R., Vasanthan, T., et al. (2015). *In situ* identification and quantification of starch-hydrolyzing bacteria attached to barley and corn grain in the rumen of cows fed barley-based diets. *FEMS Microbiol. Ecol.* 91, 1–11. doi: 10.1093/femsec/fiv077

Yokoyama, M. T., and Davis, C. L. (1971). Hydrogenation of unsaturated fatty acids by *Treponema* (*Borrelia*) strain B25, a rumen spirochete. *J. Bacteriol.* 107, 519–527. doi: 10.1128/jb.107.2.519-527.1971

Zhang, J., Shi, H., Wang, Y., Li, S., Cao, Z., Ji, S., et al. (2017). Effect of dietary forage to concentrate ratios on dynamic profile changes and interactions of ruminal microbiota and metabolites in Holstein herfers. *Front. Microbiol.* 8:2206. doi: 10.3389/fmicb.2017.02206

Zhang, D. F., and Yang, H. J. (2011). *In vitro* ruminal methanogenesis of a hay-rich substrate in response to different combination supplements of nitrocompounds, pyromellitic diimide and 2-bromoethanesulphonate. *Anim. Feed Sci. Technol.* 163, 20–32. doi: 10.1016/j.anifeedsci.2010.09.019

Zhang, R., Ye, H., Liu, J., and Mao, S. (2017a). High-grain diets altered rumen fermentation and epithelial bacterial community and resulted in rumen epithelial injuries of goats. *Appl. Microbiol. Biotechnol.* 101, 6981–6992. doi: 10.1007/s00253-017-8427-x

Zhang, R., Zhu, W., Jiang, L., and Mao, S. (2017b). Comparative metabolome analysis of ruminal changes in Holstein dairy cows fed low- or high-concentrate diets. *Metabolomics* 13, 1–15. doi: 10.1007/s11306-017-1204-0

Zhou, X. Q., Zhang, Y. D., Zhao, M., Zhang, T., Zhu, D., Bu, D. P., et al. (2015). Effect of dietary energy source and level on nutrient digestibility, rumen microbial protein synthesis, and milk performance in lactating dairy cows. *J. Dairy Sci.* 98, 7209–7217. doi: 10.3168/jds.2015-9312

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Metabolome and microbiome analysis revealed the effect mechanism of different feeding modes on the meat quality of Black Tibetan sheep

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Introduction: Black Tibetan sheep is one of the primitive sheep breeds in China that is famous for its great eating quality and nutrient value but with little attention to the relationship between feeding regimes and rumen metabolome along with its impact on the muscle metabolism and meat quality.

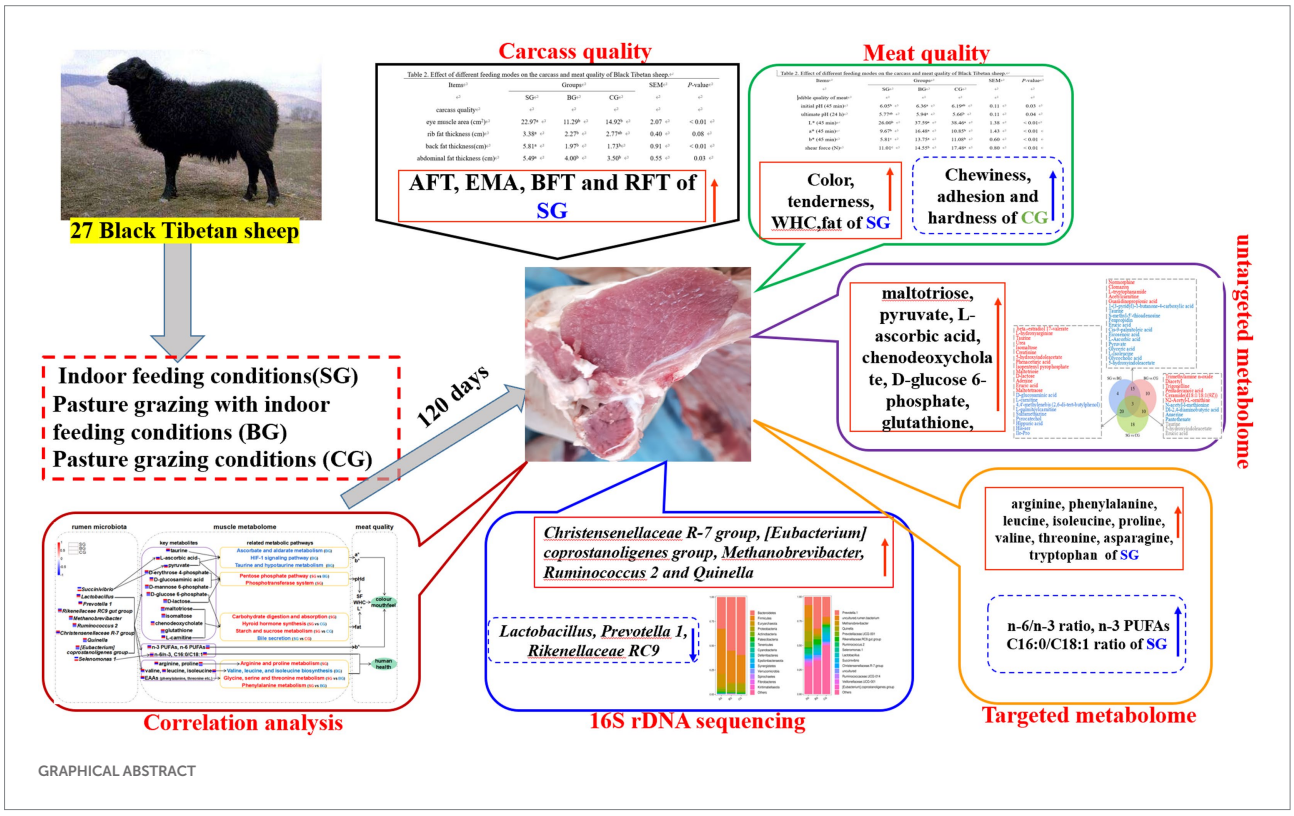
Methods: This study applies metabolomics-based analyses of muscles and 16S rDNA-based sequencing of rumen fluid to examine how feeding regimes influence the composition of rumen microbiota, muscle metabolism and ultimately the quality of meat from Black Tibetan sheep. Twenty-seven rams were randomly assigned to either indoor feeding conditions (SG, n=9), pasture grazing with indoor feeding conditions (BG, n=9) or pasture grazing conditions (CG, n=9) for 120 days.

Results: The results showed that, compared with BG and CG, SG improved the quality of Black Tibetan sheep mutton by preventing a decline in pH and increasing fat deposition to enhance the color, tenderness and water holding capacity (WHC) of the *Longissimus lumborum* (LL). Metabolomics and correlation analyses further indicated that the feeding regimes primarily altered amino acid, lipid and carbohydrate metabolism in muscles, thereby influencing the amino acid (AA) and fatty acid (FA) levels as well as the color, tenderness and WHC of the LL. Furthermore, SG increased the abundance of *Christensenellaceae R-7 group*, *[Eubacterium] coprostanoligenes group*, *Methanobrevibacter*, *Ruminococcus 2* and *Quinella*, decreased the abundance of *Lactobacillus*, *Prevotella 1* and *Rikenellaceae RC9 gut group*, and showed a tendency to decrease the abundance of *Succinivibrio* and *Selenomonas 1*. Interestingly, all of these microorganisms participated in the deposition of AAs and FAs and modified the levels of different metabolites involved in the regulation of meat quality (maltotriose, pyruvate, L-ascorbic acid, chenodeoxycholate, D-glucose 6-phosphate, glutathione, etc.).

Discussion: Overall, the results suggest that feeding Black Tibetan sheep indoors with composite forage diet was beneficial to improve the mouthfeel of meat, its color and its nutritional value by altering the abundance of rumen bacteria which influenced muscle metabolism.

KEYWORDS

Black Tibetan sheep, feeding regimes, meat quality, 16S rDNA, metabolomics



GRAPHICAL ABSTRACT

1. Introduction

Black Tibetan sheep, a characteristic breed of the Guinan County (Qinghai Province, China), is still raised by traditional grazing although this approach is not conducive to the protection and development of this particular livestock. In addition, overgrazing can easily degrade the grassland and lead to desertification, resulting in ecological imbalance (Peng et al., 2015). In order to protect grassland ecosystem in China as well as meet an increasing demand for high quality Black Tibetan sheep's meat, the animals' feeding regimes have gradually transitioned from traditional grazing to intensive feeding. This changed approach can significantly protect the ecological environment, shorten the slaughtering time and greatly increase the quantity of sheep in a short time (da Silva et al., 2020). However, such changes in feeding regimes also affect the overall health, growth characteristics and even the meat quality of sheep due to differences in diet composition (Boughalmi and Araba, 2016). For example, intensive production can increase the growth performance and carcass quality of sheep (Papi et al., 2011), while pasture grazing can improve the levels of polyunsaturated fatty acids (PUFAs) in their muscles (Wang et al., 2021). Consequently, maintaining or even improving the meat quality of Black Tibetan sheep raised in the feedlot is expected to become the core of future research.

The metabolome of muscles provides important information to characterize the nutritional and sensory properties of mutton (Muroya et al., 2020). Indeed, increasing numbers of studies have demonstrated that the levels of muscle metabolites can potentially indicate changes in meat quality. For example, a metabolite called inosine monophosphate regulates Nellore cattle beef's tenderness, along with creatine, carnosine, and conjugated linoleic acid in the *Longissimus lumborum* (LL; de Zawadzki et al., 2017). Moreover, color stability is attributed to the presence of

antioxidant or reducing compounds, such as taurine and L-glutathione (Muroya et al., 2020). Notably, in the authors' previous study, it was found that differences between White Tibetan sheep's meat quality under stall feeding and traditional grazing conditions were regulated by muscle differential metabolites (DMs; L-glutamate, inosine, adenosine, D-mannose, D-fructose, etc.) (Zhang et al., 2021). Therefore, these findings provide a basis for exploring the main metabolites and key metabolic pathways that regulate Black Tibetan sheep's meat quality under different feeding regimes.

In ruminants, the rumen microbiota independently regulates host metabolism, with changes in its composition directly influencing the host's phenotype (Zhang et al., 2022). In this context, ruminants' feeding regime and dietary components are critical factors as they determine rumen microbiota's composition and functions. For instance, Wang et al. (2021) discovered that artificial pasture grazing modified muscle metabolites that influence meat quality as well as muscle amino acid (AA) and fatty acid (FA) composition by modulating rumen microbiota. As such, it was a better choice for producing healthy mutton products (Wang et al., 2021). Furthermore, Du et al. (2021) found that a high-energy diet could enhance yak's muscle quality, including fat content and shear force of the LL. This was achieved through an increase in amylolytic bacteria and their fermentation products which were required for fatty acid synthesis (Du et al., 2021). Most importantly though, the current authors previously reported that a high-energy diet could affect Black Tibetan sheep's muscle metabolism by altering the composition of rumen microbiota, with this change improving meat quality and flavor (Zhang et al., 2022). In light of these findings, it is expected that host metabolism and animal phenotypes can be artificially modified by changing feeding regimes and diets. However, the key rumen bacteria associated with quality-related properties such as meat color and tenderness in Black Tibetan sheep are yet to be determined.

Consequently, it was hypothesized that the meat quality of this breed could be enhanced by changing feeding regimes which not only influence the rumen microbiota composition but also muscle metabolism. For this purpose, ultrahigh-performance liquid chromatography-mass spectrometry (UHPLC–MS) and gas chromatography–mass spectrometry (GC–MS) were applied to, respectively, compare changes in muscle AA and FA composition resulting from the different feeding regimes. The muscle metabolome was subsequently studied using ultrahigh-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) before determining the rumen microbiota composition based on Illumina sequencing. In addition, correlations between meat quality parameters and muscle metabolites as well as between rumen bacteria, key DMs and beneficial AAs and FAs in the LL were analyzed. This approach provided a detailed and comprehensive overview of how Black Tibetan sheep's meat quality was influenced by different feeding regimes. The findings are expected to not only provide baseline information for enhancing Black Tibetan sheep's meat quality by regulating rumen microbiota under an indoor feeding regime but also to provide an effective method for striking a balance between production and ecology.

2. Materials and methods

The study was approved by the Animal Ethics Committee of Qinghai University (QUA-2020-0709) and was carried out at the Black Tibetan Sheep Breeding Center in Guinan County of Qinghai Province, China.

2.1. Animal management and sample collection

Twenty-seven 120-day-old rams (Black Tibetan sheep) weighing 12.2 ± 0.92 kg were randomly assigned to one of the following feeding regimes: indoor feeding conditions (SG, $n=9$), pasture grazing with indoor feeding conditions (BG, $n=9$) and pasture grazing conditions (CG, $n=9$). A composite forage with a concentrate (commercial mixed ration) to roughage (silage corn and green oat hay, 1:1) ratio of 7:3 was used as feed for the lambs from the SG group (twice a day at 08:00 and 18:00), while those in the CG and BG groups grazed (08:00–18:00) on desert grassland located in Gonghe County, Qinghai Province (36.28°N, 99.88°E). The latter contained *Achnatherum splendens* and *Agropyron splendens* (sowing proportion, 50, 50%, respectively) and included two grazing areas (687 m^2/plot). In addition, the diet of the lambs from the BG group was supplemented with the composite forage after grazing. All the animals were subjected to a 7-day adaptation period before being fed (1.5 × 2.0 m^2/unit, 1 lambs/unit) individually or allowed to graze (9 lambs/plot) for 120 days with *ad libitum* access to food and water. Table 1 shows the ingredients and nutritional levels of the composite forage and pasture grass.

In accordance with animal welfare procedures, all animals were humanely slaughtered after fasting for 18 h on solid food and 2 h on liquid food. The left side of the carcasses, between the 12th and 13th ribs, were sampled before determining the color and pH values of each sample within 45 min. Moreover, rumen fluid was filtered through four layers of cheesecloth prior to collection into sterile containers. As required by standardized norms, slaughter, and sampling were conducted simultaneously by professionals. Following collection, samples were then immediately frozen in liquid nitrogen before being stored in the refrigerator at −80°C until needed for further analysis. A total of nine biological replicates and three technical replicates were obtained from each group to assess the concentrations of ammonia-N and volatile fatty acids (VFAs), AA and FA composition as well as carcass and meat quality. For the studies of rumen microbiota and muscle metabolome, six biological replicates were collected for each group.

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TABLE 1 Dietary ingredients and nutritional levels of the two different feeds (dry matter basis).

Items	Composite forage	Pasture grass
Dietary ingredients (% DM)		
Achnatherum splendens	–	50.00
Agropyron desertorum	–	50.00
Corn	43.15	–
Soybean meal	2.65	–
Rapeseed meal	12.42	–
Cottonseed meal	4.58	–
Oat silage	15.00	–
Oat hay	15.00	–
Mineral salt	0.80	–
Limestone	0.80	–
Baking soda	0.10	–
Dicalcium phosphate	0.50	–
Mineral/vitamin premix[1]	5.00	–
Total	100.00	100.00
Nutritional levels (% DM)		
Digestive energy DE (MJ/kg)	12.12	–
Crude protein	13.03	5.15
Crude ash	4.62	6.82
Crude fat	5.02	3.08
Neutral detergent fiber NDF	26.72	42.28
Acid detergent fiber ADF	16.36	30.23
Calcium	0.92	0.20
Phosphorus	1.01	0.26

[1]The premix provided the following per kg of diet: Fe (as ferrous sulfate) 4.5 g/kg; Cu (as copper sulfate) 1.0 g/kg; Zn (as zinc sulfate) 6.0 g/kg; Mn (as manganese sulfate 3.0 g/kg; Co (as cobalt sulfate) 0.02 g/kg, Se 0.02 g/kg; I 0.04 g/kg; VA 250000 IU/kg; VD 30000 IU/kg; VE 25000 IU/kg.

2.2. Carcass quality analysis of Black Tibetan sheep

At the time of carcass segmentation, the carcass quality was assessed at the 12th rib. The eye muscle area (EMA) was measured with a planimeter after being drawn on sulfuric acid paper. Abdominal fat thickness (AFT), back fat thickness (BFT) and rib fat thickness (RFT) were then directly measured with vernier calipers at 127 mm, 40 mm and 110 mm from the spinal column, respectively.

2.3. Meat quality analysis of Black Tibetan sheep

Following a 24-h postmortem phase, standard procedures were used to assess the edible quality of meat samples. A portable pH meter, previously calibrated with standards of pH 4.0 and 6.86 and having a built-in temperature compensator, was inserted into samples to determine the initial and final pH values. A Minolta-ADCI machine was then used to determine the a* (redness), b* (yellowness) and L* (lightness) values of samples. Moreover, their shear force (SF) was assessed with a Warner-Bratzler apparatus, while the elasticity, hardness, viscosity, cohesion, adhesion and chewiness of samples were measured with a texture profile analysis (TPA) machine (CT3, Brookfield).

As previously described (Zhang et al., 2021), the samples were thawed at 4°C for 12 h to calculate the thaw loss (TL) before hanging them at the same temperature for 24 h to assess the drip loss (DL). In addition, the samples were cooked in a Thermostatic Water Bath machine at 85°C for 30 min to obtain the cooking loss (CL) and cooked meat percentage (CMP). Throughout these processes, all samples were individually packed into hermetic bags. Finally, regarding the nutritional components, standard AOAC procedures were used to determine the total ash, crude fat, crude protein and moisture content of meat samples at 48 h postmortem (Lee, 1995).

2.4. Untargeted metabolomics analysis of the *Longissimus lumborum*

For untargeted metabolite analysis, 50 mg of meat samples were processed as reported before (Zhang et al., 2021). This was followed by UHPLC-QTOF-MS (1,290 Infinity, Agilent) which first involved sample separation in an UHPLC. In this case, the autosampler temperature, injection volume, column temperature and flow rate were 4°C, 2 μL, 25°C and 0.5 ml/min, respectively. Furthermore, the following liquid phase gradient was applied: 0–0.5 min, the B phase was 95%; 0.5–7 min, the B phase changed linearly from 95 to 65%, then decreased linearly to 40% in 1 min before being sustained for 1 min; the B phase was then increased to 95% in 0.1 min and sustained for 2.9 min. Quality control (QC) samples were also added into the sample queues to monitor and assess the stability of the system and the reliability of the data.

MS (AB Triple TOF 6600) was subsequently used to collect the samples' first- and second-order spectra. The chromatographic alignment, retention-time modifications and peak identification were carried out using XCMS software. Furthermore, multivariate statistical analysis was performed using SIMCA software, with heat maps subsequently generated in R. DMs, defined as those with variable importance projection (VIP) > 1 and $p < 0.05$, were also identified. Eventually, the metabolites were functionally annotated and the metabolic pathways were enriched using the Kyoto Encyclopedia of Genes and Genomes database (KEGG).[1]

1 www.genome.jp/kegg

2.5. Targeted metabolomics analysis of the *Longissimus lumborum*

2.5.1. Amino acid analysis

Fifty milligrams of meat samples were processed for AA analysis as described previously (Zhang et al., 2021). For UHPLC–MS (1,290 Infinity, Agilent) detection, sample separation was performed in an UHPLC, with an autosampler temperature of 4°C, an injection volume of 1 μL, a column temperature of 40°C and a flow rate of 250 μL/min. Furthermore, the liquid phase gradient was set as follows: 0–12 min, the B phase changed linearly from 90 to 70%; 12–18 min, the B phase changed linearly from 70 to 50%; it was then decreased to 40% in 7 min and maintained for 5 min; finally, the B phase increased linearly to 90% in 0.1 min and was sustained for 6.9 min. QC samples were also added to evaluate the stability and repeatability of the system. Finally, MS (5,500 QTRAP) was set to Multiple Reaction Monitoring (MRM), while chromatographic alignment, retention-time modifications and peak identification were carried out with the MultiQuant software to determine metabolites.

2.5.2. Fatty acid analysis

FA analysis was performed on 50 mg of meat samples as previous described (Zhang et al., 2021). In this case, for GC–MS-based (7,890/5975C, Agilent) detection, sample separation was carried out on a capillary column (30 m × 0.25 mm × 0.25 μM, DB-WAX) for which the programmed temperature was set to 40°C for 5 min before increasing to 220°C at a rate of 10°C/min for 5 min. Helium was used as the carrier gas in a split ratio of 10:1 and a flow rate of 1.0 ml/min, while the inlet, transfer line and ion source temperatures were 280°C, 250°C, and 230°C, respectively. QC was also performed as mentioned above. MS was set to Single Ion Monitoring (SIM) and operated in the Electron Impact Ionization Source (EIS), while electron energy was set at 70 eV. Eventually, chromatographic alignment, retention-time modifications and peak identification were carried out using MSD ChemStation software to determine metabolites.

2.6. Rumen function analysis

2.6.1. Rumen fermentation characteristics

Following collection of the rumen fluid, a portable pH meter was used to immediately record the pH value. The concentrations of VFAs and ammonia-N were subsequently determined by GC (Shimadzu NX 2030) and the phenol-hypochlorite assay, respectively (Broderick and Kang, 1980).

2.6.2. Rumen microbiota composition

Genomic DNA was extracted from rumen fluid samples using SDS technique before assessing the DNA's purity and concentration. The V3-V4 regions of 16S rDNA genes were then amplified by PCR using specific primers under the following conditions: initial denaturation for 1 min at 98°C, followed by 30 cycles, each with denaturation for 10 s at 98°C, annealing for 30 s at 50°C and extension for 60 s at 72°C. The PCR ended with a 5-min final extension at 72°C. AxyPrep DNA Gel Extraction Kit (AXYGEN, United States) and QuantiFluor™-ST (Promega, United States) were then used to, respectively, purify and quantify the PCR products as specified by the respective manufacturers. This was followed by the preparation of a sequencing library using an Illumina Next®Ultra™DNA Library Prep Kit (NEB, United States), with both Qubit@ 2.0 Fluorometer and Agilent Bioanalyzer 2,100 systems used to assess the quality of the library. Eventually, sequencing was performed on an Illumina HiSeq2500 platform to generate 250 bp paired-end reads.

The sequencing data generated above were filtered and merged, as previously reported, to obtain effective tags (Wang et al., 2020a). UPARSE software was then used to cluster representative sequences into operational taxonomic units (OTUs) at a 97% similarity threshold before classifying the latter into various species using the RDP classifier against the SILVA database. Alpha (Chao1, Simpson, Shannon, etc.) and beta diversities (Principal Coordinate Analysis based on unweighted unifrac) were also determined in QIIME, while both Anosim and Adonis were constructed using Bray-Curtis distance matrices. Furthermore, LEfSe-based analyses allowed microbial biomarkers within each group to be quantitatively identified before using STAMP analysis to confirm the abundance of differential species between the three groups. The metabolic function of rumen microbiota was finally predicted using the KEGG database.

2.7. Statistical analysis

Means and standard errors of the means (SEM) were calculated with one-way analysis of variance (ANOVA) using SPSS software (16.0). Significance was set at $p < 0.05$ and tendencies were reported at $0.05 \leq p \leq 0.1$. For the different feeding regimes, Pearson's correlation coefficient was further used to determine the relationship between rumen bacteria, muscle metabolome and meat quality in Black Tibetan sheep, with significant correlations indicated by $p < 0.05$ and $|r| > 0.50$.

3. Results

3.1. Carcass quality

The carcass quality of Black Tibetan sheep under different feeding regimes is shown in Table 2. The AFT, EMA and BFT were higher ($p < 0.05$) in SG than in BG and CG, although these three parameters were not different between BG and CG. In addition,

10.3389/fmicb.2022.1076675

TABLE 2 Effects of different feeding modes on the carcass and meat quality of Black Tibetan sheep.

Items	Groups			SEM	*p*-value
	SG	BG	CG		
Carcass quality					
Eye muscle area (cm^2)	22.97^a	11.29^b	14.92^b	2.07	< 0.01
Rib fat thickness (cm)	3.38	2.27	2.77	0.4	0.08
Back fat thickness(cm)	5.81^a	1.97^b	1.73^b	0.91	<0.01
Abdominal fat thickness (cm)	5.49^a	4.00^b	3.50^b	0.55	0.03
Edible quality of meat					
Initial pH (45 min)	6.05^b	6.36^a	6.19^{ab}	0.11	0.03
Ultimate pH (24 h)	5.77^{ab}	5.94^a	5.66^b	0.11	0.04
L* (45 min)	26.00^b	37.59^a	38.46^a	1.38	< 0.01
a* (45 min)	9.67^b	16.48^a	10.85^b	1.43	< 0.01
b* (45 min)	5.81^c	13.75^a	11.08^b	0.6	< 0.01
Shear force (N)	11.01^c	14.55^b	17.48^a	0.8	< 0.01
Thaw loss (%)	7.24	6.44	6.25	0.58	0.21
Drip loss (%)	4.14^c	5.10^b	6.13^a	0.46	< 0.01
Cooking loss (%)	17.39^c	29.20^b	34.69^a	1.88	< 0.01
Cooked meat percentage (%)	76.72^a	69.54^b	63.40^c	2.42	< 0.01
Hardness (g)	824.44^b	632.56^b	1832.67^a	115.75	< 0.01
Elasticity (mm)	1.54^b	2.02^a	2.13^a	0.11	< 0.01
Viscosity (mJ)	0.16	0.2	0.17	0.03	0.4
Adhesion (g)	252.44^b	182.56^b	505.78^a	46.21	< 0.01
Cohesion	0.35	0.34	0.37	0.06	0.85
Chewiness (mJ)	6.48^b	4.21^b	10.02^a	1.22	< 0.01
Nutritional quality of meat					
Moisture (%)	73.58	74.33	74.39	0.59	0.33
Ash (%)	1.10^b	1.22^{ab}	1.34^a	0.06	< 0.01
Fat (%)	4.41^a	3.78^{ab}	3.16^b	0.34	< 0.01
Protein (%)	21.36	21.5	21.52	0.44	0.93

^{a,b,c}Different superscripts within a row indicate significantly different values for *P*-value <0.05.

SG showed a tendency ($p = 0.08$) to increase the RFT. Therefore, the results indicated that Black Tibetan sheep's carcass quality under the indoor feeding regimes was the best.

3.2. Meat quality

The meat quality of Black Tibetan sheep under different feeding regimes is shown in Table 2. For the edible quality, BG had a higher ($p < 0.05$) initial pH value than SG as well as a higher ($p < 0.05$) ultimate pH value than CG. This showed that CG displayed the largest pH decline in the LL (within 24 h after slaughtering), followed by BG and SG, with the latter having the lowest range of pH decline. The a* and b* values were also significantly higher ($p < 0.01$) in BG than in SG and CG, whereas the L* value was significantly lower ($p < 0.01$) in SG compared with BG and CG. In addition, CG had the largest SF, DL, and CL, followed by BG, which had medium values, while SG had the lowest ones ($p < 0.01$). Conversely, SG had a higher CMP than CG, while BG was in the median range ($p < 0.01$). In addition, the chewiness, adhesion, and hardness were significantly lower ($p < 0.01$) in SG and BG in comparison with CG, whereas the elasticity was considerably lower ($p < 0.01$) in SG. Finally, as far as the nutritional components of the LL were concerned, the fat content was considerably higher ($p < 0.01$) but the ash content was considerably lower ($p < 0.01$) in SG than in CG. As a result, SG showed better meat edible quality [e.g., color, tenderness, and water holding capacity (WHC)] as well as greater meat fat deposition.

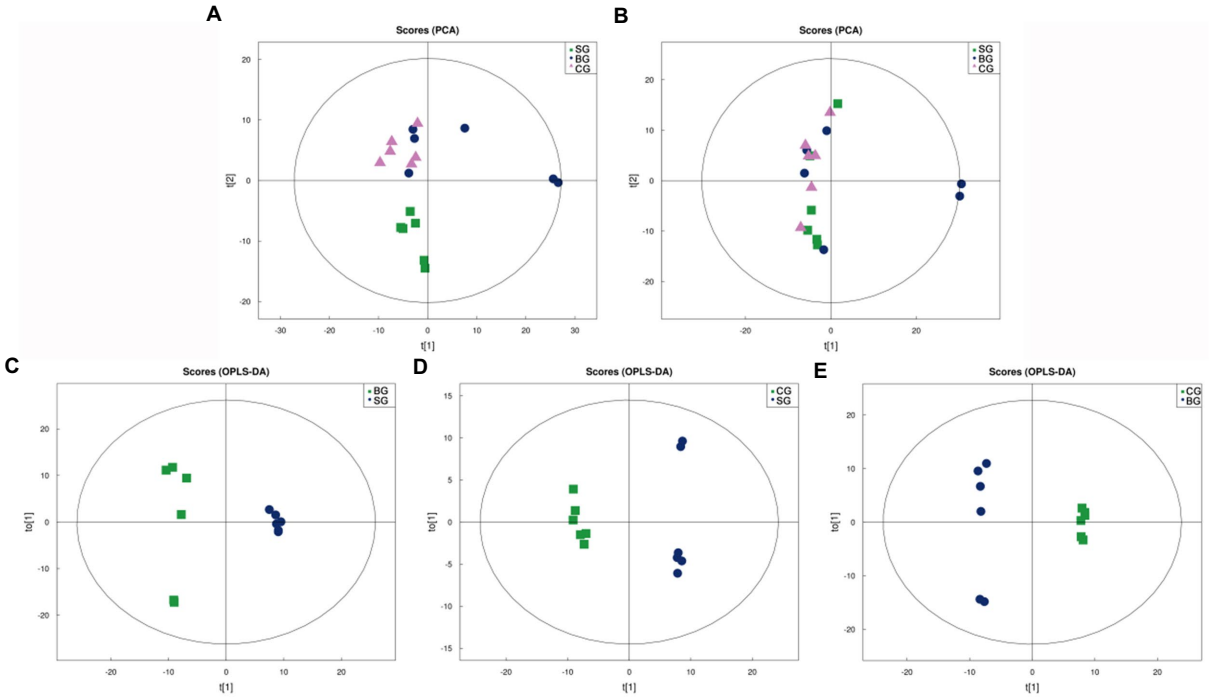

FIGURE 1
PCA score plots of all samples in the ESI+ **(A)** and ESI− **(B)** modes as well as OPLS-DA score plots of the comparison between SG and BG **(C)**, between SG and CG **(D)**, and between BG and CG **(E)** in the ESI+ mode. t[1] represents the principal component 1, t[2] **(A,B)** and to[1] **(C–E)** represent the principal component 2, and the ellipse represents the 95% confidence interval.

3.3. Analysis of muscle untargeted metabolome

As shown in Figures 1A,B, the Principal Component Analysis (PCA) scores for the three groups were plotted under positive (ESI+) and negative (ESI−) ion modes, respectively. In this case, the R^2X of the ESI+ and ESI− modes for the three groups were 0.609 and 0.532, respectively, and hence, an ESI+ mode was selected for subsequent analysis as this was more useful and reliable. It was noted that, under this mode, the SG samples clustered separately from those of BG and CG, although no clear separation was observed for the latter two. Therefore, to further optimize the three groups' separation and focus on the metabolic variations between the LL, Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) was used to build more intensive and accurate models for the analysis of the three sets of meat samples, with the resulting OPLS-DA score plots shown in Figures 1C–E (ESI+). In this case, OPLS-DA revealed good within-group aggregation along with between-group separation. R^2X, R^2Y, and Q^2 of the OPLS-DA models are also summarized in Supplementary Table S1, and all of the models showed acceptable goodness-of-fit and goodness-of-prediction. Therefore, the results indicated that the metabolic pattern in Black Tibetan sheep's LL was significantly altered by different feeding regimes.

By applying the threshold (VIP > 1, $p < 0.05$) for DMs' identification under both the ESI+ and ESI− modes, a total of 171 DMs were identified across the three groups, of which 80 were connected with KEGG metabolic pathways (Figure 2). More specifically, pairwise comparisons revealed 42 DMs (18 and 24 in the positive and negative modes, respectively) between SG and BG, 51 DMs (25 and 26 in the positive and negative modes, respectively) between SG and CG and 38 DMs (20 and 18 in the positive and negative modes, respectively) between BG and CG. Compared with BG and CG, 23 metabolite levels were also significantly different in SG (14 DMs were significantly higher, and 9 DMs were lower). Furthermore, 18 metabolite levels were different in BG (13 DMs were significantly lower, whereas 5 DMs were higher) compared with the other groups, while for the CG group, the levels of 10 metabolites were significantly different (4 DMs were significantly lower, and 6 DMs were higher). Overall, most of these DMs were lipids, AAs, carbohydrates and their derivatives.

To further determine the differential metabolic pathways in Black Tibetan sheep's LL after different feeding regimes, the Differential Abundance Score (DA score) of each comparable group was analyzed. As shown in Figure 3A, compared with BG, 18 metabolic pathways were upregulated in SG (DA score > 0.5, $p < 0.05$), with the key ones being

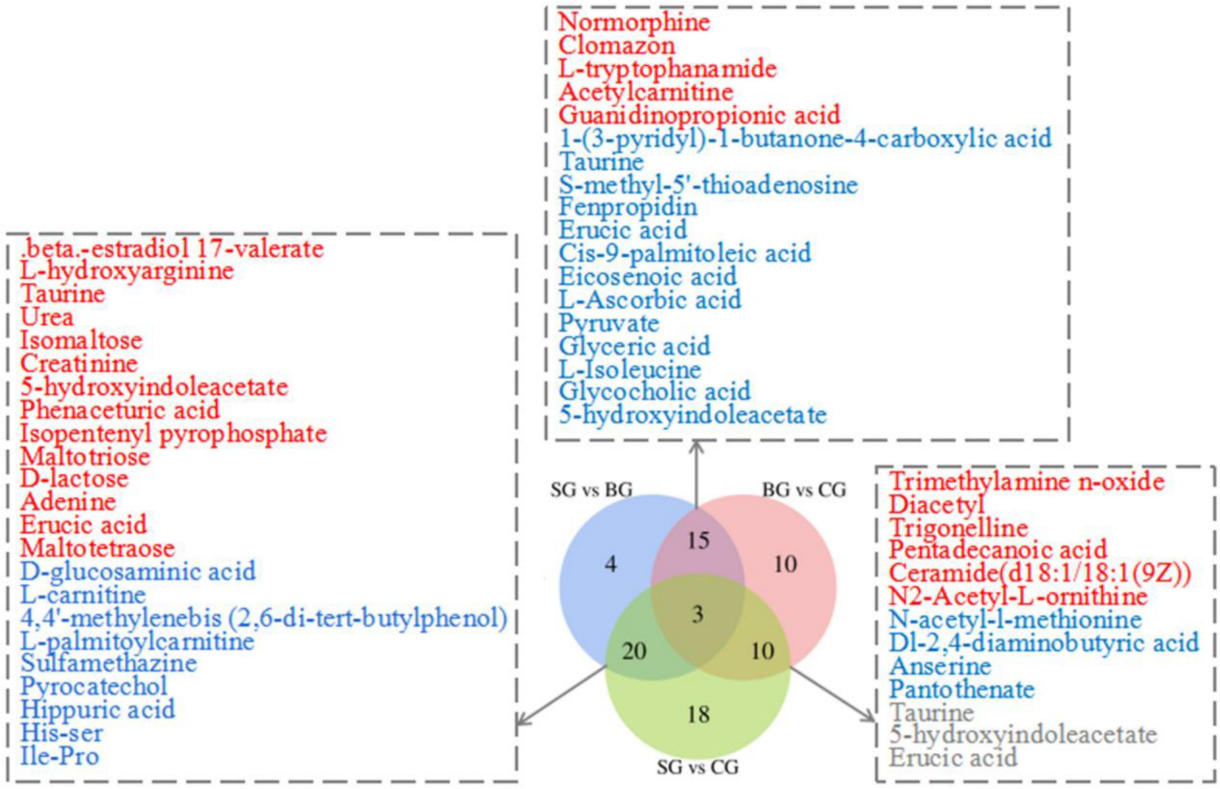

FIGURE 2
Venn diagram illustrates the overlap of DMs connected with KEGG metabolic pathways for three sets of comparisons (SG vs. BG; SG vs. CG; BG vs. CG) in the *Longissimus lumborum*. The color red and blue represent the upregulation and downregulation of metabolites, respectively.

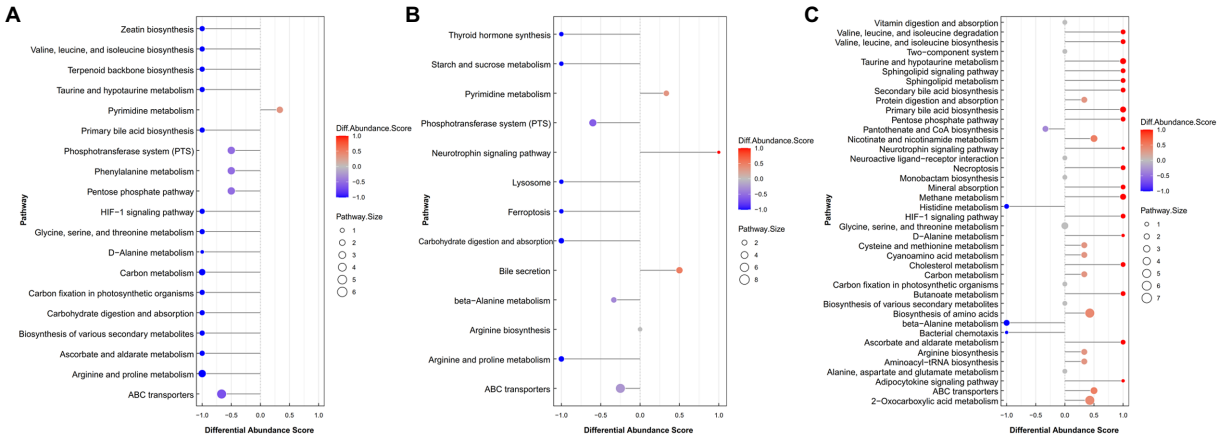

FIGURE 3
The DA score maps of all differential metabolic pathways for the comparison between SG and BG **(A)**, between SG and CG **(B)** and between BG and CG **(C)** in the *Longissimus lumborum*. The DA score captures the average and gross changes for all metabolites in a pathway. Scores of 1 and−1, respectively, indicate a decrease and an increase in all measured metabolites in the pathway for SG **(A,B)** and BG **(C)**.

phosphotransferase system (PTS), pentose phosphate pathway, ascorbate and aldarate metabolism, carbohydrate digestion and absorption, HIF-1 signaling pathway and six amino acid metabolisms (taurine, hypotaurine metabolism, etc.). Among the metabolites involved in these key pathways, the levels of 3-hydroxyphenylacetic acid, D-erythrose 4-phosphate, phenaceturic acid, urea, taurine, L-isoleucine, maltotriose, L-hydroxyarginine, L-ascorbic acid, glyceric acid, D-lactose,

pyruvate and creatinine were all higher in SG, whereas those of D-glucosaminic acid and hippuric acid were lower (Supplementary Table S3). On the other hand, compared with CG, only two metabolic pathways were downregulated in SG, while seven were upregulated (DA score > 0.5, $p < 0.05$). Bile secretion was the main downregulated pathway in SG, unlike starch and sucrose metabolism, arginine and proline metabolism, carbohydrate digestion and absorption, thyroid hormone synthesis and PTS which were all upregulated (Figure 3B). Among the metabolites involved in these key pathways, the levels of D-glucose 6-phosphate, maltotriose, L-hydroxyarginine, isomaltose, creatinine, D-mannose 6-phosphate, glutathione, D-lactose and urea were all higher in SG, while those of D-glucosaminic acid, chenodeoxycholate, L-carnitine and choline were lower (Supplementary Table S3). Finally, comparison between CG and BG showed that 3 metabolic pathways were downregulated and 20 were upregulated in the former (DA score > 0.5, $p < 0.05$). In this case, the key upregulated pathways included the HIF-1 signaling pathway, pentose phosphate pathway, ascorbate and aldarate metabolism, four amino acid metabolisms (taurine, hypotaurine metabolism, etc.) and four lipid metabolisms (sphingolipid metabolism, etc.). On the other hand, beta-alanine metabolism and histidine metabolism were downregulated in CG (Figure 3C). Among the metabolites involved in these key pathways, the levels of methylmalonic acid, glyceric acid, L-isoleucine, sphingomyelin (d18:1/18:0), taurine, ceramide [d18:1/18:1(9Z)], glycocholic acid, pyruvate, taurocholate and L-ascorbic acid were all higher in CG, while those of anserine, pantothenate and aspartic acid were lower (Supplementary Table S3).

TABLE 3 Effects of different feeding modes on the AA composition in the *Longissimus lumborum* of Black Tibetan sheep (μg/g tissue).

Items	Groups		SEM	*P*-value	
	SG	BG	CG		
Arginine	108.46	66.26	90.77	14.61	0.07
Methionine	5.87[a]	4.07[b]	5.71[a]	0.35	<0.01
Phenylalanine	44.25[a]	26.90[b]	39.47[a]	3.22	<0.01
Tyrosine	47.02[a]	27.64[b]	38.63[ab]	5.86	0.04
Leucine	74.33[a]	47.42[b]	66.43[a]	5.74	<0.01
Isoleucine	23.84[a]	17.04[b]	22.02[a]	1.32	<0.01
Proline	77.08[a]	40.81[b]	35.98[b]	10.40	0.01
Valine	54.07[a]	37.86[b]	43.56[b]	4.26	0.02
Threonine	57.63	22.92	45.43	12.53	0.08
Asparagine	43.93[a]	20.48[b]	34.95[ab]	6.26	0.03
Tryptophan	39.51[b]	54.88[a]	42.05[b]	4.91	0.04
Hydroxyproline	36.43[a]	10.80[b]	36.59[a]	7.94	0.03
Taurine	1014.74[a]	365.16[b]	678.34[ab]	188.75	0.04
Choline	22.14	190.63	50.89	64.24	0.08
Aminoadipic acid	26.24[a]	9.19[b]	16.09[ab]	5.31	0.05
EAAs[1]	344.21	255.26	300.03	33.19	0.09
NEAAs	4781.98	3533.80	4892.30	798.59	0.25
TAAs	5126.19	3789.06	5192.33	830.41	0.24

[a,b]Different superscripts within a row indicate significantly different values for *P*-value <0.05. Complete amino acid profile is provided in Supplementary Table S4. EAAs, essential amino acids; NEAAs, non-essential amino acids; TAAs, total amino acids.
[1]EAAs = (leucine + methionine + valine + isoleucine + threonine + phenylalanine + lysine + tryptophan).

3.4. Analysis of muscle targeted metabolome

3.4.1. Amino acid composition

As shown in Table 3, the concentrations of total amino acids (TAAs) and non-essential amino acids (NEAAs) in the LL did not differ between the three groups, although SG showed a tendency to increase the levels of essential amino acids (EAAs; $p = 0.09$), arginine ($p = 0.07$) and threonine ($p = 0.08$). Similarly, the levels of tyrosine, asparagine, taurine and aminoadipic acid were higher ($p < 0.05$) in SG than in BG. In addition, the levels of proline and valine were higher ($p < 0.05$) in SG than in BG and CG, the concentrations of methionine, phenylalanine, leucine, isoleucine and hydroxyproline were higher ($p < 0.01$) in SG and CG than in BG, and BG had a higher ($p < 0.05$) concentration of tryptophan compared with SG and CG.

3.4.2. Fatty acid composition

As noted from Table 4, the levels of total fatty acids (TFAs), saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and PUFAs, including the ratio of PUFAs/SFAs (P/S) in the LL, were not different between the three groups even though the ratios of n-6/n-3 and C16:0/C18:1 were significantly affected by different feeding regimes. In particular, BG showed a tendency ($p = 0.05$) to increase the concentration of n-3 PUFAs as well as CG showed a tendency ($p = 0.07$) to increase the concentration of n-6 PUFAs. The C16:0/C18:1 ratio was higher ($p < 0.05$) in BG than in SG, while the n-6/n-3 ratio was higher ($p < 0.01$) in SG and CG than in BG. In addition, SG had a higher ($p < 0.01$) C20:1 N9 concentration than BG and CG. Similarly, BG had higher ($p < 0.05$) C20:5 N3 and C22:5 N3 concentrations than SG as well as a higher ($p < 0.05$) C22:6 N3 concentration than both SG and CG. Conversely, CG had a higher ($p < 0.05$) concentration of C18:3 N6 compared with BG.

3.5. Rumen function

3.5.1. Analysis of rumen fermentation characteristics

Fermentation characteristics in Black Tibetan sheep's rumen under the three different feeding regimes were as shown in

TABLE 4 Effects of different feeding modes on the FA composition in the *Longissimus lumborum* of Black Tibetan sheep (μg/g tissue).

Items	Groups			SEM	*P*-value
	SG	BG	CG		
C18:2 N6	520.10	515.18	706.96	76.09	0.08
C18:3 N6	6.20[a]	3.19[b]	7.01[a]	1.09	0.03
C18:3 N3	26.54	30.90	52.72	9.59	0.07
C20:1 N9	22.48[a]	10.94[b]	13.22[b]	2.09	<0.01
C20:3 N6	18.68	28.17	25.19	3.65	0.10
C20:4 N6	2.81	2.07	2.56	0.24	0.06
C20:3 N3	187.83	310.29	190.08	47.26	0.07
C20:5 N3	14.40[b]	44.02[a]	21.83[ab]	9.45	0.05
C23:0	0.88	0.72	1.17	0.17	0.10
C22:5 N3	39.09[b]	88.59[a]	69.11[ab]	12.97	0.02
C22:6 N3	6.77[b]	22.67[a]	8.90[b]	4.48	0.02
TFAs	6707.74	5737.86	6832.17	2164.09	0.86
SFAs	3010.23	2747.13	3434.54	1250.24	0.86
MUFAs	2839.16	1921.01	2279.78	931.81	0.63
PUFAs	858.35	1069.72	1117.85	137.12	0.21
n-3 PUFAs	274.63	496.47	342.64	71.72	0.05
n-6 PUFAs	583.73	573.25	775.21	78.04	0.07
PUFAs/SFAs	0.34	0.45	0.39	0.16	0.80
n-6 PUFAs/n-3 PUFAs	2.12[a]	1.19[b]	2.26[a]	0.17	<0.01
C16:0/C18:1	0.58[b]	0.87[a]	0.77[ab]	0.09	0.05

[a,b]Different superscripts within a row indicate significantly different values for *p*-values <0.05. Complete fatty acid profile is provided in Supplementary Table S5. TFAs, total fatty acids, SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; n-3 PUFAs, omega-3 polyunsaturated fatty acids; n-6 PUFAs, omega-6 polyunsaturated fatty acids.

TABLE 5 Effects of different feeding modes on rumen fermentation characteristics of Black Tibetan sheep.

Items	Groups			SEM	*P*-value
	SG	BG	CG		
pH	5.71	5.59	5.75	0.12	0.38
Ammonia-N (mmol/L)	21.68[a]	8.84[b]	8.42[b]	2.40	<0.01
Acetate (mmol/L)	60.10	65.91	66.96	5.02	0.35
Propionate (mmol/L)	23.42	20.27	21.53	1.30	0.07
Butyrate (mmol/L)	18.37[a]	11.10[b]	12.57[b]	1.22	<0.01
Valerate (mmol/L)	1.35[a]	0.69[c]	0.95[b]	0.11	<0.01
Total VFAs (mmol/L)	107.71	97.95	102.52	7.27	0.42
A/P	2.57[b]	3.22[a]	3.14[a]	0.16	<0.01

[a,b,c]Different superscripts within a row indicate significantly different values for *P*-value <0.05. VFAs, volatile fatty acids; A, acetate; P, propionate.

Table 5, with no differences in the pH value observed for the three groups. However, SG had a higher ($p < 0.01$) ammonia-N concentration than BG and CG. For VFAs, SG had higher ($p < 0.01$) butyrate and valerate levels than BG and CG. Besides, SG had a lower ($p < 0.01$) ratio of acetate to propionate (A/P) than BG and CG.

3.5.2. Analysis of rumen microbiota composition

As shown in Figure 4A, 1118 OTUs were obtained across the three groups, with 1,135, 951 and 405 being assigned only to SG, BG, and CG, respectively. At the same time, significant differences were noted in bacterial diversity and richness between the three groups of samples (Supplementary Table S2). More specifically, BG had higher ($p < 0.01$) Shannon and Simpson values than SG and CG, while CG had lower ($p < 0.01$) Chao1 and Ace than SG and BG. Anosim analysis (Figure 4B) and PCoA plot (Figure 4C) further revealed significant differences and good separation between SG and the other two groups, although similar results were not obtained when comparing BG and CG. Overall, these findings highlight the changes in Black Tibetan sheep's rumen bacteria in response to differences between the three feeding regimes. Furthermore, 372 bacterial genera from 22 phyla were identified, with Figures 4D,E showing the top 15 most abundant genera and phyla, respectively.

The main differences in phyla and genera of rumen bacteria between the three groups are shown in Table 6. At the phylum level, compared with BG and CG, SG had more Euryarchaeota, Deferribacteres, Epsilonbacteraeota, Firmicutes and Synergistetes ($p < 0.01$) but less Bacteroidetes ($p < 0.01$). Moreover, Cyanobacteria, Spirochaetes and Kiritimatiellaeota were more abundant ($p < 0.01$) in BG than in SG and CG. At the genus level, *Quinella*, *Methanobrevibacter*, *Ruminococcus 2* and *[Eubacterium] coprostanoligenes group* were more abundant ($p < 0.01$) in SG compared with BG and CG, while *Lactobacillus* was less abundant ($p < 0.01$). At the same time, compared with SG and CG, BG had more *Rikenellaceae RC9 gut group* and *Prevotella 1* ($p < 0.01$) but less *Christensenellaceae R-7 group* ($p < 0.05$). Finally, CG had a higher abundance of *Prevotella 1* ($p < 0.01$) compared with SG, while the latter showed a tendency to decrease the abundance of *Selenomonas 1* ($p = 0.10$) and *Succinivibrio* ($p = 0.06$). Based on the results, functional prediction of Black Tibetan sheep's rumen microbiota suggested that composite forage diet promoted the metabolism of carbohydrates, AAs, lipids and other substances in the rumen (Figure 4F).

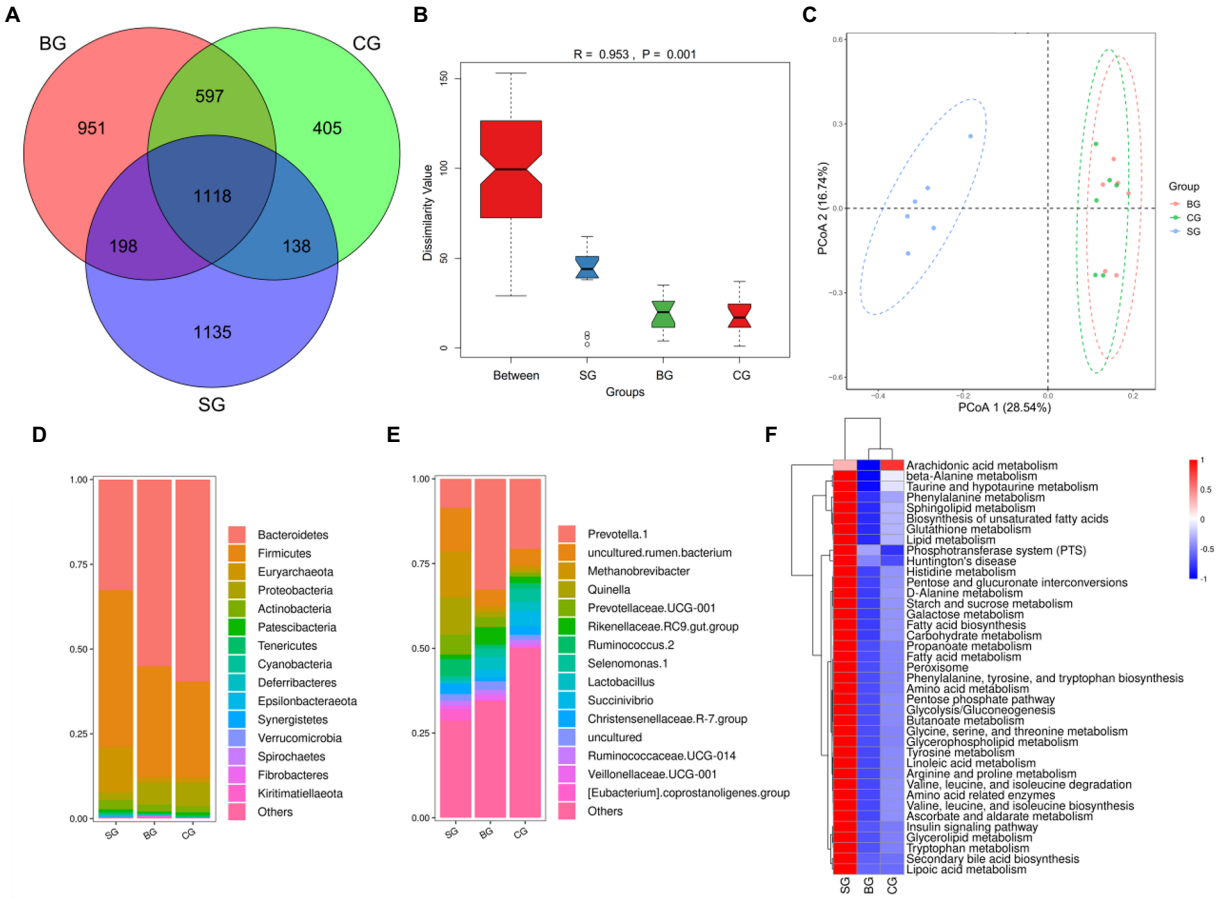

FIGURE 4
Venn diagram of OTUs showing the overlap of rumen microbiota between the three groups **(A)**. Anosim analysis **(B)** and PCoA plot **(C)** of the samples' rumen microbiota. The relative abundance of the 15 most abundant taxa at the phylum **(D)** and genus **(E)** levels among the three groups (as a percentage of the total sequences). Heatmap predicting the important differences in rumen functions **(F)**.

3.6. Correlation analysis

3.6.1. Correlation between muscle untargeted metabolome and meat quality parameters

The correlation between meat quality and muscle metabolism was assessed using the data from untargeted metabolome and the phenotypic parameters of Black Tibetan sheep's LL under different feeding regimes (Figure 5A). In this context, L*, a*, b*, SF, DL, and CL were positively correlated with D-glucosaminic acid and chenodeoxycholate but negatively correlated with maltotriose, D-lactose and isomaltose. Conversely, CMP and fat were negatively correlated with D-glucosaminic acid and chenodeoxycholate but positively correlated with maltotriose, D-lactose and isomaltose. In addition, L* was negatively correlated with D-glucose 6-phosphate and glutathione but positively correlated with L-carnitine. Negative correlations were also noted for a* with pyruvate, taurine and L-ascorbic acid as well as for DL with D-erythrose 4-phosphate, D-glucose 6-phosphate and D-mannose 6-phosphate. In the case of glutathione, it was positively correlated with fat content, while negative correlations were observed for glutathione, D-glucose 6-phosphate and D-mannose 6-phosphate with SF as well as between taurine and b*. Overall, bile secretion, pentose phosphate pathway, PTS, carbohydrate digestion and absorption as well as sucrose and starch metabolism significantly affected the values of L*, a*, b*, SF, DL, CL, CMP, and fat content in the LL. Taurine and hypotaurine metabolism, ascorbate and aldarate metabolism and HIF-1 signaling pathway also significantly affected the a* and b* values of the LL, with thyroid hormone synthesis also affecting SF, L* value and fat content.

3.6.2. Correlation of rumen bacteria with muscle amino acids, fatty acids, and differential metabolites

A strong connection was found between rumen bacteria and muscle DMs (Figure 5B) as well as the profiles of AAs and FAs (Figure 5C) in the LL. In particular, in the case of *Prevotella 1*, *Rikenellaceae RC9 gut group* and *Lactobacillus*, a positive correlation

TABLE 6 The main differential rumen bacteria at the phylum and genus levels among the three groups (accounting for the relative abundance in top 10).

Items	Groups			SEM	*P*-value
	SG	BG	CG		
Phylum level (%)					
Bacteroidetes	32.66^{b}	55.10^{a}	59.60^{a}	3.96	<0.01
Firmicutes	46.32^{a}	32.72^{b}	28.25^{b}	4.48	<0.01
Euryarchaeota	13.44^{a}	1.57^{b}	1.59^{b}	3.46	<0.01
Cyanobacteria	0.03^{b}	0.26^{a}	0.09^{b}	0.05	<0.01
Deferribacteres	0.32^{a}	0.00^{b}	0.00^{b}	0.09	<0.01
Epsilonbacteraeota	0.24^{a}	0.01^{b}	0.00^{b}	0.09	0.03
Synergistetes	0.15^{a}	0.06^{b}	0.03^{b}	0.02	<0.01
Spirochaetes	0.03^{b}	0.15^{a}	0.04^{b}	0.03	<0.01
Fibrobacteres	0.00	0.17	0.02	0.07	0.05
Kiritimatiellaeota	0.02^{b}	0.10^{a}	0.03^{b}	0.01	<0.01
Genus level (%)					
Prevotella 1	8.64^{c}	32.72^{a}	20.63^{b}	3.10	<0.01
Methanobrevibacter	13.40^{a}	1.56^{b}	1.57^{b}	3.46	<0.01
Quinella	10.98^{a}	1.61^{b}	0.73^{b}	0.64	<0.01
Rikenellaceae RC9 gut group	1.23^{b}	5.16^{a}	1.90^{b}	0.92	<0.01
Ruminococcus 2	5.08^{a}	1.24^{b}	1.63^{b}	1.37	0.03
Selenomonas 1	1.30	2.43	3.91	1.13	0.10
Lactobacillus	0.83^{b}	3.69^{a}	2.68^{a}	0.78	<0.01
Succinivibrio	0.11	2.31	4.61	1.73	0.06
Christensenellaceae R-7 group	3.06^{a}	1.16^{b}	2.48^{a}	0.58	0.02
[Eubacterium] coprostanoligenes group	3.30^{a}	0.31^{b}	0.19^{b}	0.53	<0.01

^{a,b,c}Different superscripts within a row indicate significantly different values for *P*-value <0.05.

was noted with D-glucosaminic acid, chenodeoxycholate and L-carnitine, while a negative correlation was observed for *[Eubacterium] coprostanoligenes group*, *Methanobrevibacter*, *Quinella*, *Ruminococcus 2* and *Christensenellaceae R-7 group* with those metabolites. A positive correlation was also noted for *[Eubacterium] coprostanoligenes group*, *Methanobrevibacter* and *Quinella* with glutathione as well as for *Succinivibrio* with pyruvate and L-ascorbic acid. In addition, *Lactobacillus* was negatively correlated with D-erythrose 4-phosphate, D-glucose 6-phosphate and D-mannose 6-phosphate, whereas *Methanobrevibacter* was positively correlated with those metabolites. In the case of *Quinella*, *Ruminococcus 2* and *[Eubacterium] coprostanoligenes group*, a positive correlation was observed with maltotriose, D-lactose, isomaltose and taurine, while *Prevotella 1* was negatively correlated with those metabolites. Regarding the AA profiles, *Rikenellaceae RC9 gut group*, *Prevotella 1* and *Lactobacillus* were negatively correlated with arginine, phenylalanine, leucine, isoleucine, proline, valine, threonine, asparagine, tryptophan, taurine and the total EAA content, whereas *Quinella* and *[Eubacterium] coprostanoligenes group* were positively linked to those metabolites. Furthermore, *Christensenellaceae R-7 group* was positively correlated with leucine, isoleucine and valine. Finally, as far as FA profiles were concerned, positive correlations were observed between *Selenomonas 1* and n-6 PUFAs as well as between *Christensenellaceae R-7 group* and the n-6/n-3 ratio. In addition, *Rikenellaceae RC9 gut group* and *Prevotella 1* were negatively linked to the n-6/n-3 ratio but, along with *Lactobacillus*, they were positively correlated with n-3 PUFAs and the C16:0/C18:1 ratio. On the other hand, *[Eubacterium] coprostanoligenes group*, *Quinella* and *Christensenellaceae R-7 group* were negatively linked to n-3 PUFAs, with these organisms, along with *Methanobrevibacter* and *Ruminococcus 2*, being also negatively correlated with the C16:0/C18:1 ratio.

4. Discussion

Feeding regimes have a significant effect on sheep's carcass quality (Prache et al., 2021), and, as reported in previous findings (da Silva et al., 2020; Morales Gómez et al., 2021), this study also found that Black Tibetan sheep raised indoors with composite forage diet had the best carcass quality. This had been attributed to a lower A/P ratio in the rumen alongside a combination of higher energy intake and lower energy expenditure (Boughalmi and Araba, 2016). Indoor feeding regimes can provide a higher dietary nutritional level and digestive energy for Black Tibetan sheep as, under this type of feeding, the animals require less exercise, have lower energy consumption and gain weight at a faster rate, resulting in better production performance (Aurousseau et al., 2004). In contrast, those under pasture grazing consume a lot of energy due higher physical activity, resulting in less fat deposition in muscles and subsequently, poorer carcass quality (Turner et al., 2014).

Meat color, as an important indicator of meat quality, can affect consumers' purchasing behavior and the shelf life of fresh meat (Hussain et al., 2021). In this context, higher a* as well as lower L* and b* values are indicative of better muscle color within a certain range. It has been reported that the L* value was positively correlated with muscle WHC, and a change in the refractive index of muscle surface as a result of water exudation was the main reason for the increase in the L* value (Kim et al., 2011). Therefore, for this study, the better WHC justified the lower L* value in SG compared with BG and CG. At the same time, muscle WHC is affected by the rate of pH decline and the content of intermuscular fat. Indeed, the slower the pH decline rate and the higher the intermuscular fat content, the better the muscle WHC (Moreno et al., 2020), and interestingly, similar observations were made in this study. Moreover, BG had higher a* and b* values than SG and CG, and this could have been linked to the degree of muscle oxidation. In fact, myoglobin combines with

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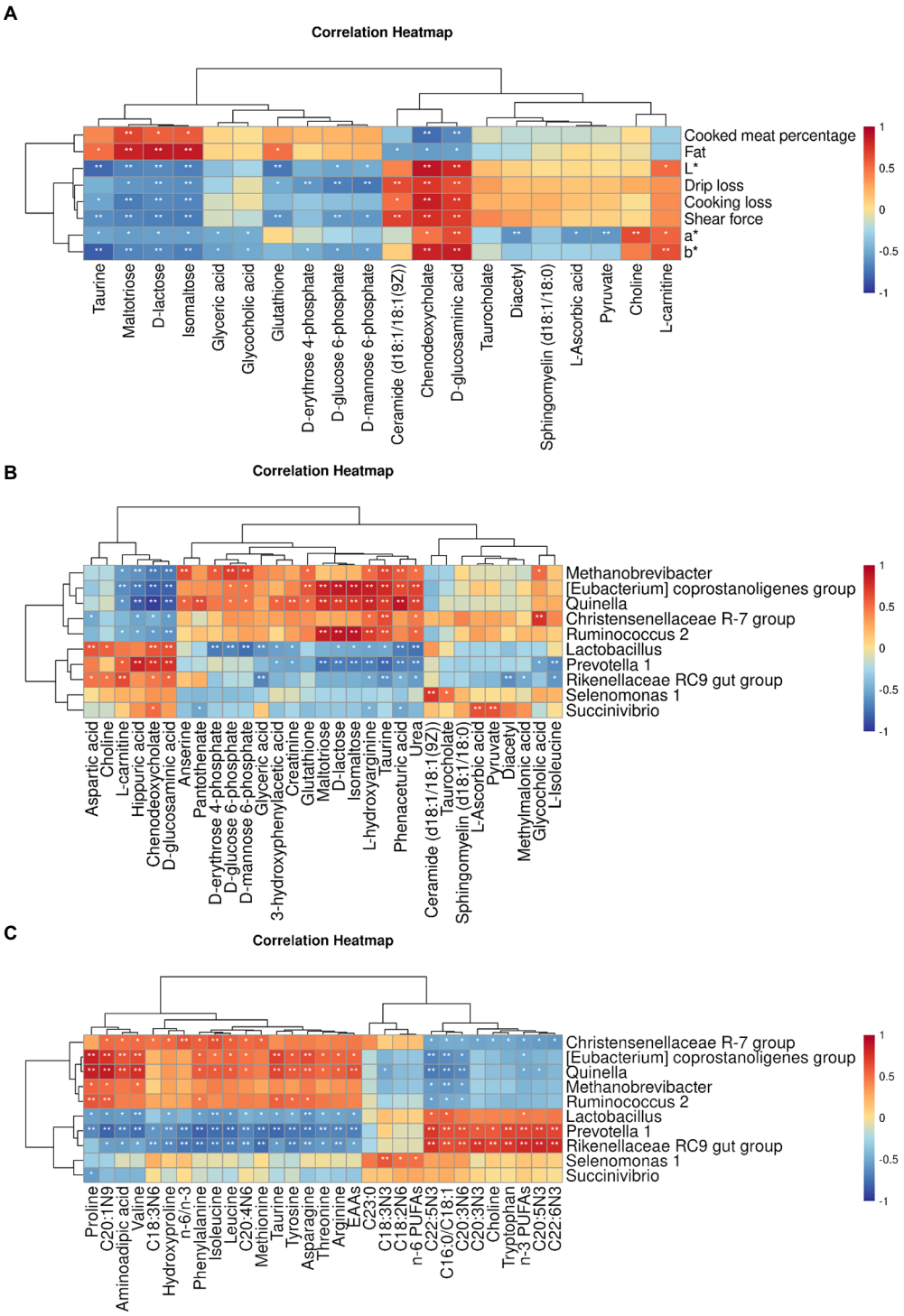

FIGURE 5
Pearson's correlation heatmap between muscle untargeted metabolome and meat quality parameters **(A)**, between rumen bacteria and muscle DMs **(B)** and between rumen bacteria and the AA and FA profiles **(C)**. The color red and blue represent positive and negative correlations, respectively. The color intensity is proportional to the correlation values. *p-value <0.05 and **p-value <0.01.

oxygen to form bright red oxymyoglobin, resulting in a higher a* value, while for b*, a change in value is associated with lipid oxidation (Hernández Salueña et al., 2019). Therefore, indoor feeding regimes can improve muscle WHC by slowing down the rate of pH decline and increasing the fat deposition, thereby reducing the L* value. On the other hand, pasture grazing with indoor feeding regimes might reduce the antioxidant capacity of muscles, resulting in increased a* and b* values.

Mutton products' tenderness determines their acceptability and satisfaction among consumers (O'Quinn et al., 2018). In this context, SF represents an indicator that directly reflects a change in tenderness (Bendall, 1978). Furthermore, according to some studies, feedlot cattle tend to be more tender than pasture cattle (Wicks et al., 2019; Torrecilhas et al., 2021). Similarly, a previous study has shown that the muscle SF of White Tibetan sheep which had been raised indoors with composite forage diet was lower compared with those which had been raised through a traditional grazing regime (Zhang et al., 2021). Despite the above factors, meat tenderness is actually dependent on a number of other factors, including the collagen and fat content of muscular tissues, the length of sarcomeres and the rate of pH decline (Veiseth et al., 2004). A rapid decrease in pH causes muscle contraction and thus, reduces muscle tenderness (Choi et al., 2005). However, breaking muscle fibers is easier to implement during the chewing process, especially with an increase in intramuscular fat content which is translated into a decrease in muscle SF (Chambaz et al., 2003). Therefore, the lower SF in SG compared with BG and CG in this study might be attributed to the slower rate of pH decline and a higher fat deposition in the LL.

Glycolysis is predominant in post-slaughtered muscle due to the cessation of blood circulation and the interruption of oxygen supply (Hou et al., 2020). The accumulation of lactic acid from this process is the main reason of the pH decline in muscles (Choe et al., 2008). Through the phosphorylation and transfer of carbon source, PTS reduces glucose phosphorylation, resulting in a reduction in cAMP (cyclic adenosine monophosphate) concentrations. As a result, this decreases glycogen metabolism by inhibiting the APK (cAMP-dependent protein kinase) signaling pathway and inactive glycogen phosphorylase's phosphorylation (Kim et al., 2004). In this study, compared with BG and CG, PTS was significantly upregulated in SG, hence suggesting that glycogen metabolism was inhibited in this group. Additionally, hexokinase (HK), which converts glucose into D-glucose 6-phosphate, is the first rate-limiting enzyme in the glycolytic pathway as the accumulation of D-glucose 6-phosphate decreases the activity of HK (Hingst et al., 2019). Furthermore, part of the glucose in the postmortem muscle is involved in the pentose phosphate pathway, with glucose-6-phosphate dehydrogenase (G6PD) being the key enzyme that transfers glucose metabolism to this pathway (Tang, 2019). In the present study, SG had a higher D-glucose 6-phosphate level compared with CG as well as an upregulated pentose phosphate pathway compared with BG. It was, therefore, speculated that SG could have inhibited glycolysis by decreasing HK's activity and increasing G6PD's activity in the LL to slow down the rate of pH decline.

Interestingly, the L* value, SF and WHC indexes (DL, CL, and CMP) were significantly linked with D-lactose and D-glucosaminic acid in this study, while SF and DL were negatively correlated with D-mannose 6-phosphate and D-glucose 6-phosphate. A negative correlation was also noted between DL and D-erythrose 4-phosphate as well as between the L* value and D-glucose 6-phosphate. All these metabolites were involved in PTS (SG vs. the other two groups) and the pentose phosphate pathway (SG vs. BG). Overall, it is suggested that indoor feeding regimes could have regulated the rate of pH decline in the LL by inhibiting glycolysis, thereby improving the muscle color, SF and WHC of Black Tibetan sheep. In particular, these events were achieved by upregulating PTS (SG vs. the other two groups), the pentose phosphate pathway (SG vs. BG) and the D-glucose 6-phosphate deposition level (SG vs. CG) in SG. However, further research (e.g., glycogen and glucose content, glycolytic potential, and enzyme activity in the LL) would still be required to support the above theory.

According to previous studies, an increase in carbohydrate metabolism can provide the host with more substrate and energy for fat synthesis in muscles (Smith et al., 2018; Du et al., 2021). Conversely, bile promotes the digestion and absorption of lipids by the liver and regulates cholesterol metabolism, thereby reducing fat deposition in muscle tissues (Boyer, 2013). In this context, thyroid hormones play an important role as they are involved in multiple metabolic pathways, including lipid catabolism, lipid anabolism and body weight regulation in mammalian systems (Sinha et al., 2019). Consequently, attenuated thyroid hormone signaling results in decreased lipid utilization by the liver, which in turn increases fat deposition in muscle tissues (Ferrandino et al., 2017). This study found that the muscle metabolites involved in carbohydrate metabolism (maltotriose, D-lactose, and isomaltose), bile secretion (chenodeoxycholate and glutathione) and thyroid hormone synthesis (glutathione) were strongly correlated with fat content. As such, the upregulation of carbohydrate digestion and absorption (SG vs. the other two groups), starch and sucrose metabolism (SG vs. CG) and thyroid hormone synthesis (SG vs. CG), along with the downregulation of bile secretion (SG vs. CG), were potential indicators of fat deposition. At the same time, the L* value, SF and WHC indexes (DL, CL and CMP) were significantly associated with maltotriose, D-lactose, isomaltose and chenodeoxycholate in the current study. In particular, the L* value and SF were negatively correlated with D-glucose 6-phosphate and glutathione. Similarly, DL was negatively correlated with D-glucose 6-phosphate, while the L* value was positively correlated with L-carnitine. All of these metabolites are involved in metabolic pathways connected with fat deposition. Overall, it is suggested that indoor feeding regimes could have regulated the fat deposition process in the LL by upregulating carbohydrate digestion and absorption (SG vs. the other two groups), starch and sucrose metabolism (SG vs. CG) and thyroid hormone synthesis (SG vs. CG), while downregulating bile secretion (SG vs. CG) to improve muscle color, SF and WHC of Black Tibetan sheep.

The oxidative stability of muscle tissues mainly depends on the balance between antioxidants and oxidative mediators (Nair et al., 2014), with the former consisting of non-enzymatic antioxidants as well as antioxidant enzymes (Wood and Enser, 1997). Vitamin C (VC), also known as ascorbic acid, is a non-enzymatic antioxidant in animals. VC can effectively scavenge reactive oxygen species (ROS) and alleviate the

inhibitory effects of stress response on the activities of antioxidant enzymes, such as catalase (CAT) and glutathione peroxidase (GSH-Px), to enhance animals' antioxidant and anti-stress capacity (Shojadoost et al., 2021). Taurine, as a sulfur-containing amino acid, can improve the antioxidant and anti-stress capacity of animals by regulating the enzymatic antioxidant defense system in cells (e.g., increasing the activities of CAT and GSH-Px in response to stress; Baliou et al., 2021). Besides, taurine exerts antioxidant effects by activating various signaling pathways (e.g., *Nrf2*) in cells (Bai et al., 2016). It is worth noting that hypoxic environments can also induce the expression of hypoxia-inducible factor (HIF-1α) in humans and animals. HIF-1α regulates the activities of antioxidant enzymes [GSH-Px, heme oxygenase (HO^{-1}), etc.], thereby increasing the antioxidant capacity and reducing the oxidative stress damage of the host (Yeligar et al., 2010). Moreover, high expression of HIF-1α might also increase the expression of most antioxidant proteins in muscle tissues by enhancing the transcription and protein expression of the *Nrf2* gene, thereby improving the organisms' antioxidant capacity (Yeligar et al., 2010). In this study, the downregulation of ascorbate and aldarate metabolism, taurine and hypotaurine metabolism as well as the HIF-1 signaling pathway suggested a decrease in the levels of ascorbate, taurine and HIF-1α in BG compared with SG and CG. At the same time, the a* value was negatively correlated with pyruvate, L-ascorbic acid and taurine, with a similar negative correlation noted between the b* value and taurine. All these metabolites were likely to be involved in ascorbate and aldarate metabolism, taurine and hypotaurine metabolism and HIF-1 signaling pathway. Overall, it is suggested that pasture grazing with indoor feeding regimes could have reduced the antioxidant capacity of the LL by downregulating the above metabolic processes and signaling pathway, thereby negatively impacting muscle color. However, more evidence (e.g., antioxidant capacity, lipid oxidation degree, and hemoglobin content in the LL) would be required to ascertain this theory.

Amino acids are important ingredients that add flavor and nutritional value to mutton (Cai et al., 2010). For example, branched-chain AAs (leucine, isoleucine, and valine) promote muscle growth by stimulating mRNA translation *via* mTORC1 (mammalian target of rapamycin complex 1) signaling (Rohini et al., 2018). Similarly, eucine, isoleucine and valine regulate carbohydrate and lipid metabolism, inhibit proteolysis and increase protein synthesis (Rohini et al., 2018), while arginine produces enzymes and proteins that oxidize substrates to regulate muscle oxidation (Wu, 2009). Furthermore, as a substrate, proline is used in the synthesis of pyruvate and glucose, which can subsequently be used to characterize muscle collagen content, while threonine gives mutton a sweet taste, making it more likeable to consumers (Madruga et al., 2010). Among their other functions, AAs further contribute to the meat's aroma by interacting with carbonyl compounds during cooking (Dinh et al., 2018). In this study, SG promoted the deposition of proline and arginine in the LL by upregulating the metabolism of these AAs compared with BG and CG. At the same time, BG reduced the deposition of branched-chain AAs in the LL by downregulating valine, leucine and isoleucine biosynthesis compared with SG and CG. In fact, there was a tendency for higher concentration of threonine in the LL of the SG group through the upregulation of glycine, serine and threonine metabolism. It was previously reported that stall feeding regimes could increase EAA deposition in sheep's LL (Zhang et al., 2021), and interestingly, the similar result was observed in this study. Thus, an indoor feeding regime is more likely to enhance Black Tibetan sheep's meat flavor and promote human health.

The composition and content of FAs are important indicators of the nutritional value of meat products. In addition, the UFAs present in mutton, especially n-3 PUFAs and n-6 PUFAs, are beneficial to human health (Simopoulos, 2008). Natural pasture grass can inhibit biohydrogenation in rumen and increase the PUFA levels in muscle tissues (Ponnampalam et al., 2016). This could actually justify a tendency for higher concentrations of n-3 PUFAs in BG and n-6 PUFAs in CG as observed in this study. However, meat with high PUFA level is also more prone to lipid oxidation during storage and processing, resulting in rancidity, odor and the deterioration of meat color. Consequently, the meat's edible quality and nutritional value are reduced, with their shelf life also shortened and altogether, these factors affect the market acceptance of meat (Scollan et al., 2014). For instance, Priolo et al. (2001) discovered that low oxidative stability of meat resulted in its pronounced browning. In the current study, increasing PUFA level in BG (n-3 PUFAs) and CG (n-6 PUFAs) might have impaired the oxidative stability of muscles and reduce meat quality. This could actually justify the lower b* value and better meat color observed for SG. An important determinant of meat's nutritional value is its n–6/n–3 ratio, which assesses whether the meat is healthy for consumers (Simopoulos, 2002). In this context, mutton's n–6/n–3 value needs to be approximately four to maintain good cardiovascular health in human (Molendi-Coste et al., 2011). Moreover, the risk of diabetes in humans is directly proportional to the dietary C16:0/C18:1 ratio. Since decreasing the C16:0/C18:1 value improves insulin sensitivity in humans (Saglimbene et al., 2017), this could be an effective method to reduce the blood sugar concentration and control diabetes. Thus, although a tendency for higher concentrations of n–3 PUFAs in BG and n–6 PUFAs in CG was observed in this study, these characteristics actually increased the risk of lipid oxidation. Meanwhile, SG had a higher n–6/n–3 ratio closer to the recommended value as well as a lower C16:0/C18:1 ratio compared with BG. This confirmed the higher meat quality obtained from indoor feeding regimes.

Increasing numbers of studies have reported that changes in rumen microbiota associated with muscle metabolites can be used to explore the mechanism for improving the meat quality of ruminants under different feeding regimes (Zhang et al., 2022). In this study, all rumen samples were dominated by Firmicutes and Bacteroidetes as reported in previous findings (Cunha et al., 2011; Li et al., 2012). Firmicutes can promote fiber breakdown (Evans et al., 2011), while Bacteroidetes can help digest complex carbohydrates (Spence et al.,

2006). Furthermore, differences in rumen bacteria at the genus level between the three groups and their association with metabolite deposition in the LL were detected. In this study, *Prevotella*, the dominant genus in all samples, comprised 8.64–32.72% of all rumen bacteria and this was consistent with previous reports (Patel et al., 2014; Xue et al., 2017). *Prevotella* was reported to play a significant role in the efficient utilization of hemicelluloses, starch degradation, and protein and peptide metabolism (Wallace et al., 1997). For instance, in a study conducted by Zhou et al., *Rikenellaceae RC9 gut group* fermented carbohydrates or proteins, potentially improving the metabolism of lipids (Liyuan et al., 2018). Similarly, another study revealed that *Succinivibrio*, an amylolytic bacteria, fermented succinate into propionate to supply energy for the host (Zhang et al., 2018). In addition, *Selenomonas* is capable of fermenting glucose to produce acetate and propionate, with *Christensenellaceae R-7 group* also involved in acetate production. *Ruminococcus* belongs to the Firmicutes phylum and mainly degrades fibrous substances, while *Lactobacillus* have been used as probiotics (Xu et al., 2017). Unexpectedly, it was found that Black Tibetan sheep's rumen from the SG group had a higher abundance of Firmicutes but a lower abundance of Bacteroidetes compared with BG and CG. In particular, indoor feeding regimes decreased the abundance of *Prevotella 1* (vs. the other two groups), *Lactobacillus* (vs. the other two groups) and *Rikenellaceae RC9 gut group* (vs. BG), showed a tendency for lower abundance of *Selenomonas 1* and *Succinivibri*, while increasing that of *Methanobrevibacter*, *Ruminococcus 2*, *Quinella* and *[Eubacterium] coprostanoligenes group* compared with BG and CG as well as that of *Christensenellaceae R-7 group* compared with BG. However, these results were inconsistent with previous findings (Wang et al., 2020a,b), and this could have been due to the differences between the ingredients of composite feedstuff used in this study and those used in other works.

Rumen bacteria may indirectly affect the muscle's metabolite deposition by interacting with the host (Wang et al., 2021). It was found that some rumen bacteria were significantly correlated with the deposition of AAs and FAs as well as lipid metabolites such as L-carnitine, acetylcarnitine, linoleic acid, and linolenic acid in muscles (Wang et al., 2021). For instance, Huws et al. (2011) discovered that *Prevotella* was related to rumen lipid metabolism, while Wang et al. (2020a) discovered that *Rikenellaceae RC9 gut group* could modulate the metabolism of lipids, energy and glucose in the host because a high abundance of *Rikenellaceae RC9 gut group* in grazing sheep's rumen could promote the deposition of glycodeoxycholic acid, alpha-linolenic acid and glycocholic acid in mutton. Interestingly, the results of the current study were consistent with the above reports. Besides *Selenomonas 1*, all differential rumen bacterial genus were significantly correlated with muscle metabolites involved in the regulation of meat quality including maltotriose, D-lactose, pyruvate, D-erythrose 4-phosphate, L-ascorbic acid, D-glucosaminic acid, isomaltose, chenodeoxycholate, D-mannose 6-phosphate, taurine, D-glucose 6-phosphate, L-carnitine and glutathione. Moreover, *Selenomonas 1*, *[Eubacterium] coprostanoligenes group*, *Prevotella 1*, *Quinella*, *Rikenellaceae RC9 gut group*, *Lactobacillus* and *Christensenellaceae R-7 group* were significantly correlated with muscle FA and AA composition. More importantly though, predicting the rumen functions of Black Tibetan sheep under the three feeding regimes suggested that indoor feeding regimes promoted the metabolism of carbohydrates, AAs, lipids and other substances in the rumen. This was, in fact, consistent with the enrichment of metabolic pathways based on muscle DMs.

SCFAs are reportedly the end-products of fermentation performed by rumen microbiota (Saleem et al., 2013). At the same time, the rumen microbiome mainly provides substrates and energy, in the form of VFAs, to the host for muscle metabolism (Nathani et al., 2015). Hence, changes in dietary nutrient components not only modify the available fermentation substrates but also rumen SCFA profiles, which subsequently affect the metabolic pathways of rumen microbiota and the LL (Ghimire et al., 2017). For example, *Ruminiclostridium_6* and *U29-B03* might participate in carbohydrate metabolism to produce VFAs, thereby facilitating IMF deposition to promote tenderness in muscles (Du et al., 2021). In this study, changes in the rumen bacteria composition of Black Tibetan sheep could explain the higher concentrations of butyrate and valerate in the SG group, with these changes resulting in the upregulated metabolism of carbohydrates, AAs, lipids and other substances in the rumen and LL. Thus, it was proposed that composite forage diet could regulate metabolite deposition in the LL by altering the rumen microbiota composition as well as the SCFA profiles to improve meat quality (Figure 6). This is because a change in feeding regime induces a change in the amount of exercise, feed intake and dietary nutrient components, with these being the main factors that affect rumen microbial fermentation and muscle metabolite deposition (Chikwanha et al., 2018). In the present study, the tendency for higher concentrations of EAAs in the LL of the SG group were likely due to the high concentrations of EAAs in the composite forage, along with the higher protein utilization efficiency of rumen microbiota (Matthews et al., 2019). In addition, it was very likely that the tendency for higher concentrations of n-3 PUFAs and n-6 PUFAs in the BG and CG groups were due to the high concentrations of PUFAs in pasture grass as well as the low biohydrogenation of the ingested PUFAs (Fernandez-Turren et al., 2020). Finally, the significant differences in rumen microbiota composition between the three groups also reflected a change in feeding pattern or diet (Shabat et al., 2016). However, more studies would be required to reveal the connection between diet components and the changes in rumen microbiota, muscle metabolome and rumen metabolites.

5. Conclusion

This study suggested that indoor feeding with composite forage diet can improve the muscle color, tenderness and WHC of Black Tibetan sheep by regulating AA, lipid and carbohydrate metabolism in muscle tissues. In addition, indoor feeding regimes also had a positive effect on the composition of FAs and AAs in the LL. Further analyses showed that indoor feeding regimes even affected the levels

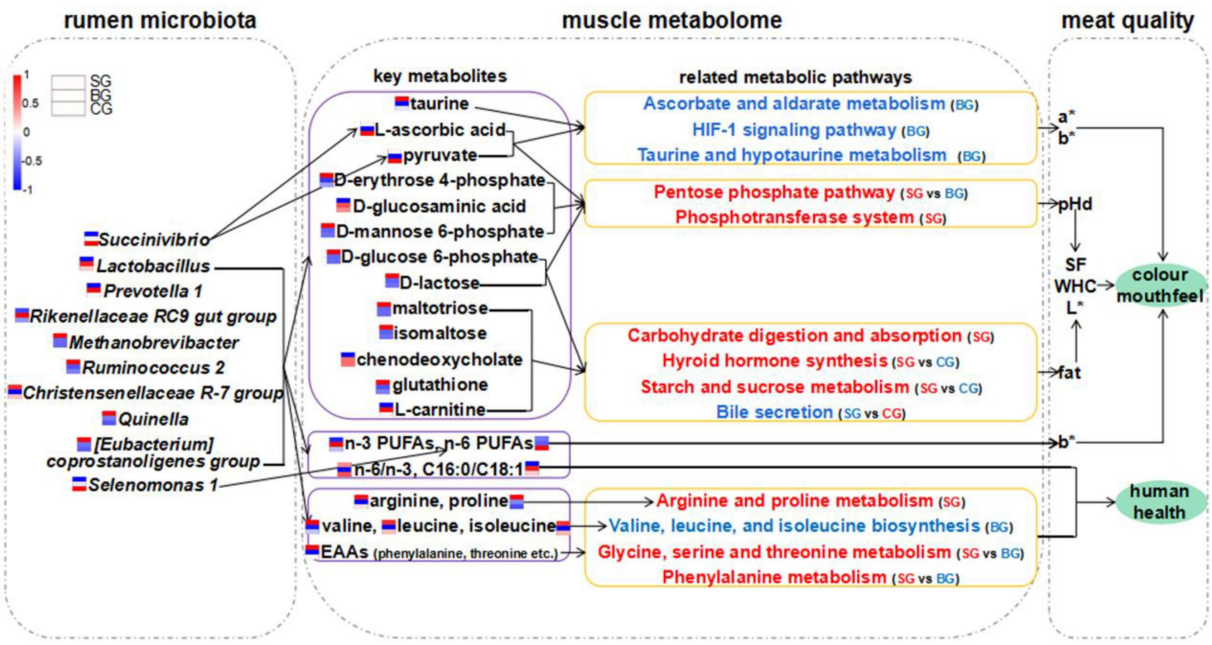

FIGURE 6
Hypothesized scheme pathways and potential mechanisms related to the changes of rumen microbiota, muscle metabolome and meat quality. Blue and red colors indicate significantly downregulated and upregulated metabolic pathways in each comparison, respectively. Expression levels of AAs, FAs, metabolites and bacteria in SG, BG, and CG were presented based on heatmaps. Red represents high expression and blue represents low expression. Complete heatmap analysis for these expression levels is provided in Supplementary Figure S1. pHd, the extent of pH decline (within 24h after slaughter); SF, shear force; WHC, water holding capability.

of important metabolites (maltotriose, pyruvate, L-ascorbic acid, chenodeoxycholate, D-glucose 6-phosphate, glutathione, etc.), FAs, AAs and related metabolic pathways involved in regulating meat quality in Black Tibetan sheep's LL. This was achieved through changes in the abundance of rumen bacteria (increased abundance of *Christensenellaceae R-7 group*, *[Eubacterium] coprostanoligenes group*, *Methanobrevibacter*, *Ruminococcus 2* and *Quinella*, decreased abundance of *Lactobacillus*, *Prevotella 1* and *Rikenellaceae RC9 gut group*, and the tendency for decreased abundance of *Selenomonas 1* and *Succinivibrio*). Altogether, the results suggested that indoor feeding regimes can improve the overall quality of Black Tibetan sheep's meat.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: NCBI SRA (accession: PRJNA895394). Access Links: http://www.ncbi.nlm.nih.gov/bioproject/895394.

Ethics statement

This study was approved by the Animal Ethics Committee of Qinghai University (QUA-2020-0709) and was carried out at the Black Tibetan Sheep Breeding Center in Guinan County of Qinghai Province, China. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

XZ and LH: conceptualization, data curation, formal analysis, investigation, methodology, software, validation, writing-original draft, and writing-review and editing. SH and LG: data curation, formal analysis, visualization, software, validation, writing-review and editing, and funding acquisition. BY, ZW, YM, SR, RM, ZA, and SI: validation, visualization, writing-review and editing, and methodology. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1076675/full#supplementary-material

References

Aurousseau, B., Bauchart, D., Calichon, E., Micol, D., and Priolo, A. (2004). Effect of grass or concentrate feeding systems and rate of growth on triglyceride and phospholipid and their fatty acids in the *M. longissimus thoracis* of lambs. *Meat Sci.* 66, 531–541. doi: 10.1016/S0309-1740(03)00156-6

Bai, J., Yao, X., Jiang, L., Qiu, T., Liu, S., Qi, B., et al. (2016). Taurine protects against As2O3-induced autophagy in pancreas of rat offsprings through Nrf2/Trx pathway. *Biochimie* 123, 1–6. doi: 10.1016/j.biochi.2016.01.002

Baliou, S., Adamaki, M., Ioannou, P., Pappa, A., Panayiotidis, M. I., Spandidos, D. A., et al. (2021). Protective role of taurine against oxidative stress (review). *Mol. Med. Rep.* 24, 1–19. doi: 10.3892/mmr.2021.12242

Bendall, J. R. (1978). Variability in rates of pH fall and of lactate production in the muscles on cooling beef carcasses. *Meat Sci.* 2, 91–104. doi: 10.1016/0309-1740(78)90010-4

Boughalmi, A., and Araba, A. (2016). Effect of feeding management from grass to concentrate feed on growth, carcass characteristics, meat quality and fatty acid profile of Timahdite lamb breed. *Small Rumin. Res.* 144, 158–163. doi: 10.1016/j.smallrumres.2016.09.013

Boyer, J. L. (2013). Bile formation and secretion. *Comprehen. Physiol.* 3, 1035–1078. doi: 10.1002/cphy.c120027

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

Cai, Z.-W., Zhao, X.-F., Jiang, X.-L., Yao, Y.-C., Zhao, C.-J., Xu, N.-Y., et al. (2010). Comparison of muscle amino acid and fatty acid composition of castrated and Uncastrated male pigs at different slaughter ages. *Ital. J. Anim. Sci.* 9, 173–178. doi: 10.4081/ijas.2010.e33

Chambaz, A., Scheeder, M. R. L., Kreuzer, M., and Dufey, P. A. (2003). Meat quality of Angus, Simmental, Charolais and Limousin steers compared at the same intramuscular fat content. *Meat Sci.* 63, 491–500. doi: 10.1016/S0309-1740(02)00109-2

Chikwanha, O. C., Vahmani, P., Muchenje, V., Dugan, M. E. R., and Mapiye, C. (2018). Nutritional enhancement of sheep meat fatty acid profile for human health and wellbeing. *Food Res. Int.* 104, 25–38. doi: 10.1016/j.foodres.2017.05.005

Choe, J. H., Choi, Y. M., Lee, S. H., Shin, H. G., Ryu, Y. C., Hong, K. C., et al. (2008). The relation between glycogen, lactate content and muscle fiber type composition, and their influence on postmortem glycolytic rate and pork quality. *Meat Sci.* 80, 355–362. doi: 10.1016/j.meatsci.2007.12.019

Choi, Y.-M., Ryu, Y.-C., Lee, S.-H., and Kim, B.-C. (2005). Relationships between myosin light chain isoforms, muscle fiber characteristics, and meat quality traits in porcine Longissimus muscle. *Food Sci. Biotechnol.* 14, 639–644.

Cunha, I. S., Barreto, C. C., Costa, O. Y. A., Bomfim, M. A., Castro, A. P., Kruger, R. H., et al. (2011). Bacteria and Archaea community structure in the rumen microbiome of goats (Capra hircus) from the semiarid region of Brazil. *Anaerobe* 17, 118–124. doi: 10.1016/j.anaerobe.2011.04.018

da Silva, P. C. G., Brandão Ferreira Ítavo, C. C., Vinhas Ítavo, L. C., De Nadai Bonin Gomes, M., Dias Feijó, G. L., Monteiro Ferelli, K. L. S., et al. (2020). Carcass traits and meat quality of Texel lambs raised in Brachiaria pasture and feedlot systems. *Anim. Sci. J.* 91:13394. doi: 10.1111/asj.13394

de Zawadzki, A., Arrivetti, L. O. R., Vidal, M. P., Catai, J. R., Nassu, R. T., Tullio, R. R., et al. (2017). Mate extract as feed additive for improvement of beef quality. *Food Res. Int.* 99, 336–347. doi: 10.1016/j.foodres.2017.05.033

Dinh, T. T. N., Legako, J. F., Miller, M. F., and Brooks, J. C. (2018). Effects of USDA quality grade and cooking on water-soluble precursors of beef flavor. *Meat Sci.* 146, 122–130. doi: 10.1016/j.meatsci.2018.08.008

Du, M., Yang, C., Liang, Z., Zhang, J., Yang, Y., Ahmad, A. A., et al. (2021). Dietary energy levels affect carbohydrate metabolism-related bacteria and improve meat quality in the *Longissimus thoracis* muscle of yak (Bos grunniens). *Front. Vet. Sci.* 8:718036. doi: 10.3389/fvets.2021.718036

Evans, N. J., Brown, J. M., Murray, R. D., Getty, B., Birtles, R. J., Hart, C. A., et al. (2011). Characterization of novel bovine gastrointestinal tract Treponema isolates and comparison with bovine digital dermatitis treponemes. *Appl. Environ. Microbiol.* 77, 138–147. doi: 10.1128/AEM.00993-10

Fernandez-Turren, G., Repetto, J. L., Arroyo, J. M., Pérez-Ruchel, A., and Cajarville, C. (2020). Lamb fattening under intensive pasture-based systems: a review. *Animals* 10:382. doi: 10.3390/ani10030382

Ferrandino, G., Kaspari, R. R., Spadaro, O., Reyna-Neyra, A., Perry, R. J., Cardone, R., et al. (2017). Pathogenesis of hypothyroidism-induced NAFLD is driven by intra- and extrahepatic mechanisms. *Proc. Natl. Acad. Sci. U. S. A.* 114, 9172–9180. doi: 10.1073/pnas.1707797114

Ghimire, S., Kohn, R. A., Gregorini, P., White, R. R., and Hanigan, M. D. (2017). Representing interconversions among volatile fatty acids in the Molly cow model. *J. Dairy Sci.* 100, 3658–3671. doi: 10.3168/jds.2016-11858

Hernández Salueña, B., Sáenz Gamasa, C., Diñeiro Rubial, J. M., and Alberdi Odriozola, C. (2019). CIELAB color paths during meat shelf life. *Meat Sci.* 157:107889. doi: 10.1016/j.meatsci.2019.107889

Hingst, J. R., Bjerre, R. D., Wojtaszewski, J. F. P., and Jensen, J. (2019). Rapid radiochemical filter paper assay for determination of hexokinase activity and affinity for glucose-6-phosphate. *J. Appl. Physiol.* 127, 661–667. doi: 10.1152/japplphysiol.00207.2018

Hou, X., Liu, Q., Meng, Q., Wang, L., Yan, H., Zhang, L., et al. (2020). TMT-based quantitative proteomic analysis of porcine muscle associated with postmortem meat quality. *Food Chem.* 328:127133. doi: 10.1016/j.foodchem.2020.127133

Hussain, Z., Li, X., Zhang, D., Hou, C., Ijaz, M., Bai, Y., et al. (2021). Influence of adding cinnamon bark oil on meat quality of ground lamb during storage at 4 °C. *Meat Sci.* 171:108269. doi: 10.1016/j.meatsci.2020.108269

Huws, S. A., Kim, E. J., Lee, M. R. F., Scott, M. B., Tweed, J. K. S., Pinloche, E., et al. (2011). As yet uncultured bacteria phylogenetically classified as Prevotella, Lachnospiraceae incertae sedis and unclassified Bacteroidales, Clostridiales, and Ruminococcaceae may play a predominant role in ruminal biohydrogenation. *Environ. Microbiol.* 13, 1500–1512. doi: 10.1111/j.1462-2920.2011.02452.x

Kim, Y. H., Frandsen, M., and Rosenvold, K. (2011). Effect of ageing prior to freezing on color stability of ovine longissimus muscle. *Meat Sci.* 88, 332–337. doi: 10.1016/j.meatsci.2010.12.020

Kim, M.-S., Park, J.-Y., Namkoong, C., Jang, P.-G., Ryu, J.-W., Song, H.-S., et al. (2004). Anti-obesity effects of α-lipoic acid mediated by suppression of hypothalamic AMP-activated protein kinase. *Nat. Med.* 10, 727–733. doi: 10.1038/nm1061

Lee, M. H. (1995). Official methods of analysis of AOAC international. *Trends Food Sci. Technol.* 6:382. doi: 10.1016/0924-2244(95)90022-5

Li, R. W., Connor, E. E., Li, C., Baldwin Vi, R. L., and Sparks, M. E. (2012). Characterization of the rumen microbiota of pre-ruminant calves using metagenomic tools. *Environ. Microbiol.* 14, 129–139. doi: 10.1111/j.1462-2920.2011.02543.x

Liyuan, Z., Xinhua, X., Qian, Z., Jia, Z., Ming, L., Miao, Y., et al. (2018). Improved glucose and lipid metabolism in the early life of female offspring by maternal dietary Genistein is associated with alterations in the gut microbiota. *Front. Endocrinol.* 9:516. doi: 10.3389/fendo.2018.00516

Madruga, M. S., Elmore, J. S., Oruna-Concha, M. J., Balagiannis, D., and Mottram, D. S. (2010). Determination of some water-soluble aroma precursors in goat meat and their enrolment on flavor profile of goat meat. *Food Chem.* 123, 513–520. doi: 10.1016/j.foodchem.2010.04.004

Matthews, C., Crispie, F., Lewis, E., Reid, M., O'Toole, P. W., and Cotter, P. D. (2019). The rumen microbiome: a crucial consideration when optimizing milk and meat production and nitrogen utilization efficiency. *Gut Microbes* 10, 115–132. doi: 10.1080/19490976.2018.1505176

Molendi-Coste, O., Legry, V., and Leclercq, I. A. (2011). Why and how meet n-3 PUFA dietary recommendations? *Gastroenterol. Res. Pract.* 2011:364040. doi: 10.1155/2011/364040

Morales Gómez, J. F., Antonelo, D. S., Beline, M., Pavan, B., Bambil, D. B., Fantinato-Neto, P., et al. (2021). Feeding strategies impact animal growth and beef color and tenderness. *Meat Sci.* 183:108599. doi: 10.1016/J.MEATSCI.2021.108599

Moreno, I., Lipová, P., Ladero, L., Fernández-García, J. L., and Cava, R. (2020). Glycogen and lactate contents, pH and meat quality and gene expression in muscle *Longissimus dorsi* from Iberian pigs under different rearing conditions. *Livest. Sci.* 240:104167. doi: 10.1016/j.livsci.2020.104167

Muroya, S., Ueda, S., Komatsu, T., Miyakawa, T., and Ertbjerg, P. (2020). Meatabolomics: muscle and meat metabolomics in domestic animals. *Meta* 10:188. doi: 10.3390/metabo10050188

Nair, M. N., Suman, S. P., Li, S., Joseph, P., and Beach, C. M. (2014). Lipid oxidation–induced oxidation in emu and ostrich myoglobins. *Meat Sci.* 96, 984–993. doi: 10.1016/j.meatsci.2013.08.029

Nathani, N. M., Patel, A. K., Mootapally, C. S., Reddy, B., Shah, S. V., Lunagaria, P. M., et al. (2015). Effect of roughage on rumen microbiota composition in the efficient feed converter and sturdy Indian Jaffrabadi buffalo (*Bubalus bubalis*). *BMC Genomics* 16:1116. doi: 10.1186/s12864-015-2340-4

O'Quinn, T. G., Legako, J. F., Brooks, J. C., and Miller, M. F. (2018). Evaluation of the contribution of tenderness, juiciness, and flavor to the overall consumer beef eating experience. *Transl. Anim. Sci.* 2, 26–36. doi: 10.1093/tas/txx008

Papi, N., Mostafa-Tehrani, A., Amanlou, H., and Memarian, M. (2011). Effects of dietary forage-to-concentrate ratios on performance and carcass characteristics of growing fat-tailed lambs. *Anim. Feed Sci. Technol.* 163, 93–98. doi: 10.1016/j.anifeedsci.2010.10.010

Patel, V., Patel, A. K., Parmar, N. R., Patel, A. B., Reddy, B., and Joshi, C. G. (2014). Characterization of the rumen microbiome of Indian Kankrej cattle (*Bos indicus*) adapted to different forage diet. *Appl. Microbiol. Biotechnol.* 98, 9749–9761. doi: 10.1007/s00253-014-6153-1

Peng, J., Liang, C., Niu, Y., Jiang, W., Wang, W., and Wang, L. (2015). Moderate grazing promotes genetic diversity of Stipa species in the inner Mongolian steppe. *Landsc. Ecol.* 30, 1783–1794. doi: 10.1007/s10980-015-0227-z

Ponnampalam, E. N., Burnett, V. F., Norng, S., Hopkins, D. L., Plozza, T., and Jacobs, J. L. (2016). Muscle antioxidant (vitamin E) and major fatty acid groups, lipid oxidation and retail color of meat from lambs fed a roughage based diet with flaxseed or algae. *Meat Sci.* 111, 154–160. doi: 10.1016/j.meatsci.2015.09.007

Prache, S., Schreurs, N., and Guillier, L. (2021). Review: factors affecting sheep carcass and meat quality attributes. *Animal* 16:100330. doi: 10.1016/J.ANIMAL.2021.100330

Priolo, A., Micol, D., and Agabriel, J. (2001). Effects of grass feeding systems on ruminant meat color and flavor: A review. *Anim. Sci.* 50, 185–200. doi: 10.1051/animres:2001125

Rohini, A., Agrawal, N., Kumar, H., and Kumar, V. (2018). Emerging role of branched chain amino acids in metabolic disorders: a mechanistic review. *PharmaNutrition* 6, 47–54. doi: 10.1016/j.phanu.2018.01.003

Saglimbene, V. M., Wong, G., Ruospo, M., Palmer, S. C., Campbell, K., Larsen, V. G., et al. (2017). Dietary n-3 polyunsaturated fatty acid intake and all-cause and cardiovascular mortality in adults on hemodialysis: the DIET-HD multinational cohort study. *Clin. Nutr.* 38, 429–437. doi: 10.1016/j.clnu.2017.11.020

Saleem, F., Bouatra, S., Guo, A. C., Psychogios, N., Mandal, R., Dunn, S. M., et al. (2013). The bovine ruminal fluid metabolome. *Metabolomics* 9, 360–378. doi: 10.1007/s11306-012-0458-9

Scollan, N. D., Dannenberger, D., Nuernberg, K., Richardson, I., MacKintosh, S., Hocquette, J.-F., et al. (2014). Enhancing the nutritional and health value of beef lipids and their relationship with meat quality. *Meat Sci.* 97, 384–394. doi: 10.1016/j.meatsci.2014.02.015

Shabat, S. K. B., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Berg Miller, M. E., et al. (2016). Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. *ISME J.* 10, 2958–2972. doi: 10.1038/ismej.2016.62

Shojadoost, B., Yitbarek, A., Alizadeh, M., Kulkarni, R. R., Astill, J., Boodhoo, N., et al. (2021). Centennial review: effects of vitamins a, D, E, and C on the chicken immune system. *Poult. Sci.* 100:100930. doi: 10.1016/j.psj.2020.12.027

Simopoulos, A. P. (2002). The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed. Pharmacother.* 56, 365–379. doi: 10.1016/S0753-3322(02)00253-6

Simopoulos, A. P. (2008). The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Exp. Biol. Med.* 233, 674–688. doi: 10.3181/0711-MR-311

Sinha, R. A., Bruinstroop, E., Singh, B. K., and Yen, P. M. (2019). Nonalcoholic fatty liver disease and hypercholesterolemia: roles of thyroid hormones, metabolites, and agonists. *Thyroid* 29, 1173–1191. doi: 10.1089/thy.2018.0664

Smith, S. B., Blackmon, T. L., Sawyer, J. E., Miller, R. K., Baber, J. R., Morrill, J. C., et al. (2018). Glucose and acetate metabolism in bovine intramuscular and subcutaneous adipose tissues from steers infused with glucose, propionate, or acetate. *J. Anim. Sci.* 96, 921–929. doi: 10.1093/jas/sky017

Spence, C., Wells, W. G., and Smith, C. J. (2006). Characterization of the primary starch utilization operon in the obligate anaerobe *Bacteroides fragilis*: regulation by carbon source and oxygen. *J. Bacteriol.* 188, 4663–4672. doi: 10.1128/JB.00125-06

Tang, B. L. (2019). Neuroprotection by glucose-6-phosphate dehydrogenase and the pentose phosphate pathway. *J. Cell. Biochem.* 120, 14285–14295. doi: 10.1002/jcb.29004

Torrecilhas, J. A., San Vito, E., Fiorentini, G., de Souza, C. P., Simioni, T. A., Lage, J. F., et al. (2021). Effects of supplementation strategies during the growing phase on meat quality of beef cattle finished in different systems. *Livest. Sci.* 247:104465. doi: 10.1016/j.livsci.2021.104465

Turner, K. E., Belesky, D. P., Cassida, K. A., and Zerby, H. N. (2014). Carcass merit and meat quality in Suffolk lambs, Katahdin lambs, and meat-goat kids finished on a grass–legume pasture with and without supplementation. *Meat Sci.* 98, 211–219. doi: 10.1016/j.meatsci.2014.06.002

Veiseth, E., Shackelford, S. D., Wheeler, T. L., and Koohmaraie, M. (2004). Factors regulating lamb longissimus tenderness are affected by age at slaughter. *Meat Sci.* 68, 635–640. doi: 10.1016/j.meatsci.2004.05.015

Wallace, R. J., McKain, N., Broderick, G. A., Rode, L. M., Walker, N. D., Newbold, C. J., et al. (1997). Peptidases of the rumen bacterium, *Prevotella ruminicola*. *Anaerobe* 3, 35–42. doi: 10.1006/anae.1996.0065

Wang, B., Luo, Y., Su, R., Yao, D., Hou, Y., Liu, C., et al. (2020a). Impact of feeding regimens on the composition of gut microbiota and metabolite profiles of plasma and feces from Mongolian sheep. *J. Microbiol.* 58, 472–482. doi: 10.1007/s12275-020-9501-0

Wang, B., Luo, Y., Wang, Y., Wang, D., Hou, Y., Yao, D., et al. (2020b). Rumen bacteria and meat fatty acid composition of Sunit sheep reared under different feeding regimens in China. *J. Sci. Food Agric.* 101, 1100–1110. doi: 10.1002/jsfa.10720

Wang, B., Wang, Y., Zuo, S., Peng, S., Wang, Z., Zhang, Y., et al. (2021). Untargeted and targeted metabolomics profiling of muscle reveals enhanced meat quality in artificial pasture grazing Tan lambs via rescheduling the rumen bacterial community. *J. Agric. Food Chem.* 69, 846–858. doi: 10.1021/acs.jafc.0c06427

Wicks, J., Beline, M., Gomez, J. F. M., Luzardo, S., Silva, S. L., and Gerrard, D. (2019). Muscle energy metabolism, growth, and meat quality in beef cattle. *Agriculture* 9:195. doi: 10.3390/agriculture9090195

Wood, J. D., and Enser, M. (1997). Factors influencing fatty acids in meat and the role of antioxidants in improving meat quality. *Br. J. Nutr.* 78, S49–S60. doi: 10.1079/BJN19970134

Wu, G. (2009). Amino acids: metabolism, functions, and nutrition. *Amino Acids* 37, 1–17. doi: 10.1007/s00726-009-0269-0

Xu, Z., Cao, L., Liu, J., Tan, H., and Deng, Z. (2017). Evaluation of the diversity of probiotic bacillus, clostridium, and Bifidobacterium using the Illumina-based sequencing method. *Probio. Antimicrob. Prot.* 10, 748–754. doi: 10.1007/s12602-017-9337-z

Xue, D., Chen, H., Zhao, X., Xu, S., Hu, L., Xu, T., et al. (2017). Rumen prokaryotic communities of ruminants under different feeding paradigms on the Qinghai-Tibetan plateau. *Syst. Appl. Microbiol.* 40, 227–236. doi: 10.1016/j.syapm.2017.03.006

Yeligar, S. M., Machida, K., and Kalra, V. K. (2010). Ethanol-induced HO-1 and NQO1 are differentially regulated by HIF-1α and Nrf2 to attenuate inflammatory

cytokine expression. *J. Biol. Chem.* 285, 35359–35373. doi: 10.1074/jbc.M110.138636

Zhang, X., Han, L., Hou, S., Raza, S. H. A., Gui, L., Sun, S., et al. (2022). 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.* 9:915558. doi: 10.3389/fnut.2022.915558

Zhang, X., Han, L., Hou, S., Raza, S. H. A., Wang, Z., Yang, B., et al. (2021). Effects of different feeding regimes on muscle metabolism and its association with meat quality of Tibetan sheep. *Food Chem.* 374:131611. doi: 10.1016/J.FOODCHEM.2021.131611

Zhang, H., Shao, M., Huang, H., Wang, S., Ma, L., Wang, H., et al. (2018). The dynamic distribution of small-tail Han sheep microbiota across different intestinal segments. *Front. Microbiol.* 9:32. doi: 10.3389/fmicb.2018.00032

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Effects of milk replacer feeding level on growth performance, rumen development and the ruminal bacterial community in lambs

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Feeding with a suitable level of milk replacer (MR) can improve the survival rate and stimulate the growth potential of early lambs. However, feeding excessive MR might be detrimental to rumen development and microbial colonization. Herein, we investigated the effects of feeding different levels of MR on rumen digestive function and ruminal microorganisms. Fourteen healthy male Hu lambs with similar birth weights and detailed pedigree records were divided into two groups to receive low (2% of average body weight per day) and high (4% of average body weight per day) levels of MR. We analyzed the effects of the MR feeding level on growth performance, fiber degradation rates, rumen fermentation parameters, enzyme activities and rumen histomorphology. We found that feeding with a high level of MR improved the average daily gain of early lambs, but decreased the starter intake, rumen weight and papillae length. We also analyzed the effects of the MR feeding level on the rumen microbiota using 16S-rRNA amplicon sequencing data. The results showed that high a MR feeding level increased the rumen microbial diversity but decreased the abundance of many carbohydrate degrading bacteria. Several bacterial genera with significant differences correlated positively with rumen cellulase activity and the acid detergent fiber degradation rate. Our results suggested that a high level of MR could improve the growth performance of early lambs in the short term; however, in the long term, it would be detrimental to rumen development and have adverse effects on the adaptation process of the microbiota to solid feed.

KEYWORDS

lambs, milk replacer, rumen development, rumen fermentation, fiber degradation, rumen microbiota

1. Introduction

Artificial rearing of lambs using milk replacer (MR) is widely applied in sheep husbandry because of insufficient milk secretion of ewes, mastitis, postpartum paralysis and other reasons (Belanche et al., 2019; McCoard et al., 2020; Arshad et al., 2021; Mialon et al., 2021). Artificial feeding to make up for the lack of breast milk can increase the survival rate of lambs, shorten the breeding cycle of ewes, and improve animal welfare. In China, Hu sheep constitute an important livestock for lamb production because of their excellent prolificacy, rapid growth, and ability to adapt to poor-quality feeds and diverse environments (Yue, 1996; He et al., 2019). However, multiparous ewes also easily lead to insufficient breastmilk for lambs. Artificial rearing is important for the utilization of surplus lambs from dairy sheep systems, and lambs from meat sheep operations that are not able to be naturally reared to generate additional revenue from meat production (McCoard et al., 2020). Therefore, the rational utilization of MR significant to improve the survival rate and growth performance of lambs.

MR is an artificial milk produced by replacing milk protein with non-milk protein based on the nutritional standards of breast milk (Li et al., 2021). The nutritional components and physical form of MR are similar to breast milk, and its quality is not easily affected by the external environment (Toral et al., 2015). Evidence suggests that feeding lambs with MR could accelerate growth performance, has long-term benefits for sheep health (Zhang et al., 2019), enhance immunity, reduce the stress reaction caused by sudden changes of diets (Amdi et al., 2021), and affect post-weaning starter intake and average daily gain (Chapman et al., 2016). The beneficial effects of MR administration in early-weaning lambs also function *via* regulating their rumen microbiota (Bhatt et al., 2009).

However, high levels of MR might be detrimental to rumen development in early lambs and affect their rumen microbiota. The rumen of a newborn ruminant is not completely developed and does not contain a fully established microflora (Ekiz et al., 2016); early lambs rumen function is similar to monogastric animals (Longenbach and Heinrichs, 1998). The period from birth to 2 months of age is a critical stage of rumen development, representing a time window to regulate microbial colonization in lambs, and the feeding strategy in the first few weeks of life has been reported to affect rumen development (Carballo et al., 2019). Although little research has been conducted on the effects of MR feeding level on the rumen development, excessive intake of MR might reduce the intake of starter feed, which is essential for rumen development. Reports suggested that feeding high levels of MR could reduce post-weaning starter digestibility, especially for NDF (neutral detergent fiber) and ADF (acid detergent fiber; Terré et al., 2006; Hill et al., 2010). Low digestibility in young ruminants fed large quantities of MR is likely to be associated with suboptimal development of the rumen (Hill et al., 2016). These changes might be closely related to the rumen microbiota. Furman et al. proposed that both deterministic effects, driven by age and diet, and stochastic effects, driven by early colonization events, shape the composition of the rumen microbiome throughout life (Furman et al., 2020). MR is usually fed quantitatively in artificially reared lambs, and the feeding level varies greatly in different farms. Therefore, it is important to clarify the effect of the MR level on the rumen microbiota and its relationship with lamb development, and to balance rumen development and microbiota colonization using an appropriate feeding MR level. In recent years, several studies have examined the length of MR feeding period on rumen fermentation and microbial diversity (Carballo et al., 2019; Zhang et al., 2019; Mao et al., 2021), but relatively few have been directed at assessing how MR feeding level can affect rumen development. The appropriate MR level for lambs and the effect of feeding MR on rumen development and microbial colonization remains unclear.

Thus, in the present study, we hypothesized that feeding early lambs with intensive MR would affect rumen function and the rumen microbiota through changes in feed structure and intake, thereby affecting performance. We analyzed the growth performance and starter intake of lambs fed with different levels of MR, and evaluated the development and functionality of the rumen *via* ruminal fiber degradation rates, rumen weight, fermentation parameters, enzyme activities and rumen histomorphology. Furthermore, we employed 16S rRNA sequencing to explore the effect of the MR feeding level on the rumen microbiota and its relationship with rumen function and development. A detailed understanding of the regulation of early rumen development (function, morphology, and colonization) could provide the basis for the rational use of early nutritional regulation strategies to improve the productivity and health of lambs.

2. Materials and methods

2.1. Experimental design and animal handling

The experimental animals were selected from a commercial sheep farm (Minqin Zhongtian Sheep Industry Co. Ltd., Gansu, China), and 14 Hu male lambs with detailed pedigree records were randomly divided into two groups according to the principle of similar body weight (mean ± SD: 3.29 ± 0.68 kg). They were divided into the low MR feeding level group (L, 2% of average body weight per day). The L group received a traditional MR feeding quantity, which was 2% of average body weight per day, following the feeding guidelines of producer (Beijing Precision Animal Nutrition Research Center, Beijing, China; Yue et al., 2011). The H group received an intensive MR feeding quantity at 4% of average body weight per day, which has been reported to have a great impact on the growth performance of early lambs (Zhang et al., 2019). The MR contains 96.91% dry matter (DM), 23.22% protein, and 13.20% fat. All lambs were kept indoors with ewes to ensure adequate colostrum intake for 3 days after birth, from 3 days old for training to consuming MR, and to 7 days old to completely replace breast milk with MR and start feeding with

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the same starter. The daily MR was subdivided in three parts, dissolved in five times the weight of warm water (40 ± 1°C) and was artificially fed at 09:00, 15:00, and 21:00. All experimental lambs were reared in single cages (1.2 m × 1 m × 1 m; 1.2 m^3) with free access to the diet [the formula of the diet met the requirements of the Standards for Feeding Sheep and Goats for Meat issued by China (NYT816-2004)] and water. The diet formula and nutritional composition are shown in Table 1 (Huang et al., 2022). The experimental lambs were slaughtered and sampled at 49 days old.

2.2. Measurement of growth performance and starter diet intake

We first examined the effect of the MR feeding level on the growth performance and starter intake of lambs. All lambs were weighed at birth and then weighed every 7 days to calculate the average body weight and average daily gain, and to adjust the MR feeding scale. Starting from 7 days old, the starter intake of each lamb was recorded daily as the difference between offered and refused feed, and the average intake was calculated.

TABLE 1 Ingredients and chemical composition of starter diet and milk replacer (air-dried basis).

Items	Starter[1]	Milk replacer
Ingredients [%]		
Alfalfa meal	18.50	
Corn	21.00	
Extruded corn	22.30	
Bran	6.00	
Soybean meal	21.50	
Extruded soybean	4.00	
Corn gluten meal	5.00	
Limestone	0.30	
Premix[2]	1.00	
NaCl	0.40	
Total	100.00	
Chemical composition		
DM (%)	90.96	96.91
DE ($MJ{\cdot}kg^{-1}$)	13.01	/
CP (%)	19.50	23.22
Fat (%)	1.33	13.20
Starch (%)	33.10	0.00
NDF (%)	18.87	0.00
ADF (%)	8.60	0.00

[1]The starter was pelleted. [2]Premix provided the following per kg of the starter: 25 mg Fe as $FeSO_4{\cdot}H_2O$; 40 mg Zn as $ZnSO_4{\cdot}H_2O$; 8 mg Cu as $CuSO_4{\cdot}5H_2O$; 40 mg Mn as $MnSO_4{\cdot}H_2O$; 0.3 mg I as KI; 0.2 mg Se as Na_2SeO_3; 0.1 mg Co as $CoCl_2$; 940 IU vitamin A;111 IU vitamin D;20 IU vitamin E, and; 0.02 mg vitamin B12.

2.3. Sample collection

All the experimental lambs were slaughtered at 49 days old after a fasting period of 12 h. After the rumen contents were mixed evenly, the rumen pH was measured immediately using an acidity meter (Sartorius PB-10, Sartorius Biotech Inc., Gottingen, Germany), the mixed rumen contents were collected in sterile tubes and stored at-80°C for rumen microbial and fiber degradation rate analysis. The contents were filtered through four layers of sterilized medical gauze, packed in cryovials, and stored in-20°C refrigerator for ruminal fermentation and enzymic activity analysis. After sampling, the rumen was weighed, segments of the ruminal tissue were collected at the location of cranial ventral sac and fixed in 4% paraformaldehyde for morphology measurements.

2.4. Measurement of the rumen fiber degradation rate

To better understand how the MR feeding level affected rumen function in lambs, we examined the ruminal NDF and ADF degradation rates after a 12 h fast. The rumen degradation rates of neutral detergent fiber (NDF) and acid detergent fiber (ADF) after 12 h of fasting were determined using the acid-insoluble ash method (Keulen and Young, 1977). The rumen contents and starter were dried at 65°C for 8 h, and then the contents of NDF, ADF, and acid-insoluble ash were determined, respectively. The NDF and ADF were determined following the method of Van Soest et al. (1991), and acid infusible ash was determined according to the method reported by Soltani et al. (2020). The degradation rates of NDF and ADF were evaluated using the acid-insoluble ash as internal markers and were calculated according to the indirect digestibility method (Keulen and Young, 1977). The calculation of the NDF or ADF degradation rate was as follows:

$$\text{NDF or ADF degradation rate (\%)} = [1-(A/B)\times(FB/FA)]\times 100,$$

where A was the acid-insoluble ash concentrations in the starter, B was the acid-insoluble ash concentrations in the rumen contents, FA was the NDF or ADF concentrations in the starter, and FB was the NDF or ADF concentrations in the rumen contents.

2.5. Measurement of the rumen metabolic phenotypes and digestive enzymatic activity

Determination of volatile fatty acids (VFA) used metaphosphorylated rumen fluid and a gas chromatographic method (Zhang Y. et al., 2021). For VFA determination, the supernatant was carefully collected and filtered through a 0.45-μm syringe filter. The clear supernatant was transferred to a vial for gas chromatography (GC). The VFA values were determined using a TRACE-1300 series GC ultra-gas chromatograph (Thermo Scientific, Milan, Italy). Total ruminal nitrogen, ammonia nitrogen, urea nitrogen, the enzymatic activity of cellulase and

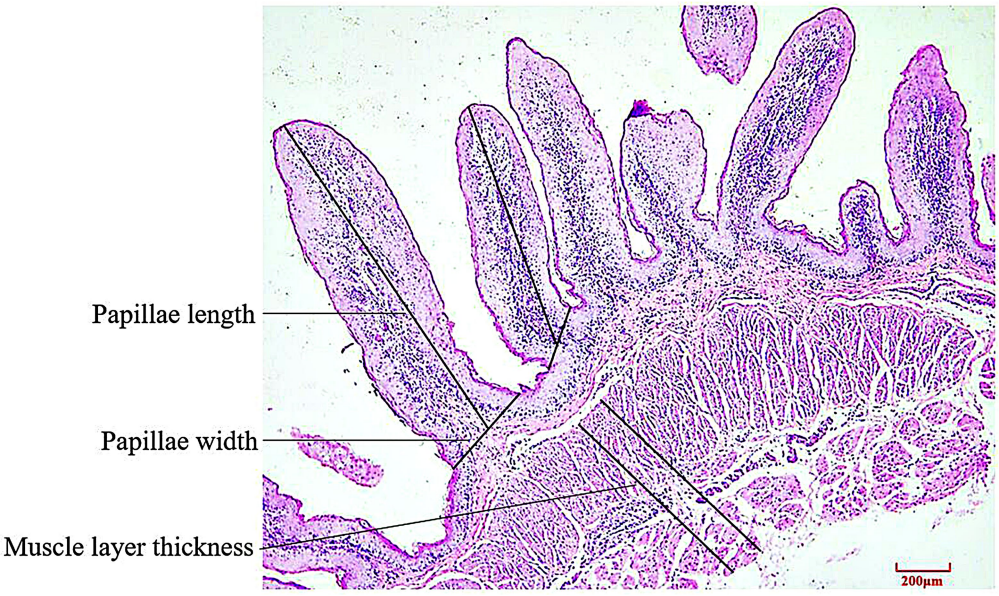

FIGURE 1
Schematic diagram of histological sections and determination of rumen tissue (HE staining).

protease were determined using commercial assay kits (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

2.6. Measurement of histomorphology in the rumen

Rumen specimens were embedded in paraffin, sectioned and stained with hematoxylin–eosin. In triplicate, 5 intact well-oriented papillae were selected for each ruminal cross section. Papillae length, papillae width and muscle layer thickness were determined using an image analysis system (Motic Image Plus 2.0, Motic China Group Co. Ltd., Xiamen, China). Papillae length was measured from the apex to the base of the papilla along its axis, papillae width was measured at bottom of papillae height and muscle layer thickness was measured from the junction between the submucosal and muscular layers to that between the muscular layer and the tunica serosa (Figure 1).

2.7. DNA extraction and high-throughput sequencing

Total DNA of rumen contents was extracted using an Omega E.Z.N.A. Stoll DNA kit (Omega Bio-Tek, Winooski, VT, United States). The sequencing process was the same as that detailed our previous study (Li et al., 2022). DNA quality and quantity were assessed using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, United States). The DNA was diluted to 50 ng/μl and amplicons were prepared for high throughput sequencing. PCR was used to amplify the V3–V4 regions of the 16S rRNA gene using the universal primers 341-F (50-CCTAYGGGRBGCASCAG-30) and 806-R (50-GGACTACNNGGGTATCTAAT-30). The reactions consisted of an initial incubation at 98°C for 3 min; followed by 21 cycles of 95°C for 30 s, 55°C for 45 s, and 72°C for 1 min; and a final extension step at 72°C for 7 min. Bar-coded amplicons were mixed at equimolar ratios, used for Illumina paired-end library preparation and cluster generation, and sequenced on an Illumina Hiseq2500 instrument (San Diego, CA, United States) to generate 250 bp paired-end reads.

2.8. Sequence and statistical analysis

Paired-end reads were merged using FLASH (V1.2.7),[1] and the merged sequences were termed raw tags. The raw tags were filtered through a quality control pipeline using the Quantitative Insight into Microbial Ecology (QIIME) tool kit. The effective tags were assigned to operational taxonomic units (OTUs) with a 97% identity threshold using the Uparse (v7.0.1001),[2] and taxonomy was assigned using SILVA[3] in mothur with a 0.80 confidence threshold. Alpha diversity analysis was applied to assess the complexity of species diversity for a sample by using four indices: Chao1, Shannon, Simpson, ACE, and Observed species. These indices were

1 http://ccb.jhu.edu/software/FLASH/
2 http://drive5.com/uparse/
3 http://www.arb-silva.de/

calculated using QIIME (Version 1.8.0). Beta diversity analysis was used to evaluate differences in species complexity in the samples. Beta diversity was calculated using principal coordinates analysis (PCoA) and cluster analysis in the QIIME software (Version 1.8.0).

The data for body weight (BW), average daily gain (ADG), starter intake, rumen degradation rate of fiber, rumen metabolic phenotypes, digestive enzymatic activity, rumen histomorphology, microbial alpha diversity values, and the bacterial abundance were analyzed statistically using t-tests in the SPSS software (version 25.0; IBM Corp., Armonk, NY, United States). Spearman correlation coefficients were used to evaluate the relationships between the most abundant genera and rumen function-related parameters using the R software (version 4.1.1). Statistical significance was set at $p < 0.05$, and $p < 0.001$ indicated an extremely significant difference.

3. Results

3.1. Body weight, average daily gain, and starter intake

There was no significant difference ($p > 0.05$) in BW between the groups; however, there was significant difference ($p < 0.05$) in the ADG at 7–14 and 14–21 day and starter intake (SI) at 42–49 day. In addition, the trend lines of BW and ADG were higher in the H group than in the L group, whereas the SI showed the opposite trend (Figure 2).

3.2. Ruminal fiber degradation rate

There were no significant differences in the NDF and ADF degradation rates between the groups ($p > 0.05$), nor were the contents of NDF and ADF different in the rumen digesta (Table 2).

3.3. Rumen weight, pH, fermentation parameters, and enzyme activities

The rumen weight of the L group was significantly higher than that of the H group ($p = 0.019$); however, there was no significant effect on other indices in the rumen of the lambs ($p > 0.05$; Table 3).

3.4. Ruminal histomorphology

The rumen papillae length of the L group was significantly higher than that of the H group ($p < 0.01$); however, there were no significant effects on rumen papillae width and muscle layer thickness of the lambs (both $p > 0.05$; Table 4).

3.5. Ruminal microbiota diversity and community structure

The present study used 16S rRNA gene sequencing of rumen samples to compare the differences in the rumen microbiota between the H and L groups. The rarefaction curves showed that adequate sequencing depth was achieved, and the number of observed species was close to saturation (Supplementary Figure S1A). The total number of OTUs was 870, with 635 shared OTUs detectable across the groups; with 176 and 59 endemic species in the H and L groups, respectively (Supplementary Figure S1B). The Shannon ($p = 0.042$) and Chao1 ($p = 0.047$) indices significantly increased as the MR level increased from 2 to 4%; however, there was no significant change in the other indices ($p > 0.05$; Table 5).

Based on PCoA analysis (Figures 3A,B), there was clustering in the unweighted UniFrac measurements according to the L and H MR levels. However, the ANOSIM analysis based on Bary-Curtis showed an insignificant difference between groups ($p = 0.574$; Figure 3C).

The dominant flora (Figure 4) in the rumen (relative level > 5%) were Proteobacteria, Firmicutes, and Bacteroidetes at the phylum level. The relative abundance of Fibrobacteres ($p = 0.033$) decreased significantly, and the relative abundance of Synergistetes ($p = 0.005$), Euryarchaeota ($p = 0.012$), and Verrucomicrobia ($p = 0.038$) increased significantly with the increasing MR feeding level.

The dominant flora (Table 6) in the rumen (relative level > 5%) in group L were *Succinivibrionaceae_UCG-001*, *Succinivibrio*, *Prevotella_7*, *Prevotella_1*, *Oribacterium*, and *Lachnospiraceae_NK3A20_group*, and the dominant flora in the rumen in group H were *Succinivibrionaceae_UCG-001*, *Succinivibrio*, *Prevotella_7*, *Prevotella_1*, and *Oribacterium*. Among them, *Succinivibrionaceae_UCG-001* was the first largest flora, accounting for 15.21 and 18.24% in L and H groups, respectively, and *Succinivibrio* is the second largest flora, accounting for 8.88 and 14.37% in L and H groups, respectively. The relative abundance of *Lachnospiraceae_NK3A20_group* ($p = 0.007$), *Succiniclasticum* ($p = 0.035$), and *Ruminococcus_1* ($p = 0.034$) was significantly higher in the L group, and the relative abundance of *Ruminococcaceae_UCG-014*, *Moryella,* and *Eubacterium_nodatum_group* in the L group exhibited tended to be higher than that in the H group ($p < 0.1$).

3.6. Correlation analysis of rumen weight, pH, fermentation parameters, enzyme activity, ruminal histomorphology and microorganisms

Correlation analyses of the relative abundances of the top 30 genus-level taxonomic composition and rumen weight, pH, fermentation parameters, enzyme activity, and rumen fiber degradation were performed (Figure 5). The results showed that

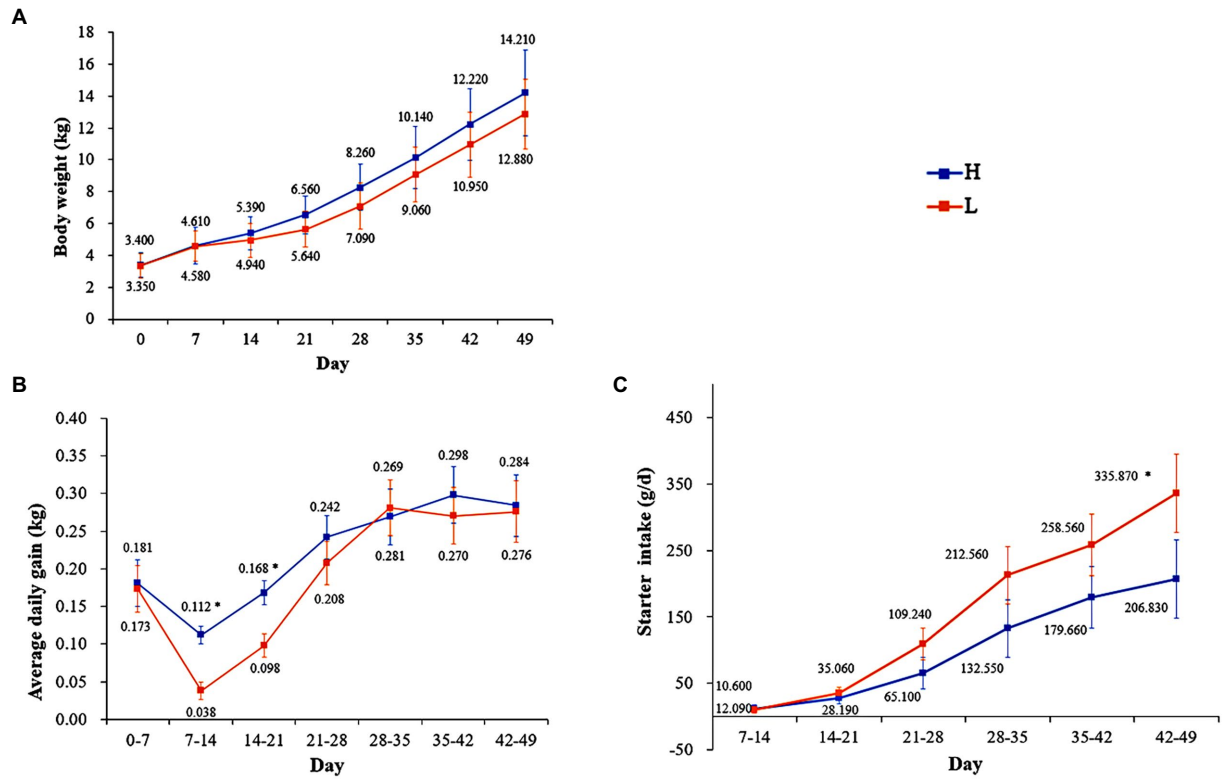

FIGURE 2
Effects of MR levels of on body weight **(A)**, average daily gain **(B)**, and starter intake **(C)**. H: high MR feeding level group, fed MR at 4% DM/kg of average body weight per day; L: low MR feeding level group, fed MR at 2% DM/kg of average body weight/d. * in the same column indicates a significant difference between two groups ($p<0.05$).

TABLE 2 Effects of MR levels of on fiber degradation rate of lambs (%).

Index*	Groups		SEM	*p* Value
	L	H		
NDF	57.20	56.8	2.70	0.915
ADF	39.60	40.60	2.10	0.746
NDFD	53.70	53.20	3.20	0.913
ADFD	30.10	26.20	5.30	0.614

*NDF-rumen neutral detergent fiber content; ADF-rumen acid detergent fiber content; NDFD-degradation rate of NDF; and ADFD - degradation rate of ADF; H: high MR feeding level group, fed MR at 4% DM/kg of average body weight per day; L: low MR feeding level group, fed MR at 2% DM/kg of average body weight per day.

the relative abundance of *Christensenellaceae_R-7_group* ($p=0.002$), *Lachnospiraceae_XPB1014_group* ($p=0.001$), *Prevotellaceae_UCG-003* ($p=0.01$), *Phocaeicola* ($p=0.001$), *Ruminococcaceae_UCG-014* ($p=0.008$), *Moryella* ($p=0.01$), and *Treponema_2* ($p=0.015$) correlated positively with cellulase activity, whereas the abundance of *Succinivibrionaceae_UCG-001* ($p=0.03$) correlated negatively with cellulase activity. The relative abundance of *Ruminococcaceae_NK4A214_group* ($p=0.023$), *Christensenellaceae_R-7_group* ($p=0.004$), *Lachnospiraceae_XPB1014_group* ($p=0.005$), *Prevotellaceae_UCG-003* ($p=0.004$), *Phocaeicola* ($p=0.005$), *Ruminococcaceae_UCG-014* ($p=0.042$), *Moryella* ($p=0.01$), and *Treponema_2* ($p=0.003$) *Ruminobacter* ($p=0.002$) correlated negatively with muscle layer thickness. The relative abundance of *Lachnospiraceae_NK3A20_group* ($p=0.047$), *Ruminococcaceae_UCG.014* ($p=0.042$), *Moryella* ($p=0.041$) correlated negatively with papillae length. *Succinivibrio* correlated positively with the ADF degradation rate ($p=0.039$) and the total VFA concentration ($p=0.024$), whereas the NDF ($p=0.02$) and ADF ($p=0.042$) degradation rates correlated positively with *Sharpea*. The valerate ($p=0.004$) concentration and rumen weight ($p=0.016$) correlated positively with *Prevotella_7*. The propionate concentration correlated negatively with *Prevotella_1* ($p<0.017$) and *Roseburia* ($p=0.041$). The isovalerate concentration ($p=0.001$), isobutyrate concentration ($p=0.023$), and pH ($p=0.07$) correlated positively with *Prevotella_1*. Total nitrogen correlated negatively correlated with *Fibrobacter* and *Roseburia* (both $p=0.04$). Protease correlated positively with *Eubacterium_nodatum_group* ($p=0.035$) and *Ruminococcaceae_NK4A214_*group ($p=0.049$), whereas it correlated negatively with *Roseburia* ($p=0.023$).

4. Discussion

To obtain a higher price for lambs and more income from a flock, it is essential to have the minimum loss of lambs and optimum growth during the neonatal phase (Poonia et al., 2015).

TABLE 3 Effects of MR levels on the rumen weight, pH, fermentation parameters and enzyme activities of lambs.

Index	Groups		SEM	*p* Value
	L	H		
Rumen weight (g)	244.56[a]	160.49[b]	21.90	0.019
PH	6.56	6.70	0.07	0.207
Total VFA* (mmol/L)	65.39	51.17	8.14	0.240
Acetate (%)	50.58	52.89	1.57	0.319
Propionate (%)	32.89	29.52	1.47	0.132
Isobutyrate (%)	2.73	3.58	0.29	0.062
Butyrate (%)	6.64	6.75	0.60	0.898
Isovalerate (%)	4.29	4.88	0.60	0.500
Valerate (%)	2.86	2.38	0.36	0.369
Cellulase (U/mg protein)	72.58	114.53	33.17	0.389
Protease (U/mg protein)	0.54	0.52	0.17	0.939
Total nitrogen (mg/ml)	4724.96	5111.40	390.47	0.497
Ammonia nitrogen (mg/ml)	96.49	100.98	4.90	0.530
Urea nitrogen (mg/ml)	35.37	34.12	5.35	0.871

*The sum of all individual volatile fatty acids (VFA). In the same row, different small letter superscripts indicate a significant difference ($p < 0.05$), H: high MR feeding level group, fed MR at 4% DM/kg of average body weight per day; L: low MR feeding level group, fed MR at 2% DM/kg of average body weight per day.

TABLE 4 Effects of MR levels on the rumen ruminal histomorphology of lambs.

Index	Groups		SEM	*p* Value
	L	H		
Papillae length	1964.36[a]	1032.59[b]	173.66	< 0.010
Papillae width	422.04	440.29	41.47	0.668
Muscle layer thickness	941.43	948.10	110.83	0.953

In the same row, different small letter superscripts indicate a significant difference ($p < 0.05$), H: high MR feeding level group, fed MR at 4% DM/kg of average body weight per day; L: low MR feeding level group, fed MR at 2% DM/kg of average body weight/d.

Meanwhile, appropriate feeding strategies to promote rumen development and microbiota establishment might be beneficial to the performance of lambs in the later period. Despite the accumulated knowledge regarding nutritional regulatory strategies during the early life of ruminants, the effects of the MR feeding level on rumen development, microbial colonization, and the regulatory mechanisms of host-microbial interactions in pre-ruminants are largely unknown.

Up to 3 weeks old, the growth performance of lambs was significantly improved by the higher level of MR feeding, although the low-level feeding group had a higher starter feed intake. The intake of solid feed in lambs before 3 weeks old is low, and the digestion and utilization efficiency of plant-based feed was much lower than that of MR (Li et al., 2022). Thus, at this stage, milk or MR provides most of the nutrients needed for growth and development, and the better bioavailability of protein and energy along with minerals, enzymes, and growth factors results in increased weight gains (Lee et al., 2009). Studies have also shown that high levels of MR can increase the ADG of lambs before weaning (Zhang et al., 2019). After the were 4 weeks old, with the rapid improvement of starter intake and digestive function, the effect of the MR feeding level on daily gain was no longer significant, indicating that the development of digestive function and the increase of starter intake are very important for the transition from liquid milk to plant feed in young lambs.

TABLE 5 Rumen microbial richness and diversity indexes.

Index	Groups		SEM	*p* Value
	L	H		
Observed	374.57	436.43	25.58	0.113
Shannon	4.61[b]	5.11[a]	0.16	0.042
Simpson	0.89	0.91	0.01	0.245
Chao1	406.24[b]	476.61[a]	22.44	0.047
ACE	411.87	481.28	22.53	0.500

In the same row, different small letter superscripts indicate a significant difference ($p < 0.05$), H: high MR feeding level group, fed MR at 4% DM/kg of average body weight per day; L: low MR feeding level group, fed MR at 2% DM/kg of average body weight per day.

It should be noted that although high levels of MR feeding improved the growth performance of early lambs, it had adverse effects on rumen development. In the present study, the rumen weight and papillae length at 49 days old was significantly lower in the high feeding level group, and the lower intake of starter was the main factor limiting rumen development. Studies have shown that feeding large volumes of milk delays solid feed intake, which might compromise rumen development before and during weaning (Jasper and Weary, 2002). An adequate starter intake can stimulate the development of the rumen, which is necessary to establish the rumen microbiota (Khan et al., 2016). In early lambs, MR bypasses the rumen to the abomasum (Zhang et al., 2019), therefore, the solid feed that enters the rumen is the decisive factor for rumen fermentation parameters. Interestingly, we did not find any differences in the main rumen fermentation parameters, enzyme activities, and fiber degradation rates between the groups in the present study. Considering that the two groups of lambs have the same diet composition, these results indicated that the composition of the starter diet was more important for rumen fermentation function than the starter intake.

Interestingly, we found that the MR feeding level had a profound influence on the rumen microbiota, which was reflected in the α-diversity, β-diversity, and taxa abundance. In this study, despite the increasing of starter intake, the Shannon and Chao1 indices of the rumen microbiota decreased in L group. Although highly diverse microbiota is generally considered beneficial for host health and is regarded as a sign of a mature gut (Konopka,

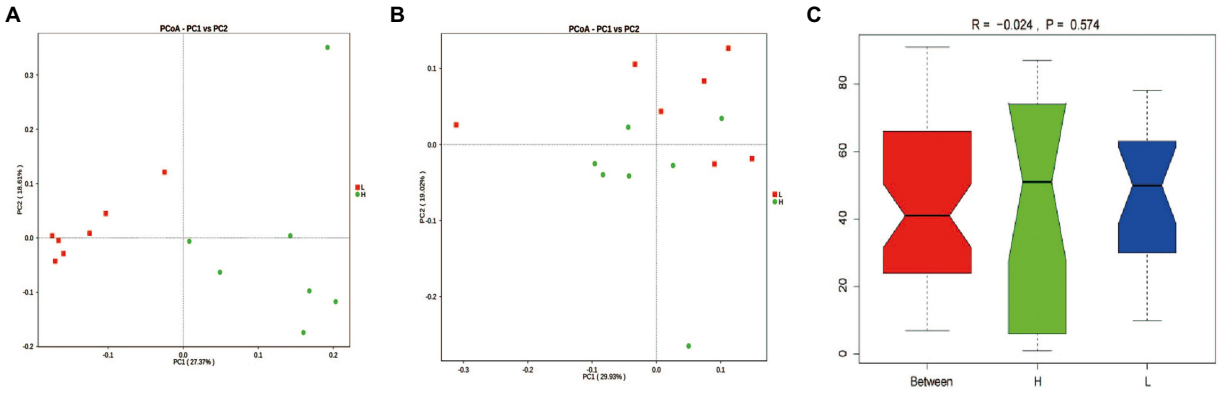

FIGURE 3
Rumen microbial OTU development. **(A)** Unweighted UniFrac and **(B)** Weighted UniFrac distances based on the relative abundance of microbial OTUs, **(C)** Bary-Curtis Anosim analysis. H: high MR feeding level group, fed MR at 4% DM/kg of average body weight per day; L: low MR feeding level group, fed MR at 2% DM/kg of average body weight per day.

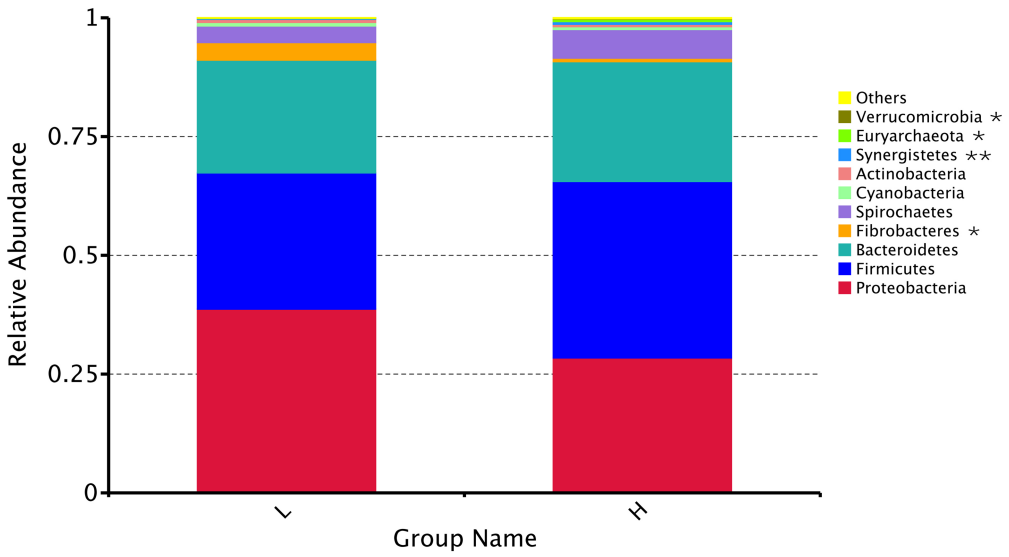

FIGURE 4
Relative abundance of the top 10 rumen microbial compositions at the phylum level. H: high MR feeding level group, fed MR at 4% DM/kg of average body weight per day; L: low MR feeding level group, fed MR at 2% DM/kg of average body weight per day. * indicates a significant difference ($p<0.05$), and ** indicates an extremely significant difference ($p<0.01$) between two groups.

2009; Le Chatelier et al., 2013), many studies have indicated that early starter intake decreased the ruminal bacterial diversity (Meale et al., 2016; Wang et al., 2016; Li et al., 2020). These findings were consistent with the results obtained from this study. We found that many bacteria genera and species disappeared in L group. The main reason may be that the increased starter intake promotes the establishment of predominant microflora and the depletion of transient bacterial species and genera. With the increase of plant-based feed intake, carbohydrate degrading bacteria occupied the widest niche, which suppressed the colonization of foreign flora, and most of the 'disappeared' bacteria are aerobic bacteria or bacteria without fermentation function (Wang et al., 2016). Besides, on average, the phyla Proteobacteria, Firmicutes, and Bacteroidetes were predominant in all samples. These findings agreed with previous studies on the ruminal microbiota (Morgavi et al., 2015). Proteobacteria are a large group of bacteria that ferment carbohydrates to ethanol, playing an important role in rumen metabolism, such as the formation and fermentation of biofilms (Zeng et al., 2017). Firmicutes represent the core bacterial component that is predominant within the rumen, mainly comprising diverse fibrolytic and cellulolytic bacterial genera. Bacteroidetes express relatively large numbers of genes encoding carbohydrate-active enzymes; thus, promoting the breakdown of structural polysaccharides in the rumen and also

TABLE 6 Genus-level taxonomic composition of the top 30 rumen microbial communities (%).

Genus	Groups		SEM	*p* Value
	L	H		
Succinivibrionaceae_UCG-001	15.21	18.24	2.99	0.632
Succinivibrio	8.88	14.37	3.31	0.430
Prevotella_7	6.03	10.29	1.74	0.234
Prevotella_1	9.48	5.40	1.78	0.267
Oribacterium	7.15	7.40	1.18	0.922
Lachnospiraceae_NK3A20_group	5.56[a]	3.05[b]	0.51	0.007
Ruminobacter	3.67	2.85	2.10	0.855
Succiniclasticum	3.98[a]	1.97[b]	0.56	0.035
Treponema_2	3.40	1.88	0.78	0.352
Fibrobacter	0.74	3.70	1.29	0.268
Sphaerochaeta	2.64	1.61	0.42	0.237
Ruminococcus_1	2.97[a]	1.15[b]	0.44	0.034
Succinivibrionaceae_UCG-002	0.02	2.69	1.30	0.325
Megasphaera	0.76	1.93	0.44	0.196
Dialister	0.24	2.18	0.86	0.274
Ruminococcaceae_UCG-014	1.65	0.72	0.25	0.065
Selenomonas	0.91	1.15	0.22	0.600
Rikenellaceae_RC9_gut_group	0.79	1.23	0.41	0.611
Mitsuokella	0.44	1.37	0.37	0.228
Roseburia	0.77	0.93	0.16	0.636
Syntrophococcus	0.93	0.56	0.13	0.154
Ruminococcaceae_NK4A214_group	1.01	0.41	0.20	0.139
Sharpea	0.72	0.54	0.22	0.701
Ruminococcus_2	0.61	0.40	0.11	0.374
Moryella	0.72	0.16	0.16	0.074
Eubacterium_nodatum_group	0.70	0.16	0.15	0.076
Prevotellaceae_UCG-001	0.37	0.38	0.08	0.956
Lachnospiraceae_XPB1014_group	0.61	0.02	0.27	0.281
Pyramidobacter	0.46	0.16	0.10	0.131
Erysipelotrichaceae_UCG-004	0.05	0.55	0.22	0.282
Other	0.07[a]	0.04[b]	0.01	0.012

In the same row, different small letter superscripts indicate a significant difference ($P < 0.05$), and H-high MR feeding level group and L-low MR feeding level group, H: high MR feeding level group, fed MR at 4% DM/kg of average body weight per day; L: low MR feeding level group, fed MR at 2% DM/kg of average body weight per day.

fermenting amino acids into acetate (Zhang Y. K. et al., 2021). Notably, the high MR feeding level decreased the relative abundance of Fibrobacteres, which were reported as major bacterial degraders of lignocellulosic material in the herbivore gut (Ransom-Jones et al., 2012). High MR feeding level decreased the starter intake, and the reduced available substrates may be the main reason for the reduction of lignocellulosic degrading bacteria. Besides, the high MR feeding level increased the relative abundance of Synergistetes, Euryarchaeota, and Verrucomicrobia, which have been reported to be associated with inflammation in the gastrointestinal epithelium (Forterre et al., 2014; Sichert et al., 2020; McCracken and Nathalia Garcia, 2021), suggesting that excessive feeding of MR might have adverse effects on gastrointestinal health by altering the microbiota.

In particular, we found that the MR feeding level affected the abundance of many carbohydrate-degrading bacteria. The abundance of *Lachnospiraceae_NK3A20_group*, *Succiniclasticum*, *Ruminococcus_1*, *Ruminococcaceae_UCG-014*, and *Moryella* decreased with the increasing MR level. *Lachnospiraceae* ferment diverse plant polysaccharides to short-chain fatty acids and

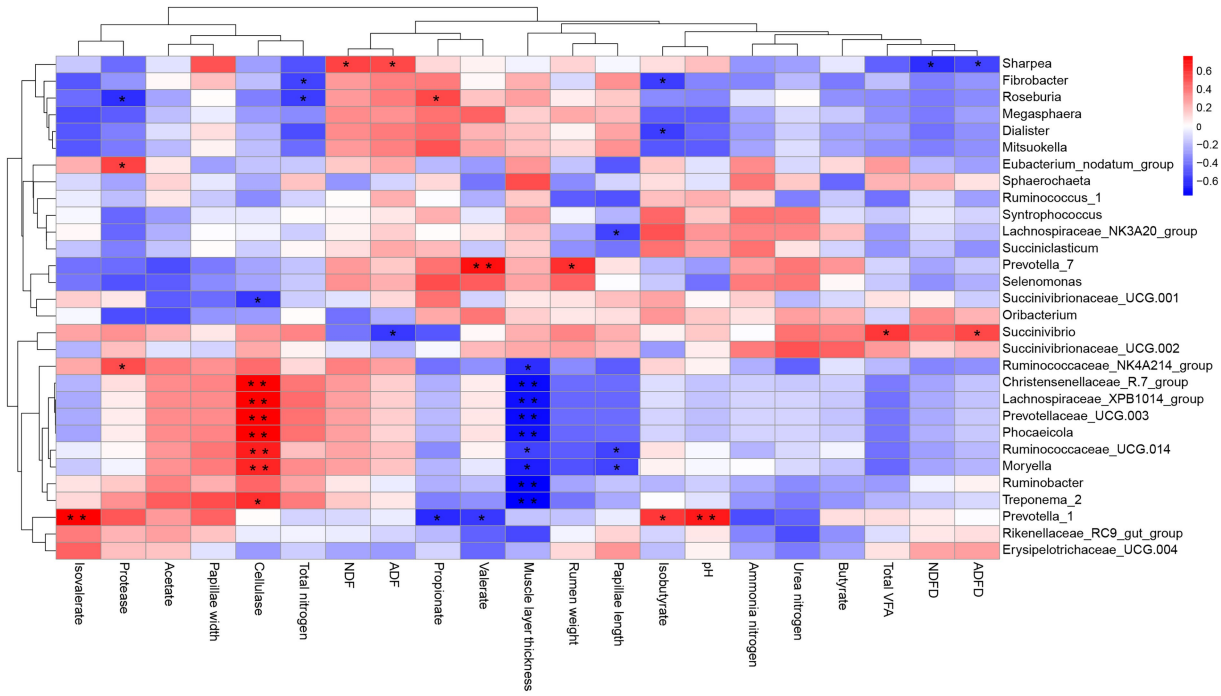

FIGURE 5
Correlation between the top 30 genus-level taxonomic compositions and rumen weight, pH, fermentation parameters, and enzyme activity. *p<0.05, **p<0.01.

alcohols (Boutard et al., 2014). *Succiniclasticum*, which ferment succinate quantitatively to propionate, were isolated from a high dilution of rumen ingesta obtained from a dairy cow fed a production diet containing grass silage as the main roughage source (Van Gylswyk, 1995). *Ruminococcus_1* plays an important role in plant fiber degradation (Devillard et al., 2004). *Moryella* has been reported to be associated with cellulose or its metabolites (Tian et al., 2019). A key deterministic factor framing the process of microbial succession is diet, especially because the abundance of certain bacteria changes in response to a fiber-based diet (Furman et al., 2020). The pre-ruminant rumen microbiota is highly active and ready to ferment a solid diet from the first week of life (Malmuthuge et al., 2019), and the increased abundance of certain important fiber degrading bacteria might be an adaptive mechanism of the rumen microbiota in response to increased solid diet intake. Typically, under *ad libitum* feed intake, as the starter intake increases, the rate of digesta passage from the rumen increases and nutrient digestibility decreases (Bourquin et al., 1990). In this study, the starter intake was significantly lower in the high MR feeding level group; however, the rumen degradation rates of NDF and ADF did not increased, which mighty be related to the decreased abundance of these carbohydrate-degrading bacteria.

We further analyzed the relationship between rumen fermentation parameters and bacterial abundance, and found that many bacterial genera with significant differences, such as *Ruminococcaceae_UCG-014* and *Moryella*, correlated significantly and positively with rumen cellulase activity, and *Succinivibrio* correlated positively with rumen ADF degradation rate and the total VFA concentration. Considering the small sample size and the vast differences in microbiome composition between the different groups, we cannot infer specific links through correlation due to large fluctuations in the microbiome between groups. This would likely yield inaccurate correlations due to strong habitat filtering (Berry and Widder, 2014) and the heteroscedasticity of the data. Although the correlation relationship does not necessarily indicate a direct causal effect, the observed multiple significant correlations between relative abundances of microbial taxa and rumen fermentation parameters provide some insight into potential host-microbiotic interactions in the rumen, suggesting that changes in the microbiota, especially the abundance of carbohydrate-degrading bacteria, may be the mechanisms for the rumen to adapt to changes in food structure and starter intake and can affect rumen function. There has been a study indicating that VFAs produced by the early microbiome were associated with the rumen tissue metabolism and the development of the epithelium with the host transcriptome and microRNAome (Malmuthuge et al., 2019). However, further studies are needed to determine the long-term consequences of the adverse effects of a high MR feeding level on rumen weight and the microbiota of lambs. A suitable balance between promoting rumen development and enhancing weight gain in early stage needs to be determined by selecting appropriate levels of MR feeding.

5. Conclusion

Increasing the feeding level of MR from 2 to 4% of the body weight of lambs significantly increased the weight gain, but decreased the intake of starter and had adverse effects on rumen development. A high MR feeding level increased the rumen microbial diversity, but decreased the abundance of many rumen carbohydrate catabolizing bacteria. This study provides new insights into the regulation of early rumen development (function, morphology, and microbial colonization) of lambs. The results suggests that the adverse effect of excessive MR feeding on rumen development and the microbiota should not be ignored; and a suitable balance between promoting rumen development and enhancing weight gain in the early stage needs to be identified by selecting appropriate levels of MR feeding.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA890401.

Ethics statement

The animal study was reviewed and approved by the Gansu Agricultural University's Academic Committee and the National Natural Science Foundation of China (Approval no. 31760682) according to guidelines established by the Biological Studies Animal Care and Use Committee of Gansu Province.

Author contributions

YH, GW, and QZ: methodology, investigation, and writing-original draft. ZC, XZ, XW, and DZ: validation and visualization. CL: funding acquisition. WW: supervision and project administration. PC, ZM, and CL: writing-review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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References

Amdi, C., Pedersen, M. L. M., Klaaborg, J., Myhill, L. J., Engelsmann, M. N., Williams, A. R., et al. (2021). Pre-weaning adaptation responses in piglets fed milk replacer with gradually increasing amounts of wheat. *Br. J. Nutr.* 126, 375–382. doi: 10.1017/s0007114520004225

Arshad, M. A., Hassan, F. U., Rehman, M. S., Huws, S. A., Cheng, Y., and Din, A. U. (2021). Gut microbiome colonization and development in neonatal ruminants: strategies, prospects, and opportunities. *Anim Nutr* 7, 883–895. doi: 10.1016/j.aninu.2021.03.004

Belanche, A., Cooke, J., Jones, E., Worgan, H. J., and Newbold, C. J. (2019). Short- and long-term effects of conventional and artificial rearing strategies on the health and performance of growing lambs. *Animal* 13, 740–749. doi: 10.1017/S1751731118002100

Berry, D., and Widder, S. (2014). Deciphering microbial interactions and detecting keystone species with co-occurrence networks. *Front. Microbiol.* 5:219. doi: 10.3389/fmicb.2014.00219

Bhatt, R. S., Tripathi, M. K., Verma, D. L., and Karim, S. A. (2009). Effect of different feeding regimes on pre-weaning growth rumen fermentation and its influence on post-weaning performance of lambs. *J Anim Physiol Anim Nutr (Berl)* 93, 568–576. doi: 10.1111/j.1439-0396.2008.00845.x

Bourquin, L. D., Garleb, K. A., Merchen, N. R., and Fahey, G. C. (1990). Effects of intake and forage level on site and extent of digestion of plant cell wall monomeric components by sheep. *J. Anim. Sci.* 27, 177–182. doi: 10.1016/0168-1591(90)90017-8

Boutard, M., Cerisy, T., Nogue, P. Y., Alberti, A., Weissenbach, J., Salanoubat, M., et al. (2014). Functional diversity of carbohydrate-active enzymes enabling a bacterium to ferment plant biomass. *PLoS Genet.* 10:e1004773. doi: 10.1371/journal.pgen.1004773

Carballo, O. C., Khan, M. A., Knol, F. W., Lewis, S. J., Stevens, D. R., Laven, R. A., et al. (2019). Impact of weaning age on rumen development in artificially-reared lambs. *J. Anim. Sci.* 97, 3498–3510. doi: 10.1093/jas/skz148

Chapman, C. E., Erickson, P. S., Quigley, J. D., Hill, T. M., Bateman, H. G., Suarez-Mena, F. X., et al. (2016). Effect of milk replacer program on calf performance and digestion of nutrients with age of the dairy calf. *J. Dairy Sci.* 99, 2740–2747. doi: 10.3168/jds.2015-10372

Devillard, E., Goodheart, D. B., Karnati, S. K., Bayer, E. A., Lamed, R., Miron, J., et al. (2004). Ruminococcus albus 8 mutants defective in cellulose degradation are deficient in two processive endocellulases, Cel48A and Cel9B, both of which possess a novel modular architecture. *J. Bacteriol.* 186, 136–145. doi: 10.1128/jb.186.1.136-145.2004

Ekiz, B., Kocak, O., Yalcintan, H., and Yilmaz, A. (2016). Effects of suckling duration on growth, slaughtering and carcass quality characteristics of Kivircik lambs. *Trop. Anim. Health Prod.* 48, 395–401. doi: 10.1007/s11250-015-0964-7

Forterre, P., Krupovic, M., Raymann, K., and Soler, N. (2014). Plasmids from euryarchaeota. *Microbiol Spectr* 2:2014. doi: 10.1128/microbiolspec.PLAS-0027-2014

Furman, O., Shenhav, L., Sasson, G., Kokou, F., Honig, H., Jacoby, S., et al. (2020). Stochasticity constrained by deterministic effects of diet and age drive rumen microbiome assembly dynamics. *Nat. Commun.* 11:1904. doi: 10.1038/s41467-020-15652-8

He, B., Jin, S., Cao, J., Mi, L., and Wang, J. (2019). Metatranscriptomics of the Hu sheep rumen microbiome reveals novel cellulases. *Biotechnol. Biofuels* 12:153. doi: 10.1186/s13068-019-1498-4

Hill, T. M., Bateman, H. G., Aldrich, J. M., and Schlotterbeck, R. L. (2010). Effect of milk replacer program on digestion of nutrients in dairy calves. *J. Dairy Sci.* 93, 1105–1115. doi: 10.3168/jds.2009-2458

Hill, T. M., Quigley, J. D., Bateman, H. G., Suarez-Mena, F. X., Dennis, T. S., and Schlotterbeck, R. L. (2016). Effect of milk replacer program on calf performance and digestion of nutrients in dairy calves to 4 months of age. *J. Dairy Sci.* 99, 8103–8110. doi: 10.3168/jds.2016-11239

Huang, Y., Wang, G., Li, C., Wang, W., Zhang, X., Wang, X., et al. (2022). Periodical changes of feces microbiota and its relationship with nutrient digestibility in early lambs. *Animals (Basel)* 12:1770. doi: 10.3390/ani12141770

Jasper, J., and Weary, D. M. (2002). Effects of ad libitum milk intake on dairy calves. *J. Dairy Sci.* 85, 3054–3058. doi: 10.3168/jds.S0022-0302(02)74391-9

Keulen, J. V., and Young, B. A. (1977). Evaluation of acid-insoluble ash as a natural marker in ruminant digestibility studies. *J. Anim. Sci.* 44, 282–287. doi: 10.2527/jas1977.442282x

Khan, M. A., Bach, A., Weary, D. M., and von Keyserlingk, M. A. G. (2016). Invited review: transitioning from milk to solid feed in dairy heifers. *J. Dairy Sci.* 99, 885–902. doi: 10.3168/jds.2015-9975

Konopka, A. (2009). What is microbial community ecology? *ISME J.* 3, 1223–1230. doi: 10.1038/ismej.2009.88

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

Lee, H. J., Khan, M. A., Lee, W. S., Yang, S. H., Kim, S. B., Ki, K. S., et al. (2009). Influence of equalizing the gross composition of milk replacer to that of whole milk on the performance of Holstein calves. *J. Anim. Sci.* 87, 1129–1137. doi: 10.2527/jas.2008-1110

Li, C., Zhang, Q., Wang, G., Niu, X., Wang, W. M., Li, F., et al. (2020). The functional development of the rumen is influenced by weaning and associated with ruminal microbiota in lambs. *Anim. Biotechnol.* 33, 612–628. doi: 10.1080/10495398.2020.1812618

Li, C., Wang, G., Zhang, Q., Huang, Y., Li, F., and Wang, W. (2022). Developmental changes of nutrient digestion in young lambs are influenced by weaning and associated with intestinal microbiota. *Anim. Biotechnol.* 1-15, 1–15. doi: 10.1080/10495398.2022.2025817

Li, A., Yang, Y., Qin, S., Lv, S., Jin, T., Li, K., et al. (2021). Microbiome analysis reveals gut microbiota alteration of early-weaned Yimeng black goats with the effect of milk replacer and age. *Microb. Cell Factories* 20:78. doi: 10.1186/s12934-021-01568-5

Longenbach, J. I., and Heinrichs, A. J. (1998). A review of the importance and physiological role of curd formation in the abomasum of young calves. *Anim. Feed Sci. Technol.* 73, 85–97. doi: 10.1016/S0377-8401(98)00130-8

Malmuthuge, N., Liang, G., and Guan, L. L. (2019). Regulation of rumen development in neonatal ruminants through microbial metagenomes and host transcriptomes. *Genome Biol.* 20:172. doi: 10.1186/s13059-019-1786-0

Mao, H., Zhang, Y., Yun, Y., Ji, W., Jin, Z., Wang, C., et al. (2021). Weaning age affects the development of the ruminal bacterial and archaeal community in hu lambs during early life. *Front. Microbiol.* 12:577. doi: 10.3389/fmicb.2021.636865

McCoard, S. A., Omar, C. C., Knol, F. W., Axel, H., Khan, M. A., Nina, H., et al. (2020). Impact of early weaning on small intestine, metabolic, immune and endocrine system development, growth and body composition in artificially reared lambs. *J. Anim. Sci.* 98:356. doi: 10.1093/jas/skz356

McCracken, B. A., and Nathalia Garcia, M. (2021). Phylum Synergistetes in the oral cavity: A possible contributor to periodontal disease. *Anaerobe* 68:102250. doi: 10.1016/j.anaerobe.2020.102250

Meale, S. J., Li, S., Azevedo, P., Derakhshani, H., Plaizier, J. C., Khafipour, E., et al. (2016). Development of ruminal and fecal microbiomes are affected by weaning but not weaning strategy in dairy calves. *Front. Microbiol.* 7:582. doi: 10.3389/fmicb.2016.00582

Mialon, M. M., Boivin, X., Durand, D., Boissy, A., Delval, E., Bage, A. S., et al. (2021). Short-and mid-term effects on performance, health and qualitative behavioural assessment of Romane lambs in different milk feeding conditions. *Animal* 15:100157. doi: 10.1016/j.animal.2020.100157

Morgavi, D. P., Rahahao-Paris, E., Popova, M., Boccard, J., and Boudra, H. (2015). Rumen microbial communities influence metabolic phenotypes in lambs. *Front. Microbiol.* 6:060. doi: 10.3389/fmicb.2015.01060

Poonia, R. K., Mehta, S. C., Singh, S., Singh, R., and Patil, N. V. (2015). Analysis of milk constituents of indian sheep, goat, camel and buffalo. *Personal- und Anstaltsfragen; Beiträge zur Irrenpflege* 21:122. doi: 10.5958/0973-9718.2015.00028.8

Ransom-Jones, E., Jones, D. L., McCarthy, A. J., and McDonald, J. E. (2012). The Fibrobacteres: an important phylum of cellulose-degrading bacteria. *Microb. Ecol.* 63, 267–281. doi: 10.1007/s00248-011-9998-1

Sichert, A., Corzett, C. H., Schechter, M. S., Unfried, F., Markert, S., Becher, D., et al. (2020). Verrucomicrobia use hundreds of enzymes to digest the algal polysaccharide fucoidan. *Nat. Microbiol.* 5, 1026–1039. doi: 10.1038/s41564-020-0720-2

Soltani, E., Naserian, A. A., Khan, M. A., Ghaffari, M. H., and Malekkhahi, M. (2020). Effects of conditioner retention time during pelleting of starter feed on nutrient digestibility, ruminal fermentation, blood metabolites, and performance of Holstein female dairy calves. *J. Dairy Sci.* 103, 8910–8921. doi: 10.3168/jds.2020-18345

Terré, M., Devant, M., and Bach, A. (2006). Performance and nitrogen metabolism of calves fed conventionally or following an enhanced-growth feeding program during the preweaning period. *Livest. Sci.* 105, 109–119. doi: 10.1016/j.livsci.2006.05.001

Tian, F., Wu, J., and Center, B. (2019). Effects of grazing and barn feeding on biological information of rumen bacterial communities in pengbo semi-fine wool sheep. *Chinese J. Anim. Vet. Sci.* 50, 2252–2263. doi: 10.11843/j.issn.0366-6964.2019.11.009

Toral, P. G., Chilliard, Y., Rouel, J., Leskinen, H., Shingfield, K. J., and Bernard, L. (2015). Comparison of the nutritional regulation of milk fat secretion and composition in cows and goats. *J. Dairy Sci.* 98, 7277–7297. doi: 10.3168/jds.2015-9649

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

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

Wang, W., Li, C., Li, F., Wang, X., Zhang, X., Liu, T., et al. (2016). Effects of early feeding on the host rumen transcriptome and bacterial diversity in lambs. *Sci. Rep.* 6:32479. doi: 10.1038/srep32479

Yue, G. H. (1996). Reproductive characteristics of Chinese Hu sheep. *Anim. Reprod. Sci.* 44, 223–230. doi: 10.1016/0378-4320(96)01562-X

Yue, X., Diao, Q., Ma, C., Deng, K., Tu, Y., Jiang, C., et al. (2011). Effects of feeding levels of a milk replacer on digestion and metabolism of nutrients, and serum biochemical indexes in lambs. *Sci. Geol. Sin.* 44, 4464–4473. doi: 10.3864/j.issn.0578-1752.2011.21.015

Zeng, Y., Zeng, D., Ni, X., Zhu, H., Jian, P., Zhou, Y., et al. (2017). Microbial community compositions in the gastrointestinal tract of Chinese Mongolian sheep using Illumina MiSeq sequencing revealed high microbial diversity. *AMB Express* 7:75. doi: 10.1186/s13568-017-0378-1

Zhang, Q., Li, C., Niu, X., Zhang, Z., and Li, F. (2018). An intensive milk replacer feeding program benefits immune response and intestinal microbiota of lambs during weaning. *BMC Vet. Res.* 14:366. doi: 10.1186/s12917-018-1691-x

Zhang, Q., Li, C., Niu, X., Zhang, Z., Li, F., and Li, F. (2019). The effects of milk replacer allowance and weaning age on the performance, nutrients digestibility, and ruminal microbiota communities of lambs. *Anim. Feed Sci. Technol.* 257:114263. doi: 10.1016/j.anifeedsci.2019.114263

Zhang, Y. K., Zhang, X. X., Li, F. D., Li, C., Li, G. Z., Zhang, D. Y., et al. (2021). Characterization of the rumen microbiota and its relationship with residual feed intake in sheep. *Animal* 15:100161. doi: 10.1016/j.animal.2020.100161

Zhang, Y., Zhang, X., Li, F., Li, C., Zhang, D., Li, X., et al. (2021). Exploring the ruminal microbial community associated with fat deposition in lambs. *Animals (Basel)* 11:3584. doi: 10.3390/ani11123584

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Litter size influences rumen microbiota and fermentation efficiency, thus determining host early growth in goats

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Introduction: Multiple litters are accompanied by low birth weight, low survival rates, and growth rates in goats during early life. Regulating rumen microbiota structure can indirectly or directly affect host metabolism and animal growth. However, the relationship between high litter size and rumen microbiome, rumen fermentation, and growth performance in goat kids is unclear.

Methods: In the present study, thirty 6-month-old, female goats were investigated, of which 10 goats were randomly chosen from single, twin and triplet goats respectively, and their birth weight was recorded. From birth, all goats were subjected to the same feed and management practices. Individual weaning and youth body weight were measured, and the rumen fluid samples were collected to characterize the bacterial communities and to determine the ruminal volatile fatty acids (VFA), free amino acids (AA), and free fatty acids (FA) concentration of those young goats.

Results and Discussion: Compared with the single and twin goats, triplet goats have lower weaning and youth body weight and average daily gain (ADG). Ruminal propionate, butyrate, and total VFA were decreased in triplet goats. Meanwhile, ruminal AA, such as branched chain amino acids (BCAA), essential amino acids (EAA), unsaturated fatty acids (UFA), and monounsaturated fatty acids (MUFA) were decreased, while saturated fatty acids (SFA) and odd and branched chain fatty acids (OBCFA) were increased in triplet goats. Our results also revealed that litter size significantly affected the rumen bacterial communities, and triplet goats had a lower the Firmicutes: Bacteroidota ratio, the abundance of Firmicutes phylum, Rikenellaceae family, and *Rikenellaceae RC9 gut group*, and had a higher proportion of Prevotellaceae family, and several genera of Prevotellaceae, such as *Prevotella*, and *unclassified f Prevotellaceae*. Furthermore, Spearman's correlation network analysis showed that the changes in the rumen bacteria were associated with changes in rumen metabolites. In conclusion, this study revealed that high litter size could bring disturbances to the microbial communities and decrease the rumen fermentation efficiency and growth performance, which can be utilized to better understand variation in microbial ecology that will improve growth performance in triplet goats.

KEYWORDS

multiple litters, rumen microbiome, rumen fermentation, growth performance, goat

Introduction

The goat is one of the oldest domesticated animal species, and more than 1,000 breeds of goats around the world (MacHugh and Bradley, 2001; Lai et al., 2016). It provides a range of products and plays economically important roles in human productive activity (Haenlein, 2004; Pulina et al., 2018). In the goat industry, litter size is one of the most important economic traits (Akpa et al., 2011),

and improvement of the reproductive traits are associated with large profits for farmers. However, the number of fetuses in the litter could affect the fetal growth rates and subsequent birth weight of goats (Gootwine et al., 2007; Zhang et al., 2008). Moreover, lambs born in multi-fetus litters have lower pre- and postnatal survival rates of the goats (Christley et al., 2003). Therefore, the detrimental impact of multiple litters on the birth weight and growth performance of goat kids should be more thoroughly studied.

Trillions of microbes colonize in the rumen and play crucial roles on metabolism and ruminant growth. This microbial cohort contains cellulolytic, hemicellulolytic, amylolytic, proteolytic, and lipolytic species, which ferment feedstuff to yield volatile fatty acids (VFA), free fatty acids (FA), and bacteria protein (O'Hara et al., 2020). Those products of rumen microbial fermentation can meet most of the host's energy and nutrition needs (Matthews et al., 2019). Recent studies found that the presence of a microbiome in the gut of fetal lambs (Bi et al., 2021), and maternal gut and reproductive tract microbiome may affect the fetal microbiome and health (Rowe et al., 2020; Hummel et al., 2021). Furthermore, studies also found that modulating the pregnant sow's microbiome could improve reproductive efficiency (Jiang et al., 2019) and piglet survival rate (Ma et al., 2020). However, little is known regarding the impact of litter size on early life programming of rumen microbiome and its implication for goat growth.

In this study, a total of 30 newborn female kids were selected from 30 litters, one female kid per litter. Ten were randomly selected from single (litter size = 1[LZ1]), twins (litter size = 2[LZ2]), and triplets (litter size = 3[LZ3]) for sampling, respectively. The aim of this study was to (1) evaluate the effects of litter size on weaning and youth growth performance of goat kids, (2) investigate the impact of litter size on rumen fermentation [VFA, amino acids (AA), and FA] and rumen microbial communities of young goats, and (3) reveal the relationship between the litter size related rumen microbiota and microbial metabolites, and growth performance traits of young goats.

Materials and methods

Ethics statement

In the present study, all animal procedures were approved by the Institutional Animal Care and Use Committee of Northwest A&F University.

Animals and sample collection

Field experiment were performed at a Saanen goat farm in Baoji, Shaanxi (34°41′N, 109°09′E). Thirty ewes delivering in 1 week (third birth), including 10 ewes producing single, 10 ewes producing twins, and 10 ewes producing triplets, with each litter including at least one female newborn, were selected. After born, one female kid was chosen from each litter, 30 female kids in total, were randomly selected from these 30 litters for sampling. Briefly, 10 female kids were randomly selected from single, twins, and triplets, respectively, and named as LZ1, LZ2, and LZ3. And the birth weight of those kids was weighed within 12 h after birth. And then, all kids were transferred to the kid barn with bottle-feeding of mixed milk collected from ewes. During pre-weaning phase (~90 days old), all goats were feed milk, alfalfa hay, and concentration mixture. After weaning, the goats were fed TMR with a 60:40 forage to concentration ratio. The animals were fed three times daily at 0730, 1300, and 1900 h. Water was available *ad libitum*. The detailed feeding programs and ingredient compositions are shown in Supplementary Table S1.

The body weight, wither height, body length, and heart girth of weaning and 6-month-old (188.9 ± 0.4 days old, mean ± SE) of those three group goats were measured. And the rumen fluid sample of those young goats were collected *via* esophageal tube before morning feeding. Briefly, the first ~50 ml of rumen fluid was discarded to avoid saliva contamination, and the text 30 ml rumen fluid strained through four layers of sterile cheesecloth under a constant flux of CO_2. Then the rumen fluid was aliquoted and stored at −80°C for further analysis.

Dry matter intake measurements

The feed intake of all goats was measured 1 week before sampling (171.9 ± 0.4 days old). In brief, feed offered to and refused by each goat was recorded continuously for 7 days. The feed samples were dried at 65°C for 48 h to obtain dry matter content of ration. Daily dry matter intake (DMI) per goat was calculated by multiplying daily as-fed intake by dry matter content of ration.

Ruminal VFA assay

Ruminal VFA (acetate, propionate, butyrate, valerate, isobutyrate, and isovalerate) analysis were performed through separation and quantification by a GC (Agilent 7820A, Santa Clara, CA, United States) with a capillary column (AE-FFAP of 30 m × 0.25 mm × 0.33 μm; ATECH Technologies Co., Lanzhou, China; Li et al., 2014). Briefly, thawed rumen fluid samples were centrifuged for 10 min at 13,500 rpm at 4°C. To remove the protein, a 1.5 ml supernatant was mixed with 300 μl 25%w/v metaphosphoric solution. After standing for 4 h at 4°C, the mixture was centrifuged for 10 min at 13,500 rpm at 4°C. Then 1 ml supernatant was moved into 200 μl 25% crotonic acid, and the mixture was collected into an EP tube passed through a 0.45-μm filter.

Ruminal free AA assay

The detection of ruminal free amino acids abundance were conducted using LC–MS/MS (Exion LC AC, QTRAP 5500, AB SCIEX, Framingham, MA, United States) according to method described as previously described (Chen et al., 2022). In brief, in order to precipitate proteins rumen fluid samples were mixed through with sulfosalicylic acid (10%, wt/vol) and centrifuged at 3,000 × *g* for 10 min at 4°C. Then the supernatant fluid was collected and filtered through a 0.45-μm filter.

Ruminal FA assay

Ruminal fatty acids composition was analyzed using the method of Sun and Gibbs (2012). The freeze-dried sample (0.3–0.4 g) was methylated with 4 ml of 0.5 mol/L NaOH/methanol for 15 min at 50°C. Then the mixture was added 4 ml 2% HCl/methanol (v/v) and vortexed for 5 min, followed by a water bath (50°C) for 60 min. The extract was dissolved in 2 ml of heptane and then introduced to the GC (Agilent Technologies 7820A GC system, Santa Clara, CA, United States) equipped with a fused silica capillary column (SP-2560, 100 m × 0.25 mm × 0.2 μm; Supelco Inc., Bellefonte, PA, United States).

Bacterial DNA extraction and PCR amplification

The total DNA was extracted using the QIAamp DNA Stool Mini kit (QIAGEN, Germany) according to the manufacturer's protocol. The DNA concentration was determined by using a Nanodrop-2000 (Thermo Fisher Scientific, United States), and the purity was monitored on 1% agarose gel electrophoresis. The DNA was stored at −80°C until further processing. The amplicon library preparation was performed by polymerase chain reaction amplification of the V3-V4 region of the 16S rRNA gene using the primer pairs: 338F (5′-ACTCCTACGGGAG GCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The PCR program was carried as previously described (Xue et al., 2018). Paired-end (2 × 300 bp) sequencing was performed on Illumina platform (Illumina, United States) according to the standard protocols (Caporaso et al., 2012).

16S rRNA sequencing analysis

The raw sequences were merged with FLASH (v1.2.11; Magoč and Salzberg, 2011) and quality filtered with fastp (0.19.6; Chen et al., 2018). Sequences were demultiplexing using QIIME2 v2021.8, and the construction of an amplicon sequence variant (ASV) table using DADA2 (Callahan et al., 2016). Bacterial 16S ASVs were assigned a taxonomy using the SILVA database v138 as the reference.

The alpha diversity diversities of the bacterial communities were determined using various diversity indices (Sobs, ACE, Chao 1, Shannon, Simpson) and calculated using the procedures within QIIME 2. The NMDS (non-metric multidimensional scaling) graphs were performed based on Bray-Curtis distance and statistical significance was determined using analysis of similarities (ANOSIM) with 999 permutations at the ASV level.

Statistical analysis

Differential DMI, growth performance traits (body weight, ADG, wither height, body length, and heart girth), and ruminal metabolites (VFA, AA, and FA) were tested by one-way ANOVA among three groups, and Student's *t*-test was used between two groups. Orthogonal polynomials were used to determine the linear effects of increasing the litter size.

The taxa with different relative abundances among those three groups were identified by a Kruskal–Wallis test, and Wilcoxon rank-sum test was used between two groups with a false discovery rate (FDR) value <0.05 to correct the p values. The differences were statistically significant at $p < 0.05$ or a tendency of difference at $p < 0.10$. Spearman's rank correlation coefficients were used to examine the correlations between bacterial abundance and rumen metabolites concentrations.

Results

Growth performance

In the present study, although there was no significant difference in birth weight between these three groups ($p > 0.05$). Numerically, the LZ3 group (2.94 ± 0.20 kg) with lower birth weight compared to LZ1 (3.21 ± 0.14 kg) and LZ2 (3.20 ± 0.12 kg) group (Supplementary Figure S1A).

The growth performance traits were shown in Figure 1. With the increased litter size, the weaning weight, ADG, and wither height of goat kids were linearly decreased ($p < 0.05$). Moreover, compared to the LZ1 group, the weaning weight, ADG, and wither height were significantly lower in LZ3 group (*t*-test, $p < 0.05$).

And, there was no significant difference in DMI between the three groups ($p = 0.729$, Supplementary Figure S1B) at the youth period. With the increased litter size, the youth body weight, and ADG of young goats were linearly decreased ($p < 0.05$), the body length and heart girth had a tendency to decrease ($p < 0.1$). Furthermore, compare to the LZ1 group, LZ3 group had a lower body weight, ADG, heart girth (*t*-test, $p < 0.05$), and body length (*t*-test, $p = 0.093$) at the youth period.

Ruminal fermentation parameters

As shown in Table 1, with the increased litter size, the concentration of ruminal propionate, total VFA of young goats were linearly decreased ($p < 0.05$), the ruminal butyrate concertation had a tendency to decrease ($p = 0.098$). In addition, compare to the LZ1 group, LZ3 group had a lower propionate, total VFA (*t*-test, $p < 0.05$), and butyrate concentration (*t*-test, $p = 0.093$).

Then the difference in ruminal AA and FA were identified between the three groups. We found that the concentration of some essential amino acids (EAA), such as His, Trp, Lys, Phe, Val and total EAA concentration, were linearly decreased with the increased litter size (Table 2, $p < 0.05$). In addition, compared to the LZ1 group, the concentration of His, Cys, Val, Trp, Phe, Lys, and total EAA were significantly decreased in LZ3 group (*t*-student, $p < 0.05$), and the concentration of some AA, such as Leu, Pro, and total branched chain amino acids (BCAA) of LZ3 group, tended to be decreased than LZ1 group (*t*-student, $p < 0.1$).

As shown in Table 3, the proportion of iso C14:0, anteiso C15:0, C15:0, saturated fatty acids (SFA), odd and branched chain fatty acids (OBCFA) and the ratio of SFA to UFA were linearly increased with the increased litter size ($p < 0.05$). And the proportion of C16:0, C18:1, C18:1t11, C18:1c9, unsaturated fatty acids (UFA) and monounsaturated fatty acids (MUFA) were linearly decreased with the increased litter size ($p < 0.05$). In addition, we found that the proportion of iso C14:0, anteiso C15:0, C15:0, OBCFA were significantly increased and C16:0, C18:1t11, C18:1c9, C18:1, and MUFA were significantly decreased in the LZ3 group compared to the LZ1 group.

Together, our results revealed that with the increased litter size, the growth performance and rumen fermentation efficiency were linearly decreased.

Ruminal microbial diversity and structure

Based on the above results, the difference in rumen microbiota between LZ3 group and LZ1, LZ2 group were identified, to evaluate whether alterations of ruminal fermentative capacity were influence by rumen microbiota.

After size filtering, quality control, and chimera removal using the QIIME 2 pipeline, an average of 45,662 reads were generated for bacteria from a total 1,369,847 quality reads from 30 rumen fluid samples, with an average length of 416 bp. The calculated Good's coverage exceeded 99.9% for all samples.

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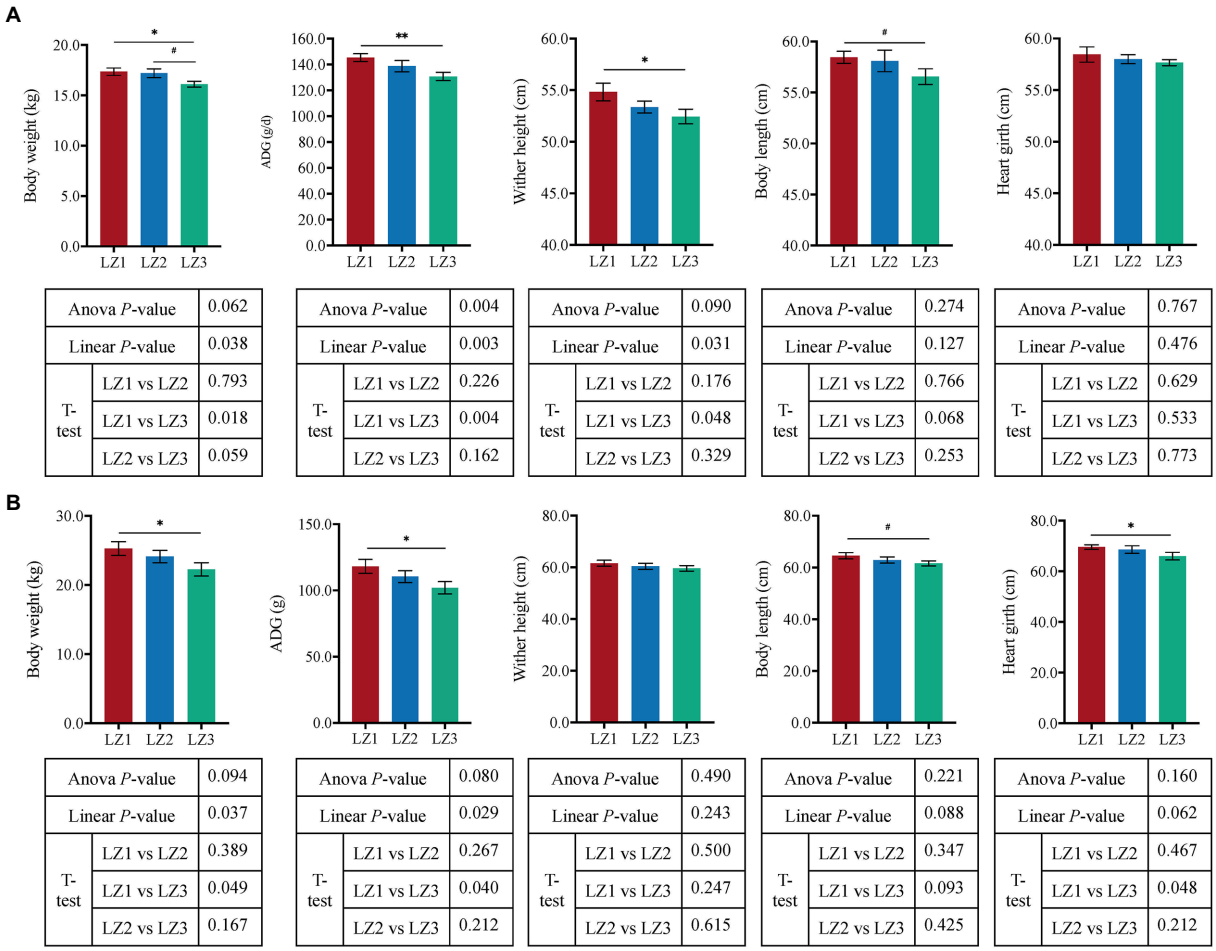

FIGURE 1
Effect of litter size on growth performance of weaning **(A)** and young **(B)** goats. Data are presented as mean± SEM, $^{\#}p<0.1$, $^{*}p<0.05$, $^{**}p<0.01$.

TABLE 1 Effect of litter size on ruminal VFA (mM) concentration of young goats.

Items	Groups			SEM	ANOVA	Linear	*T*-test		
	LZ1	LZ2	LZ3				LZ1 vs. LZ2	LZ1 vs. LZ3	LZ2 vs. LZ3
Acetate	65.41	61.94	60.81	1.711	0.533	0.296	0.453	0.136	0.821
Propionate	23.32	18.44	15.85	1.244	0.041	0.015	0.084	0.030	0.679
Isobutyrate	0.83	0.82	0.74	0.040	0.571	0.317	0.839	0.376	0.378
Butyrate	10.89	9.99	8.41	0.587	0.255	0.098	0.546	0.093	0.279
Isovalerate	1.48	1.56	1.35	0.074	0.566	0.562	0.652	0.472	0.346
Valerate	0.95	0.86	0.84	0.038	0.487	0.268	0.335	0.252	0.872
Total VFA	102.89	93.59	88.01	2.981	0.058	0.019	0.140	0.021	0.343

Different letters (a, b) within a row means values with significantly different ($p<0.05$). LZ1, single goat; LZ2, twin goats; LZ3, triplet goats; SEM, standard error of mean; VFA, volatile fatty acids.

To compare bacterial community diversity across different groups, alpha-diversity and beta-diversity were evaluated. There were no significant differences in Sobs index, Ace index, Chao 1 index, Shannon index, and Simpson index among the three groups (Table 4, $p>0.05$). And there were no significant difference in alpha-diversity indices between LZ1 and LZ3 group ($p>0.05$). In a beta-diversity analysis (NMDS based on Bray-Curtis), the LZ1 and LZ2 group were clustered together and could not be distinguished ($p=0.477$), whereas the LZ3 group was clearly distinguished from the LZ1 and LZ2 group (Figure 2, $p<0.05$).

Ruminal microbial composition

In total, 21 phyla, 32 classes, 71 orders, 120 families, and 237 genera were identified. More specifically, Firmicutes (50.2%), Bacteraioidota

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TABLE 2 Effect of litter size on ruminal free AAs (μmol/L) concentration of young goats.

Items	Groups			SEM	ANOVA	Linear	*T*-test		
	LZ1	LZ2	LZ3				LZ1 vs. LZ2	LZ1 vs. LZ3	LZ2 vs. LZ3
Asp	676.37	648.45	477.92	50.957	0.383	0.192	0.844	0.178	0.196
Glu	493.67	386.59	308.05	43.990	0.365	0.168	0.432	0.197	0.400
Ser	510.03	350.57	272.34	41.714	0.111	0.042	0.172	0.052	0.347
His	218.90	151.60	88.51	20.520	0.079	0.026	0.248	0.015	0.131
Gly	623.72	435.52	314.51	54.070	0.121	0.044	0.221	0.05	0.221
Thr	312.16	238.87	180.60	31.639	0.165	0.064	0.192	0.09	0.499
Arg	55.66	73.68	69.06	7.211	0.629	0.482	0.335	0.539	0.860
Ala	1241.27	842.05	689.11	103.339	0.139	0.056	0.182	0.063	0.412
Tyr	325.64	247.81	184.84	25.144	0.142	0.051	0.271	0.059	0.203
Cys	103.17	71.43	50.32	8.397	0.074	0.025	0.169	0.022	0.236
Val	498.42	400.84	304.39	43.533	0.119	0.043	0.216	0.042	0.253
Met	228.32	199.71	147.64	22.096	0.253	0.102	0.399	0.106	0.264
Trp	52.81[a]	54.77[a]	25.69[b]	4.886	0.039	0.022	0.765	0.007	0.017
Phe	223.70	193.13	142.13	18.803	0.140	0.050	0.296	0.045	0.189
Ile	409.59	371.27	279.01	38.193	0.284	0.117	0.443	0.116	0.270
Leu	520.57	442.25	349.68	43.029	0.168	0.064	0.269	0.058	0.287
Lys	1146.40	907.48	691.25	100.219	0.109	0.039	0.199	0.043	0.236
Pro	182.17	124.67	116.13	11.297	0.070	0.036	0.054	0.058	0.783
BCAA	1541.47	1214.36	933.08	115.570	0.184	0.069	0.318	0.056	0.268
EAA	3971.65	3033.60	2277.97	294.093	0.125	0.044	0.254	0.040	0.226
NEAA	4155.95	3107.08	2413.21	314.136	0.140	0.052	0.236	0.055	0.277

Different letters (a, b) within a row means values with significantly different ($p < 0.05$). LZ1, single goat; LZ2, twin goats; LZ3, triplet goats; SEM, standard error of mean; Asp., aspartic acid; Glu, glutamic acid; Ser, serine; His, histidine; Gly, glycine; Thr, threonine; Cys, cysteine; Val, valine; Met, methionine; Trp, tryptophan; Phe, phenylalanine; Ile, Isoleucine; Leu, leucine; Lys, lysine; Pro, proline; BCAA, branched chain amino acids; EAA, essential amino acids; NEAA, non-essential amino acids.

(43.1%), and Patescibacteria (3.4%) were the predominant phyla. The abundance of Firmicutes in LZ3 group was significantly lower than LZ2 group, while the abundance of Bacteraioidota in LZ3 group was significantly higher compared to LZ1 and LZ2 group ($p < 0.05$). Meanwhile, the Firmicutes: Bacteraioidota ratio was significantly decreased in the LZ3 group (Supplementary Figure S2, $p < 0.05$).

As shown in Figure 3, Prevotellaceae (24.8%) was the most abundant family in LZ3 group. While, F082 was the most abundant family in LZ1 (13.7%) and LZ2 (11.5%) group. Prevotellaceae abundance in LZ3 group was significantly higher than that in LZ1 and LZ2 group ($p < 0.05$). Conversely, the relative abundance of Rikenellaceae in LZ3 group was significantly lower than that in LZ1 group ($p < 0.05$). At the genus level, *norank f F082* (11.6%), *Prevotella* (10.9%), *Ruminococcus* (9.6%), *Rikenellaceae RC9 gut group* (8.5%), *Christensenellaceae R-7 group* (6.8%), *Oscillospiraceae NK4A214 group* (6.6%), norank f *Muribaculaceae* (4.6%), *Candidatus Saccharimonas* (3.4%), *unclassified c Clostridia* (2.6%), and *unclassified f Selenomonadaceae* (2.5%) were the dominant (Figure 4). The abundance of *Prevotella*, *unclassified f Prevotella* and *Prevotellaceae UCG-001* in LZ3 group were significantly higher than that in LZ1 and LZ2 group ($p < 0.05$). *Succiiniclasticum* abundance in the LZ3 group were significantly higher than that in LZ1 group ($p < 0.05$). Conversely, the abundance of *Rikenellaceae RC9 group* and *unclassified c Clostridia* were significantly lower than that in LZ1 or LZ2 group ($p < 0.05$).

The correlation between ruminal metabolites and ruminal microbiota.

As shown in Figure 5A, those LZ3 enriched bacteria were negatively correlated with ruminal VFA concentration and growth performance traits of young goats. For example, the relative abundance of *Prevotellaceae UCG-001* was negatively correlated with ADG, wither height, and ruminal butyrate concentration ($p < 0.05$). The relative abundance of *Prevotella*, and *Succinniclasticum* were negatively correlated with ruminal propionate, butyrate, valerate, and total VFA concentration ($p < 0.05$). And *Rikenellaceae RC9 gut group* had a significantly positively correlated with ruminal isobutyrate and butyrate concentration ($p < 0.05$).

And then, we analyzed the correlation between ruminal AA, FA, and litter size related bacteria genera (Figures 5B,C). The results showed that those LZ3 enriched bacteria were significantly negatively correlated with ruminal AA, UFA, and MUFA, such as *Prevotella* and ruminal BCAA, total EAA, anteiso C15:0, and OBCFA were negatively correlated but positively correlated with C18:1t11, UFA, and MUFA ($p < 0.05$). *unclassified f Prevotellaceae* were significantly negatively associated with almost all ruminal AA, such as Leu, His and total BCAA, and C18:1t11 but positively associated with OBCFA ($p < 0.05$).

TABLE 3 Effect of litter size on ruminal FAs (g/100 g of total FAs, relative abundance >0.1%) of young goats.

Items	Groups			SEM	ANOVA	Linear	*T*-test		
	LZ1	LZ2	LZ3				LZ1 vs. LZ2	LZ1 vs. LZ3	LZ2 vs. LZ3
C12:0	1.19	1.38	1.66	0.133	0.438	0.207	0.491	0.193	0.501
iso-C14:0	0.75	1.28	1.32	0.110	0.060	0.036	0.046	0.008	0.898
C14:0	3.72	3.70	4.63	0.339	0.523	0.356	0.976	0.343	0.369
iso-C15:0	2.07	2.84	2.64	0.166	0.121	0.132	0.053	0.109	0.676
anteiso-C15:0	4.38^{b}	6.12^{a}	5.87^{a}	0.270	0.013	0.024	0.003	0.018	0.739
C15:0	1.96	2.47	2.60	0.126	0.106	0.048	0.073	0.033	0.719
C16:0iso	2.14	2.48	2.73	0.178	0.456	0.217	0.401	0.225	0.62
C16:0	43.66	40.35	40.55	0.704	0.083	0.061	0.061	0.037	0.913
anteiso-C17:0	2.46	2.46	2.31	0.075	0.741	0.506	0.998	0.201	0.556
C18:0	5.45	7.57	7.05	0.499	0.204	0.194	0.094	0.081	0.741
C18:1t11	3.84^{a}	2.96^{b}	2.26^{b}	0.175	0.001	<0.001	0.015	0.002	0.074
C18:1c9	14.57^{a}	11.29^{b}	10.97^{b}	0.516	0.005	0.003	0.002	0.009	0.789
C18:1c11	2.88	1.59	2.99	0.289	0.084	0.912	0.014	0.902	0.089
C18:2cis9,12	5.62	5.04	5.34	0.367	0.799	0.728	0.501	0.771	0.768
C18:1	21.26^{a}	16.90^{b}	17.49^{b}	0.604	0.002	0.003	<0.001	0.015	0.646
SFA	71.25^{b}	75.60^{a}	74.71^{a}	0.676	0.010	0.018	0.003	0.052	0.558
UFA	28.75^{a}	24.40^{b}	25.29^{b}	0.676	0.010	0.018	0.003	0.052	0.558
SFA/UFA	2.52^{b}	3.05^{a}	3.03^{a}	0.096	0.027	0.020	0.005	0.054	0.949
SCFA	0.35	0.78	0.51	0.085	0.089	0.295	0.046	0.172	0.33
MCFA	62.22	64.26	63.94	0.773	0.517	0.364	0.253	0.356	0.897
LCFA	37.43	34.96	35.27	0.762	0.354	0.244	0.139	0.268	0.895
MUFA	22.80^{a}	17.97^{b}	18.60^{b}	0.601	<0.001	0.001	<0.001	0.005	0.623
PUFA	6.50	6.43	6.68	0.300	0.947	0.846	0.915	0.818	0.756
OBCFA	15.33	18.98	19.44	0.833	0.082	0.043	0.053	0.034	0.842

Different letters (a, b) within a row means values with significantly different ($p < 0.05$). LZ1, single goat; LZ2, twin goats; LZ3, triplet goats; SEM, standard error of mean; SFA, saturated fatty acids; UFA, unsaturated fatty acids; SCFA, short chain fatty acids; MCFA, medium chain fatty acids; LCFA, long chain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; OBCFA, odd and branched chain fatty acids.

TABLE 4 Alpha-diversity comparisons in the rumen microbiota among the different groups.

Index	Groups			SEM	Kruskal–Wallis test	Wilcoxon rank sum test		
	LZ1	LZ2	LZ3			LZ1 vs. LZ2	LZ1 vs. LZ3	LZ2 vs. LZ3
Sobs	566.5	611.5	645.6	21.92	0.309	0.341	0.119	0.676
Ace	571.2	616	650.7	22.21	0.309	0.341	0.119	0.676
Chao1	571.5	615.2	650.1	22.26	0.309	0.341	0.119	0.676
Shannon	5.11	5.3	5.31	0.080	0.487	0.309	0.305	0.909
Simpson	0.025	0.016	0.02	0.003	0.321	0.108	0.790	0.425

LZ1, single goat; LZ2, twin goats; LZ3, triplet goats; SEM, standard error of mean.

Discussion

Litter size is a critical and complicated economic trait within the goat industry. Small ruminants have a great frequency of multiple births, and past research has shown that the average litter size in goats varies from 1.30 to 2.37 (de Lima et al., 2020). However, few studies focus on the effect of high fertility on rumen fermentation and growth performance of offspring. In the present study, we investigated the relationship between litter size and rumen fermentation, microbiota community, and growth performance of young goats. We found that LZ3 goats (triplets) have lower growth performance and rumen fermentation efficiency. Moreover, rumen microbiota structure and

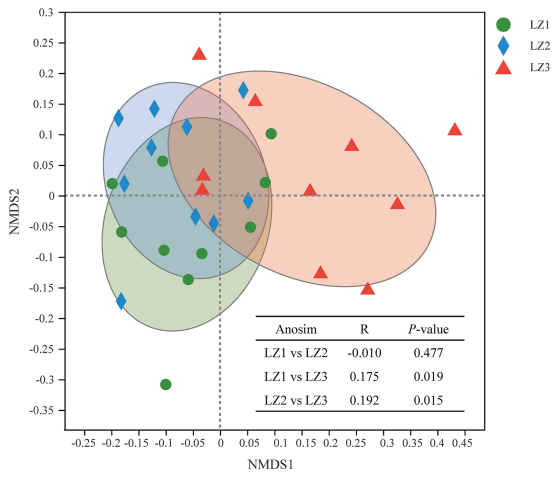

FIGURE 2
Non-metric multidimensional scaling plot based on ASV level to identify differences in microbial community structure among different groups.

composition between LZ3 group and LZ1, LZ2 group were different. And these differences in rumen microbiome were associated with a decrease in rumen metabolites, such as propionate, total VFA, and EAA.

Rumen microbiota plays a fundamentally important role in the development of rumen function and metabolism at the host. Previous studies confirmed the correlations of ruminal microbial features with the ruminants' phenotypic characteristics, such as rumen fermentation products (Kamke et al., 2016; Wallace et al., 2019), feed efficiency (Myer et al., 2015; Shabat et al., 2016; Xue M.-Y. et al., 2022), and milk production (Jami et al., 2014; Xue et al., 2020). According to our current study, triplet kids lead to significant alterations in the microbiota structure of rumen, which had lower pre-weaning and youth growth performance.

Similar to previous studies (Jami et al., 2013; Li et al., 2019; Tong et al., 2022), we also observed that Firmicutes, and Bacteraioidota were the predominant bacterial phylum in the ruminal ecosystem. The abundance of Firmicutes, Bacteraioidota, and the ratio of Firmicutes to Bacteraioidota were different in different litter sizes. The phyla Firmicutes and Bacteraioidota were known for polysaccharide

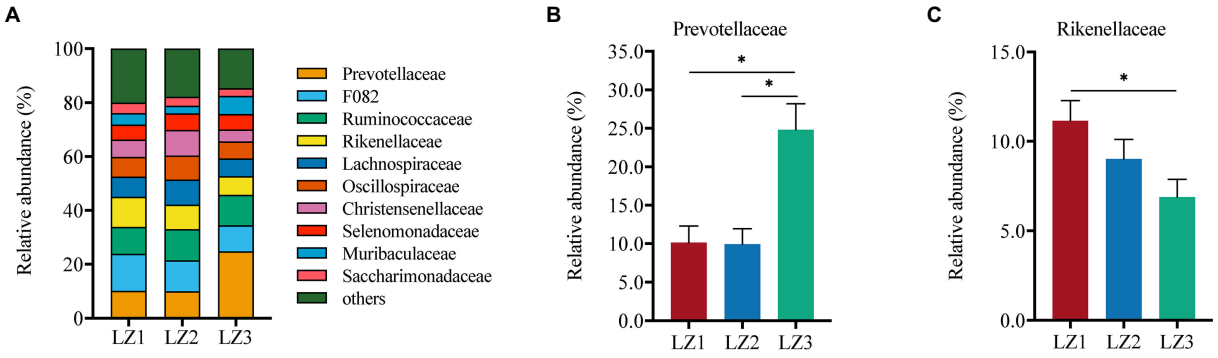

FIGURE 3
Compositions of the rumen microbiota among the different groups at family level. **(A)** Relative abundance of major family, **(B)** Prevotellaceae, **(C)** Rikenellaceae. Data are presented as mean±SEM, *p<0.05, **p<0.01.

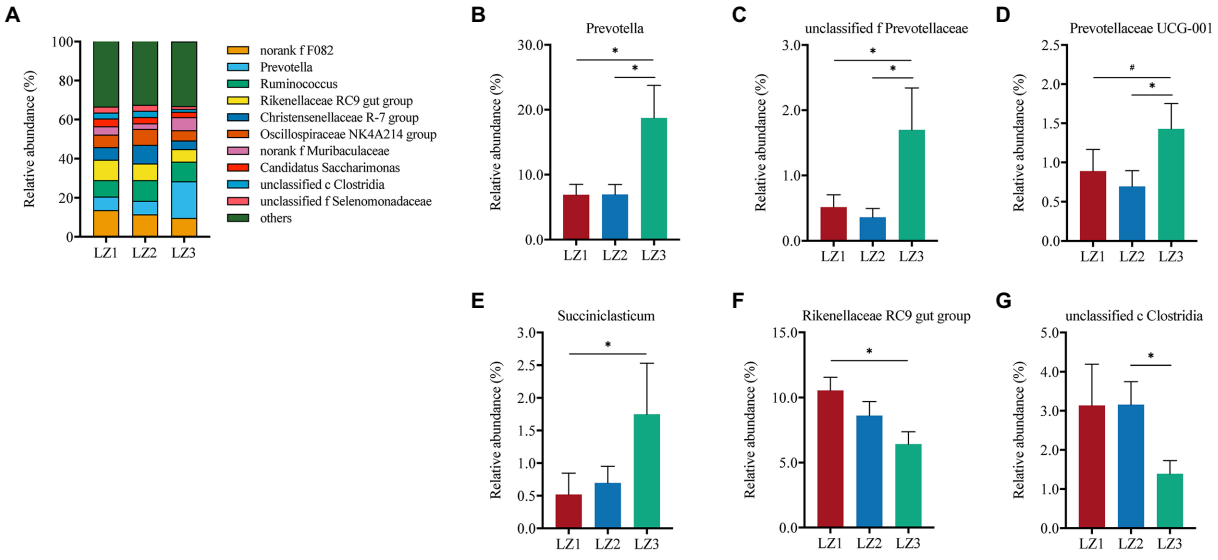

FIGURE 4
Composition of the rumen microbiota among the different groups at genus level. **(A)** Relative abundance of major genus, **(B)** *Prevotella*, **(C)** *unclassified f Prevotellaceae*, **(D)** *Prevotellaceae UCG-001*, **(E)** *Succiniclasticum*, **(F)** *Rikenellaceae RC9 gut group*, **(G)** *unclassified c Clostridia*. Data are presented as mean±SEM, #p<0.1, *p<0.05.

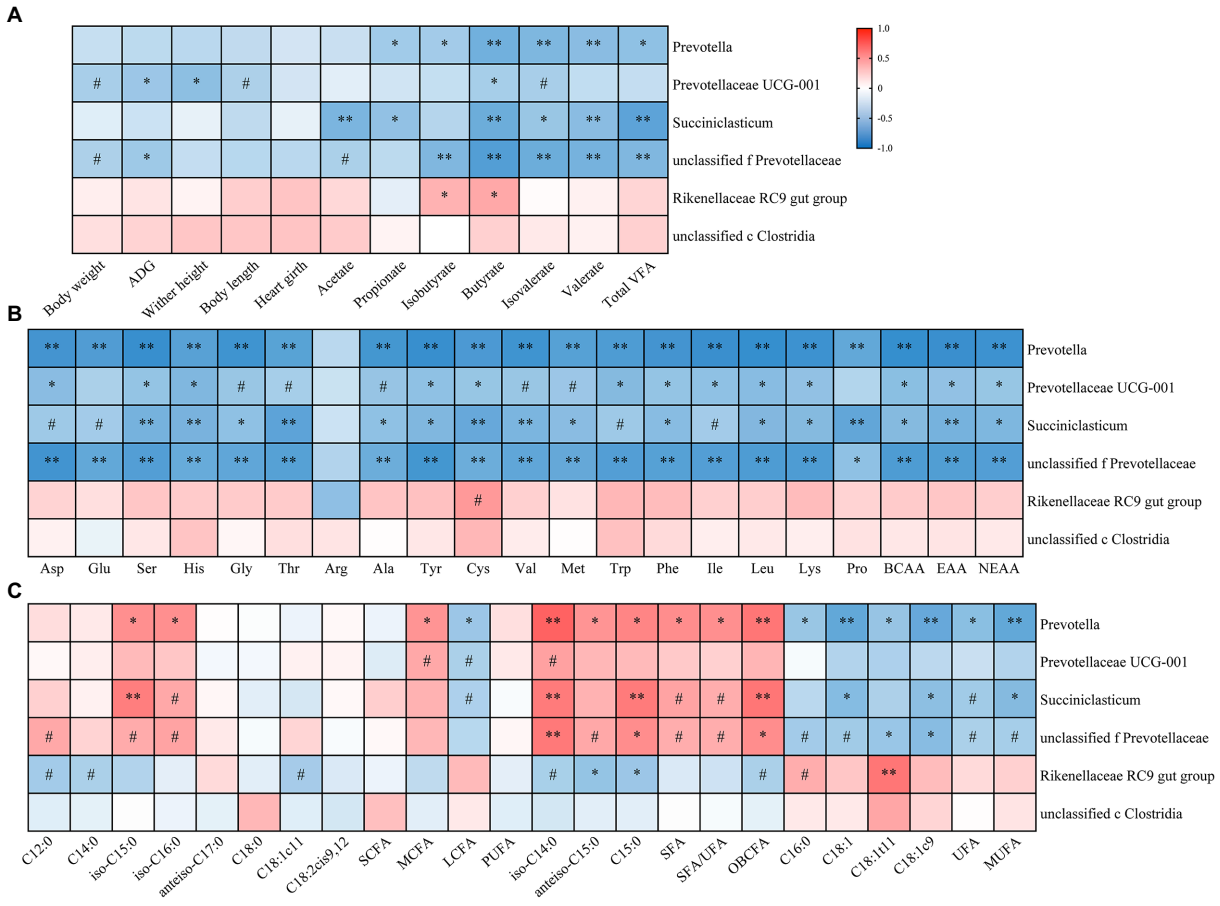

FIGURE 5
Heatmap showing association between growth performance, ruminal metabolites and rumen microbiota of young goats. The relationship between rumen microbiota and growth performance, rumen VFA **(A)**, AA **(B)**, and FA **(C)** of young goats. The color gradient represents the values of correlation coefficients. Red and blue indicate positive and negative correlations, respectively. $^{\#}p<0.1$, $^{*}p<0.05$, $^{**}p<0.01$.

fermentation. And Wang et al. (2019) found that an improved intestinal Firmicutes: Bacteraioidota ratio could allow the host to absorb more energy from the diet and to store energy.

Moreover, we found that rumen fermentation products, such as total VFA, propionate, butyrate, EAA, and BCAA concentrations, which were decreased in triplet goats, had negatively associated with those members of Prevotellaceae family. Rumen VFA and EAA are the major energy and nutrients for animal growth, health and production (Maeng et al., 1976; Van Houtert, 1993; Xue et al., 2019). His and Trp were the limiting AA for growing goats (Onodera, 2003; Ma et al., 2010), and some cell experiments demonstrated that the addition of His activated the mTOR pathway, which up-regulated phosphorylation of the downstream protein and ultimately prompted protein synthesis (Appuhamy et al., 2012). In the current study, the abundance of Prevotellaceae family and several genera of it, such as *Prevotella*, *unclassified f Prevotellaceae*, *Prevotellaceae* UCG-001, were higher in triplet goats. *Prevotella* species had a documented role in metabolism of carbohydrates, such as hemicellulose, starch, xylan, and pectin (Kabel et al., 2011; De Filippis et al., 2019), and nitrogen (Kim et al., 2017). Recent studies suggested the abundance of *Prevotellaceae* family was negatively correlated with feed efficiency in ruminants (Paz et al., 2018; Zhang et al., 2021). Rumen *Prevotella*, and *Prevotellaceae UCG-001* abundance were reported to be negatively correlated with propionate and AA concentrations (Bi et al., 2018; Tong et al., 2020; Liu et al., 2022; Xue B. et al., 2022), and higher in inefficient animals (Carberry et al., 2012; Mccann et al., 2014).

Dietary lipid plays an important role in rumen metabolism and animal production. Moreover, dietary FA are extensively hydrogenated and isomerized by rumen microbes. These transformations directly determine milk and meat FA composition, which is a criterion of the nutritional quality of the products (Chilliard et al., 2007). We observed that ruminal OBCFA and its main constituent FA, anteiso C15:0, were higher in triplet goats. OBCFA are synthesized by ruminal bacteria using the precursors, such as propionate, valerate, BCAA, and incorporated in their cell membrane (Vlaeminck et al., 2006). A previous study showed that rumen cellulolytic bacteria contain relatively high amounts of OBCFA (Fievez et al., 2012). This is consistent with our finding that those members of *Prevotellaceae* family (mainly cellulolytic bacteria), which were enriched in the rumen of triplet goats, were positively associated with OBCFA. Therefore, these triplets enriched bacteria may produce less ruminal VFA, and AA, and utilize more propionate, and BCAA to produce OBCFA, which leads to less energy and nutrient supply for animal growth in triplet goats.

Ruminal butyrate concentration, the proportion of C16:0, C18:1 components (such as C18:1, C18:1t11, and C18:1c9), and MUFA were lower in triplet goats. UFA, especially MUFA, have taxic effects on rumen methanogenic archaea, which lead to the reduction of methane production (Beauchemin et al., 2008). An *in vitro* study found that oleic acid (C18:1c9)

supplementation in diet can reduce methane production (Wu et al., 2016). Moreover, the results showed that the abundance of *Rikenellaceae RC9 gut group* was lower in the rumen of triplet goats, which were positively correlated with ruminal butyrate, C16:0, C18:1t11, and negatively correlated with ruminal anteiso C15:0 and OBCFA. *Rikenellaceae RC9 gut group* can reportedly degrade structural carbohydrates and starch in the rumen (Asma et al., 2013), and be involved in the production of VFA (Graf, 2014) and the scavenging of H_2. A recent study found that the *Rikenellaceae RC9 gut group* abundance was associated with an increase of the feed efficiency trait (Andrade et al., 2022). These bacteria may play roles in rumen fermentation and animal growth. Based on the results, it would be promising to develop probiotics that promote the "single-enriched" and inhibit the "triplets-enriched" bacterial taxa to improve rumen efficiency and growth rates in young goats.

Conclusion

In conclusion, the present study disclosed the differences in rumen microbiota composition, rumen fermentation, and growth performance between single, twin, and triplet goats. Our study revealed that triplet goats have lower growth performance and rumen fermentation efficiency. The Prevotellaceae family, and several genera of Prevotellaceae, such as *Prevotella*, were higher in the rumen of triplet goats, whereas the abundance of *Rikenellaceae RC9 gut group* was lower in the rumen of triplet goats. Our results revealed the change in rumen microbiome composition may affect the rumen metabolism, thus slowing the growth rates in triplet goats. Overall, our study generated relevant information for manipulating rumen microbiome and improving rumen function that will increase the feed efficiency and growth performance in goats.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of repository/repositories and accession number can be found in the NCBI sequence read archive, accession number PRJNA918955.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Northwest A&F University.

Author contributions

DW and JYa designed the research. DW, JYu, YL, and YW performed the experiment. DW, GT, and LC analyzed the data. DW wrote the manuscript. YC and XL undertook revision work. JYa finalized the manuscript. All authors read and approved the final version of the manuscript.

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Conflict of interest

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

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References

Akpa, G., Alphonsus, C., Dalha, S., Yakubu, H., and Garba, Y. (2011). Relationship between litter size and parity of doe in smallholder goat herds in Kano and its environs, Nigeria. *Afr. J. Agric. Res.* 6, 6212–6216. doi: 10.5897/AJAR11.365

Andrade, B. G., Bressani, F. A., Cuadrat, R. R., Cardoso, T. F., Malheiros, J. M., de Oliveira, P. S., et al. (2022). Stool and ruminal microbiome components associated with methane emission and feed efficiency in nelore beef cattle. *Front. Genet.* 13:812828. doi: 10.3389/fgene.2022.812828

Appuhamy, J. R. N., Knoebel, N. A., Nayananjalie, W. D., Escobar, J., and Hanigan, M. D. (2012). Isoleucine and leucine independently regulate mTOR signaling and protein synthesis in MAC-T cells and bovine mammary tissue slices. *J. Nutr.* 142, 484–491. doi: 10.3945/jn.111.152595

Asma, Z., Sylvie, C., Laurent, C., Jérôme, M., Christophe, K., Olivier, B., et al. (2013). Microbial ecology of the rumen evaluated by 454 GS FLX pyrosequencing is affected by starch and oil supplementation of diets. *FEMS Microbiol. Ecol.* 83, 504–514. doi: 10.1111/1574-6941.12011

Beauchemin, K. A., Kreuzer, M., O'Mara, F., and McAllister, T. A. (2008). Nutritional management for enteric methane abatement: a review. *Aust. J. Exp. Agric.* 48, 21–27. doi: 10.1071/EA07199

Bi, Y., Tu, Y., Zhang, N., Wang, S., Zhang, F., Suen, G., et al. (2021). Multiomics analysis reveals the presence of a microbiome in the gut of fetal lambs. *Gut* 70, 853–864. doi: 10.1136/gutjnl-2020-320951

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

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* 6, 1621–1624. doi: 10.1038/ismej.2012.8

Carberry, C. A., Kenny, D. A., Han, S., McCabe, M. S., and Waters, S. M. (2012). Effect of phenotypic residual feed intake and dietary forage content on the rumen microbial community of beef cattle. *Appl. Environ. Microbiol.* 78, 4949–4958. doi: 10.1128/AEM.07759-11

Chen, J., Lei, X. J., Wang, L., Zhang, Y. L., Wang, D. D., Zhao, L. C., et al. (2022). Effects of rumen-protected leucine on production performance and starch digestion in the small intestine of lactating goats. *Anim. Feed Sci. Technol.* 287:115270. doi: 10.1016/j.anifeedsci.2022.115270

Chen, S., Zhou, Y., Chen, Y., and Gu, J. (2018). fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 34, i884–i890. doi: 10.1093/bioinformatics/bty560

Chilliard, Y., Glasser, F., Ferlay, A., Bernard, L., Rouel, J., and Doreau, M. (2007). Diet, rumen biohydrogenation and nutritional quality of cow and goat milk fat. *Eur. J. Lipid Sci. Technol.* 109, 828–855. doi: 10.1002/ejlt.200700080

Christley, R., Morgan, K., Parkin, T., and French, N. (2003). Factors related to the risk of neonatal mortality, birth-weight and serum immunoglobulin concentration in lambs in the UK. *Prev. Vet. Med.* 57, 209–226. doi: 10.1016/s0167-5877(02)00235-0

De Filippis, F., Pasolli, E., Tett, A., Tarallo, S., Naccarati, A., De Angelis, M., et al. (2019). Distinct genetic and functional traits of human intestinal *Prevotella copri* strains are associated with different habitual diets. *Cell Host Microbe* 25, 444.e443–453.e443. doi: 10.1016/j.chom.2019.01.004

de Lima, L. G., de Souza, N. O. B., Rios, R. R., de Melo, B. A., dos Santos, L. T. A., Silva, K. D. M., et al. (2020). Advances in molecular genetic techniques applied to selection for litter size in goats (*Capra hircus*): a review. *J. Appl. Anim. Res.* 48, 38–44. doi: 10.1080/09712119.2020.1717497

Fievez, V., Colman, E., Castro-Montoya, J., Stefanov, I., and Vlaeminck, B. (2012). Milk odd-and branched-chain fatty acids as biomarkers of rumen function—an update. *Anim. Feed Sci. Technol.* 172, 51–65. doi: 10.1016/j.anifeedsci.2011.12.008

Gootwine, E., Spencer, T., and Bazer, F. (2007). Litter-size-dependent intrauterine growth restriction in sheep. *Animal* 1, 547–564. doi: 10.1017/S1751731107691897

Graf, J. (2014). "The family Rikenellaceae" in *The Prokaryotes*. eds. E. Rosenberg, E. DeLong, S. Lory, E. Stackebrandt and F. Thompson (Berlin: Springer)

Haenlein, G. (2004). Goat milk in human nutrition. *Small Rumin. Res.* 51, 155–163. doi: 10.1016/j.smallrumres.2003.08.010

Hummel, G., Woodruff, K., Austin, K., Knuth, R., Lake, S., and Cunningham-Hollinger, H. (2021). Late gestation maternal feed restriction decreases microbial diversity of the placenta while mineral supplementation improves richness of the fetal gut microbiome in cattle. *Animals* 11:2219. doi: 10.3390/ani11082219

Jami, E., Israel, A., Kotser, A., and Mizrahi, I. (2013). Exploring the bovine rumen bacterial community from birth to adulthood. *ISME J.* 7, 1069–1079. doi: 10.1038/ismej.2013.2

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

Jiang, X., Lu, N., Xue, Y., Liu, S., Lei, H., Tu, W., et al. (2019). Crude fiber modulates the fecal microbiome and steroid hormones in pregnant Meishan sows. *Gen. Comp. Endocrinol.* 277, 141–147. doi: 10.1016/j.ygcen.2019.04.006

Kabel, M. A., Yeoman, C. J., Han, Y., Dodd, D., Abbas, C. A., de Bont, J. A., et al. (2011). Biochemical characterization and relative expression levels of multiple carbohydrate esterases of the xylanolytic rumen bacterium *Prevotella ruminicola* 23 grown on an ester-enriched substrate. *Appl. Environ. Microbiol.* 77, 5671–5681. doi: 10.1128/AEM.05321-11

Kamke, J., Kittelmann, S., Soni, P., Li, Y., Tavendale, M., Ganesh, S., et al. (2016). Rumen metagenome and metatranscriptome analyses of low methane yield sheep reveals a *Sharpea*-enriched microbiome characterised by lactic acid formation and utilisation. *Microbiome* 4, 56–16. doi: 10.1186/s40168-016-0201-2

Kim, J. N., Méndez-García, C., Geier, R. R., Iakiviak, M., Chang, J., Cann, I., et al. (2017). Metabolic networks for nitrogen utilization in *Prevotella ruminicola* 23. *Sci. Rep.* 7:7851. doi: 10.1038/s41598-017-08463-3

Lai, F.-N., Zhai, H.-L., Cheng, M., Ma, J.-Y., Cheng, S.-F., Ge, W., et al. (2016). Whole-genome scanning for the litter size trait associated genes and SNPs under selection in dairy goat (*Capra hircus*). *Sci. Rep.* 6, 1–12. doi: 10.1038/srep38096

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

Li, F., Li, Z., Li, S. D., Ferguson, J., Cao, Y., Yao, J., et al. (2014). Effect of dietary physically effective fiber on ruminal fermentation and the fatty acid profile of milk in dairy goats. *J. Dairy Sci.* 97, 2281–2290. doi: 10.3168/jds.2013-6895

Liu, Y., Wu, H., Liu, C., and Zhou, Z. (2022). Rumen microbiome and metabolome of high and low residual feed intake angus heifers. *Front. Vet. Sci.* 9:812861. doi: 10.3389/fvets.2022.812861

Ma, C., Zhang, W., Gao, Q., Zhu, Q., Song, M., Ding, H., et al. (2020). Dietary synbiotic alters plasma biochemical parameters and fecal microbiota and metabolites in sows. *J. Funct. Foods* 75:104221. doi: 10.1016/j.jff.2020.104221

Ma, H., Zhang, W., Zhu, X., Song, W., Liu, J., and Jia, Z. (2010). Effects of rumen-protected tryptophan on growth performance, fibre characteristics, nutrient utilization and plasma essential amino acids in cashmere goats during the cashmere slow growth period. *Livest. Sci.* 131, 227–233. doi: 10.1016/j.livsci.2010.04.005

MacHugh, D. E., and Bradley, D. G. (2001). Livestock genetic origins: goats buck the trend. *Proc. Natl. Acad. Sci. U. S. A.* 98, 5382–5384. doi: 10.1073/pnas.111163198

Maeng, W., Van Nevel, C., Baldwin, R., and Morris, J. (1976). Rumen microbial growth rates and yields: effect of amino acids and protein. *J. Dairy Sci.* 59, 68–79. doi: 10.3168/jds.S0022-0302(76)84157-4

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

Matthews, C., Crispie, F., Lewis, E., Reid, M., O'Toole, P. W., and Cotter, P. D. (2019). The rumen microbiome: a crucial consideration when optimising milk and meat production and nitrogen utilisation efficiency. *Gut Microbes* 10, 115–132. doi: 10.1080/19490976.2018.1505176

Mccann, J. C., Wiley, L. M., David, F. T., Rouquette, F. M., Tedeschi, L. O., and Dan, Z. (2014). Relationship between the rumen microbiome and residual feed intake-efficiency of Brahman bulls stocked on bermudagrass pastures. *PLoS One* 9:e91864. doi: 10.1371/journal.pone.0091864

Myer, P. R., Smith, T. P., Wells, J. E., Kuehn, L. A., and Freetly, H. C. (2015). Rumen microbiome from steers differing in feed efficiency. *PLoS One* 10:e0129174. doi: 10.1371/journal.pone.0129174

O'Hara, E., Neves, A. L., Song, Y., and Guan, L. L. (2020). The role of the gut microbiome in cattle production and health: driver or passenger? *Annu. Rev. Anim. Biosci.* 8, 199–220. doi: 10.1146/annurev-animal-021419-083952

Onodera, R. (2003). Essentiality of histidine in ruminant and other animals including human beings. *Asian Australas. J. Anim. Sci.* 16, 445–454. doi: 10.5713/ajas.2003.445

Paz, H. A., Hales, K. E., Wells, J. E., Kuehn, L. A., Freetly, H. C., Berry, E. D., et al. (2018). Rumen bacterial community structure impacts feed efficiency in beef cattle. *J. Anim. Sci.* 96, 1045–1058. doi: 10.1093/jas/skx081

Pulina, G., Milán, M., Lavín, M., Theodoridis, A., Morin, E., Capote, J., et al. (2018). Invited review: current production trends, farm structures, and economics of the dairy sheep and goat sectors. *J. Dairy Sci.* 101, 6715–6729. doi: 10.3168/jds.2017-14015

Rowe, M., Veerus, L., Trosvik, P., Buckling, A., and Pizzari, T. (2020). The reproductive microbiome: an emerging driver of sexual selection, sexual conflict, mating systems, and reproductive isolation. *Trends Ecol. Evol.* 35, 220–234. doi: 10.1016/j.tree.2019.11.004

Shabat, S. K. B., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Berg Miller, M. E., et al. (2016). Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. *ISME J.* 10, 2958–2972. doi: 10.1038/ismej.2016.62

Sun, X., and Gibbs, S. (2012). Diurnal variation in fatty acid profiles in rumen digesta from dairy cows grazing high-quality pasture. *Anim. Feed Sci. Technol.* 177, 152–160. doi: 10.1016/j.anifeedsci.2012.08.013

Tong, F., Wang, T., Gao, N. L., Liu, Z., Cui, K., Duan, Y., et al. (2022). The microbiome of the buffalo digestive tract. *Nat. Commun.* 13:823. doi: 10.1038/s41467-022-28402-9

Tong, J.-J., Zhang, H., Jia, W., Yun, L., Mao, S.-Y., Xiong, B.-H., et al. (2020). Effects of different molecular weights of chitosan on methane production and bacterial community structure *in vitro*. *J. Integr. Agric.* 19, 1644–1655. doi: 10.1016/S2095-3119(20)63174-4

Van Houtert, M. (1993). The production and metabolism of volatile fatty acids by ruminants fed roughages: a review. *Anim. Feed Sci. Technol.* 43, 189–225. doi: 10.1016/0377-8401(93)90078-X

Vlaeminck, B., Fievez, V., Cabrita, A., Fonseca, A., and Dewhurst, R. (2006). Factors affecting odd-and branched-chain fatty acids in milk: a review. *Anim. Feed Sci. Technol.* 131, 389–417. doi: 10.1016/j.anifeedsci.2006.06.017

Wallace, R. J., Sasson, G., Garnsworthy, P. C., Tapio, I., Gregson, E., Bani, P., et al. (2019). A heritable subset of the core rumen microbiome dictates dairy cow productivity and emissions. *Sci. Adv.* 5:eaav8391. doi: 10.1126/sciadv.aav8391

Wang, S., Yao, B., Gao, H., Zang, J., Tao, S., Zhang, S., et al. (2019). Combined supplementation of *lactobacillus fermentum* and *Pediococcus acidilactici* promoted growth performance, alleviated inflammation, and modulated intestinal microbiota in weaned pigs. *BMC Vet. Res.* 15:239. doi: 10.1186/s12917-019-1991-9

Wu, D., Xu, L., Tang, S., Guan, L., He, Z., Guan, Y., et al. (2016). Influence of oleic acid on rumen fermentation and fatty acid formation *in vitro*. *PLoS One* 11:e0156835. doi: 10.1371/journal.pone.0156835

Xue, M., Sun, H., Wu, X., Guan, L. L., and Liu, J. (2018). Assessment of rumen microbiota from a large dairy cattle cohort reveals the pan and core bacteriomes contributing to varied phenotypes. *Appl. Environ. Microbiol.* 84:e00970-00918. doi: 10.1128/AEM.00970-18

Xue, M., Sun, H., Wu, X., Guan, L., and Liu, J. (2019). Assessment of rumen bacteria in dairy cows with varied milk protein yield. *J. Dairy Sci.* 102, 5031–5041. doi: 10.3168/jds.2018-15974

Xue, M.-Y., Sun, H.-Z., Wu, X.-H., Liu, J.-X., and Guan, L. L. (2020). Multi-omics reveals that the rumen microbiome and its metabolome together with the host metabolome contribute to individualized dairy cow performance. *Microbiome* 8, 64–19. doi: 10.1186/s40168-020-00819-8

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

Xue, M.-Y., Xie, Y.-Y., Zhong, Y., Ma, X.-J., Sun, H.-Z., and Liu, J.-X. (2022). Integrated meta-omics reveals new ruminal microbial features associated with feed efficiency in dairy cattle. *Microbiome* 10, 32–14. doi: 10.1186/s40168-022-01228-9

Zhang, C., Yang, L., and Shen, Z. (2008). Variance components and genetic parameters for weight and size at birth in the boer goat. *Livest. Sci.* 115, 73–79. doi: 10.1016/j.livsci.2007.06.008

Zhang, Y., Zhang, X., Li, F., Li, C., Li, G., Zhang, D., et al. (2021). Characterization of the rumen microbiota and its relationship with residual feed intake in sheep. *Animal* 15:100161. doi: 10.1016/j.animal.2020.100161

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Effect of dietary biochanin A on lactation performance, antioxidant capacity, rumen fermentation and rumen microbiome of dairy goat

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Biochanin A (BCA), an isoflavone phytoestrogen, is a secondary metabolite produced mainly in leguminous plants. The objective of this study was to evaluate the effect of BCA on lactation performance, nitrogen metabolism, and the health of dairy goat. Thirty mid-lactation Saanen dairy goats were divided into three groups randomly: control, 2 g/d BCA group, and 6 g/d BCA group. After 36 days of feeding, 30 dairy goats were transferred to individual metabolic cages. Subsequently, milk yield, feed intake, total feces, and urine excretion were recorded and samples were collected continuously for 3 days. Blood and ruminal fluid samples were collected over the subsequent 4 days. Milk yield, milk protein, fat content, and the feed conversion ratio of dairy goat were significantly increased by the BCA treatment. The levels of serum 17β-estradiol, growth hormone, insulin-like growth factor 1, glutathione peroxidase activity, and total antioxidant capacity were also increased significantly by BCA, indicating that BCA enhanced the antioxidant capacity of dairy goat. Amino acid degradation was significantly inhibited, while the ammonia nitrogen content was reduced significantly by BCA. Total volatile fatty acids was significantly increased by BCA supplementation. In addition, the relative abundance of *Verrucomicrobiota* was decreased significantly. However, the growth of nitrogen metabolism and cellulolytic bacteria was significantly increased under BCA treatment, including *Prevotella sp.*, *Treponema sp.*, *Ruminococcus flavefaciens*, and *Ruminobacter amylophilus*. In conclusion, supplementation with BCA improved the milk production performance, nitrogen metabolism, rumen fermentation and antioxidant capacity, and regulated the rumen microbiome of dairy goat.

KEYWORDS

antioxidant capacity, biochanin A, dairy goat, lactation performance, rumen microbiome, ruminal fermentation

Introduction

Biochanin A (BCA), an isoflavone phytoestrogen with various functions (Wenjin et al., 2021), is a secondary metabolite produced mainly in leguminous plants such as red clover and chickpea (Křížová et al., 2019). The BCA content in red clover (*Trifolium pratense L.*) leaves during the flowering stage ranges from 4.57 to 6.86 mg/g dry matter (DM), with an

average concentration of 5.76 mg/g DM, which accounts for approximately 40% of the total isoflavones (Lemeziene et al., 2015). Chickpea (*Cicer arietinum L.*) sprouts contain lower concentration of BCA than red clover, with a maximum concentration of 2.34 mg/g DM (Gao et al., 2015). Sonication of peanuts (*Arachis hypogaea*) in 80% aqueous ethanol yielded a BCA content of 0.964 mg/100 g DM (Chukwumah et al., 2009). Young plants of *Astragalus glycyphyllos L.* and *Astragalus cicer L.* had BCA contents of 8.81 and 11.4 mg/100 g DM, respectively (Butkute et al., 2018). Other natural sources of BCA include alfalfa (Kagan et al., 2015) and soybeans (Hu et al., 2021). Recent studies have demonstrated that BCA possesses numerous biological properties, such as anti-inflammatory, anti-microbial, and antioxidant activities (Jalaludeen et al., 2016; Sadri et al., 2017). Furthermore, BCA has been shown to be a potential drug for protection against osteoporosis (Lee and Choi, 2005), prevention and treatment of atherosclerotic cardiovascular disease (Yu et al., 2020), and alleviation of symptoms in postmenopausal women (Galal et al., 2018). Therefore, BCA is an important source of novel health foods and natural products.

Hyper-ammonia-producing bacteria (HAB) play a vital role in amino acid (AA) deamination in the rumen (Flythe and Kagan, 2010). *In vitro*, BCA was shown to possess antimicrobial activity against HAB isolated from the rumen fluid of bovines and caprines; however, it has to act synergistically with other heat-stable antibacterial compounds to inhibit HAB growth (Flythe and Kagan, 2010; Flythe et al., 2013). In addition, BCA exhibited selective antimicrobial activity against amylolytic and cellulolytic bacteria, and increased *Lactobacilli*, decreased gram-positive *cocci*, and directly inhibited the growth of *Fibrobacter succinogenes S85*, *Ruminococcus flavefaciens 8*, and *Ruminococcus albus 8* (Harlow et al., 2017b, 2018). These results were attributed to the enhancement of the activities of native rumen antimicrobials (e.g., bacteriocins) by BCA (Flythe et al., 2013; Harlow et al., 2017b). Studies have also shown that BCA can inhibit lactic acid production and increase lactate-utilizing bacteria and lactic acid metabolism, thereby ameliorating pH decline, promoting rumen fermentation, and increasing the concentrations of acetate, propionate, and total volatile fatty acids (VFAs) (Harlow et al., 2017b, 2018). Furthermore, BCA could be a substitute for antibiotics, such as monensin, for reducing rumen acidosis (Harlow et al., 2017c, 2021). Supplementing steers fed dried distillers' grains (DDG) with BCA improved crude protein (CP) digestibility of DDG and increased average daily gain, as the addition of BCA decreased ammonia production and improved the quality of absorbed protein by inhibiting HAB (Flythe et al., 2013; Harlow et al., 2017a). Similar results were observed with red clover whereby the average daily gain depended on the interaction between BCA and other isoflavones (Harlow et al., 2020).

In our lab, it was observed that BCA inhibited the activity of proteolytic and ureolytic bacteria and improved the efficiency of microbial protein synthesis (Liu et al., 2020). Therefore, BCA is hypothesized to be a potential novel urease inhibitor. However, to date, there is limited information on the *in vivo* effects of BCA on dairy livestock. Therefore, the objective of this study was to evaluate the effects of BCA on production performance, health, and rumen microbiome of dairy goats.

TABLE 1 Ingredient and chemical composition of total mixed diet fed to dairy goats.

Ingredient composition	% of DM	Chemical composition	% of DM
Oat grass	11.42	NE_L[2] (Mcal/kg)	1.92
Alfalfa hay	30.06	DM	80.76
Steam-flaked corn	13.49	ADF	28.77
Soybean meal	12.13	NDF	48.70
Cottonseed meal	1.49	CP	16.83
Dried distiller's grains	1.49	Ether extract	2.52
Bran	6.02	–	–
Wheat shorts	0.60	–	–
Preminx[1]	1.49	–	–
Sprayed corn bran	1.50	–	–
Corn gluten meal	0.84	–	–
Cron bran	6.01	–	–
Yeast	3.84	–	–
Corn silage	8.97	–	–
$NaHCO_3$	0.21	–	–
Mycotoxin adsorbent	0.19	–	–
Urea	0.25	–	–

[1]The premix provided the following per kg of diets: vitamin A 650 K international unit (IU), vitamin D 350 K IU, vitamin E 4,000 IU, Zn 2,500 mg, Cu 200 mg, Mn 1,200 mg.
[2]NE_L = lactation net energy, as calculated with reference to nutrient requirements of dairy cattle (NRC) (2007) nutrient requirements of goats.

Materials and methods

Animal management

Animal experiments were approved by the Animal Care and Use Committee of the Institute of Animal Science of the Chinese Academy of Agricultural Sciences (approval no. IAS2020-97). Thirty mid-lactation health Saanen dairy goats (body weights of 63.5 ± 5.6 kg at 147 ± 3 d of lactation) from Weihe Dairy Farm (Qiangyang, China) were randomly divided into three groups (n = 10): basal diet group (control), basal diet with 2 g/d BCA per goat group (BL), and basal diet with 6 g/d BCA per goat group (BH). To make sure the 6 g/d group indeed ingested 6 g/d BCA a day per goat, BCA was mixed with a small amount of basic diet and fed to each goat separately every morning. After each goat had finished all the basic diet with BCA, the left diet would be given to them. The doses used were based on previous report (Harlow et al., 2017c). The duration of the experiment was 50 d in autumn (September–November), with 43 d permitted for diet acclimatization and 7 d allocated for sample collection. The dairy goats were placed in individual metabolic cages on day 36 of the acclimatization period. The goats were fed, *ad libitum*, a basal diet and total mixed diet (TMR), and were allowed approximately 5% orts twice a day at 08:00 and 14:30. Dairy goats were milked twice daily, at 07:00 and 18:00. The dietary ingredients (% DM) and chemical composition (% DM) of TMR are shown in **Table 1**.

Sample collection

During days 44–46 of the experimental period, the feed intake of each goat was recorded daily. Samples of TMR and orts were

collected and stored at −20°C until further analysis. The dairy milk yields obtained from all the goats were recorded from day 44 to 46 of the experimental period. Milk samples were collected at 07:00 and 18:00 and mixed at a ratio of 1:1. A 40 mL subsample of milk was transferred to vials preserved with 2-bromo-2-nitropropane-1-3-diol and stored at 4°C for composition analysis. Approximately 80 mL subsamples, without any treatment, were stored in two 50 mL centrifuge tubes at −20°C. Urine and fecal samples were collected daily from days 44 to 46 of the experimental period. The urine was collected in a plastic container containing 200 mL of diluted sulfuric acid which was used to maintain the pH at <3. Urine volumes were recorded daily, with 5% of the urine samples stored in vials. Urine samples from each goat were mixed for three consecutive days and stored at −20°C until nitrogen balance and purine derivative analysis were performed. The total fecal output of each goat was collected into large metal nets that were placed under the metabolic cages and weighed daily. A 5% aliquot of each goat feces was transferred into self-sealed plastic bags daily, mixed for three consecutive days, and stored at −20°C until chemical analysis was performed.

On days 47 and 48 of the experimental period, approximately 1 h after the morning feed, blood samples were collected from the jugular vein using 10 mL vacutainer tubes containing sodium heparin. The tubes were immediately centrifuged at 3,500 × *g* at 4°C for 15 min to separate the plasma (Mendowski et al., 2020). The plasma samples were transferred to 2 mL centrifuge tubes to determine the plasma antioxidant capacity, biochemical parameters, and endocrine indices. On days 49 and 50, rumen fluid samples were collected through the ruminal cannula *via* suction using a hose 1 h after the morning feed (Mendowski et al., 2020). Rumen fluid was filtered through four layers of cheesecloth, and the pH was determined immediately. A 10 mL subsample of ruminal fluid was acidified immediately using 2 mL of metaphosphoric acid (25%, m/v) to determine the VFA, and ammonia-nitrogen (NH_3-N) contents. A 40 mL subsample without metaphosphoric acid was collected into a 50 mL centrifuge tube and stored at −20°C for ruminal bacterial diversity analysis. All the samples of every goat and data point was included in the analysis.

Sample analysis

Samples of TMR, orts, and feces were dried at 65°C in a forced-air oven to a constant weight to measure the DM, CP, ether extract, acid detergent fiber (ADF), and neutral detergent fiber (NDF) contents by wet chemistry. The DM was determined using a forced-air oven (AOAC method 930.15). The nitrogen content was determined using a Kjeldahl auto-analyzer (VELP DK20, Usmate, Italy), and the CP was calculated as $N \times 6.25$ (AOAC method 990.03). The ether extract content was determined using the AOAC International method 920.39 (AOAC method 920.39). The ADF and NDF contents were measured according to a previously described method (Van Soest et al., 1991). Milk subsamples preserved in 2-bromo-2-nitropropane-1-3-diol were analyzed for true protein, fat, lactose, and total milk solids using infrared spectroscopy (Foss FT 120, Denmark). The nitrogen content in the milk, fecal, and urine samples was determined using the Kjeldahl method (AOAC method 990.39). The NH_3-N concentration in rumen fluids and urine was measured using a phenol-hypochlorite assay. The rumen AA contents were determined as previously described (Liu et al., 2020). In the rumen fluid preserved with metaphosphoric acid, the VFA content was analyzed using gas chromatography (Agilent 7890A, USA). The allantoin, xanthine, uric acid, and hypoxanthine contents in the urine samples were measured as previously described (Chen and Gomes, 1992).

The total protein, albumin, globulin, creatinine, and β-hydroxybutyric acid content and the activities of alanine and aspartate aminotransferase in the plasma of dairy goats were analyzed using a Beckman AU680 Automatic Biochemical Analyzer (Beckman Coulter Inc., FL, USA). The total AA content, NH_3 concentration, glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase, and total antioxidant capacity (T-AOC) in plasma were quantified using total AA (A026-1-1), blood ammonia (A086-1), GSH-Px (A005-1), SOD (A001-3-2), catalase (A007-1-1), and T-AOC (A01502-1) assay kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), respectively. The levels of growth hormone, prolactin, estradiol, and insulin in plasma were determined using a DFM-96 Gamma Radioimmunoassay Counter (Zhongheng Electromechanical Technology Development Co., Ltd, Hefei, China). Insulin-like growth factor 1 (IGF-1) was determined using a bovine IGF-1 ELISA kit (Shanghai Ketao Biotechnology Co. LTD, Shanghai, China) according to the manufacturer's instructions.

The total DNA of rumen microorganisms was extracted using the hexadecyltrimethylammonium bromide method and measured using Nanodrop One (Thermo Scientific, MA, USA). The sequencing library was constructed using the MGIEasy kit (BGI, China) and the BGISEQ-500, with read lengths of 2 × 100 base pairs (bp), was used to sequence the libraries. Quality control analysis of the original data was performed using the TrimGalore software to remove low-quality reads with lengths less than 50 bp and average base mass less than 20 (Mehdipour et al., 2020). The relevant data were then compared with the host (sheep, alfalfa, corn, and soybean) database using BM Tagger to remove the host data (Uritskiy et al., 2018). The sequence was then annotated with the species using the Kraken 2 software (Kopylova et al., 2012) and the GTDB-r89 database (Waschulin et al., 2022). The relative abundance of species was analyzed by principal component analysis and the diversity analysis was performed using MicrobiomeAnalyst (Chong et al., 2020). The species composition and community structure of the rumen microbiome were tested for significance using linear discriminant analysis effect size analysis.

Calculations and statistical analysis

Feed conversion ratio was calculated as previously described (Sears et al., 2020) using the following equation:

Feed conversion ratio (%) = 3.5% FCM/DM intake;

3.5% FCM = [0.4324 × milk yield (kg)] + [16.216 × milk fat yield (kg)],

where FCM = fat-corrected milk; DM = dry matter.

Data were analyzed using the GLM model of SAS v.9.4 (SAS Institute Inc., Cary, NC, USA) as follows:

$$Y_{ij} = \mu + B_i + T_j + \varepsilon_{ij},$$

where Y_{ij} = dependent variable; μ = overall mean; B_i = fixed effect of random block (i = 1 to 5); T_j = fixed effect of BCA level (0, 2, or 6 g); and ε_{ij} = experimental error.

Differences among treatments were considered significant at $P < 0.05$, based on Tukey's multiple comparisons of SAS v.9.4 (SAS

Institute Inc., Cary, NC, USA). Results were expressed as least squares means and the means ± standard error of the mean (SEM).

Results

Intake, milk yield and composition, and feed conversion ratio

Milk yield in BH was 25.34%, which was significantly greater than that in control group ($P = 0.04$), however no significant difference was observed between the control and BL groups ($P = 0.70$; Table 2). Milk protein and total solid contents were increased significantly in BH by 9.73 and 6.38% ($P < 0.01$), respectively. The feed conversion ratios of dairy goats in BL and BH were increased significantly by 34.92 and 31.75% ($P < 0.01$), respectively. However, body weight, dry matter intake (DMI), milk fat, and lactose contents were not significantly affected by BCA supplementation.

Rumen fermentation parameters

As shown in Table 3, the total VFA, acetate, propionate, butyrate, valerate, and isovalerate contents in the rumen of dairy goats were increased significantly with BCA supplementation ($P < 0.05$). However, the pH and NH_3-N concentration in the rumen were decreased significantly with BCA supplementation ($P < 0.05$).

Antioxidant capacity and endocrine indexes in plasma

The GSH-Px activity in plasma was significantly increased with the increase in BCA treatment (Table 4), with GSH-Px in BL increasing by 14.83% and BH increasing by 44.81% ($P = 0.02$). The T-AOC in BH was significantly higher ($P = 0.03$) than that in BL. However, SOD and catalase activities were not significantly affected by BCA supplementation. Furthermore, the total protein, albumin, globulin, uric acid, creatinine, and β-hydroxybutyric acid contents, in addition to the alanine and aspartate aminotransferase activities, were not significantly affected by BCA supplementation (Table 5).

The levels of 17β-estradiol and growth hormone in the plasma of dairy goats in the BH group were significantly higher than those in the BL group ($P < 0.05$; Table 6). The IGF-1 content was significantly increased by BCA supplementation ($P < 0.05$). Compared with control, the IGF-1 content in BL and BH was increased by 28.26 and 26.79%, respectively. However, plasma prolactin and insulin levels were not significantly affected by BCA supplementation.

Rumen microbiome

Rumen microbial diversity was not significantly affected by BCA supplementation (Figure 1A). However, the relative abundance of microbial flora was influenced by BCA supplementation (Figure 1B). The quantity of *Verrucomicrobiota* bacteria was significantly decreased, while that of nitrogen metabolism and cellulolytic bacteria was significantly increased, including *Prevotella sp.*, *Treponema sp.*, *Ruminococcus flavefaciens*, and *Ruminobacter amylophilus* ($P < 0.05$; Figure 1C). Thus, BCA regulated the flora of the rumen microbiome, promoting nitrogen metabolic efficiency in the rumen of dairy goats.

TABLE 2 Effects of biochanin A on body weight, dry matter intake, milk yield, and composition.

Items	Treatments			SEM	*P*-value
	Control	BL	BH		
Body weight, kg	66.67	65.32	65.17	1.10	0.860
Dry matter intake, kg/d	1.88	1.45	1.90	0.10	0.119
Milk yield, kg/d	1.46^{b}	1.40^{b}	1.83^{a}	0.076	0.028
Milk composition, %	–	–	–	–	–
Protein	3.64^{b}	4.01^{ab}	4.32^{a}	0.096	0.020
Fat	4.39	4.74	4.73	0.096	0.326
Lactose	4.50	4.59	4.72	0.038	0.072
Total solid	12.54^{b}	13.34^{ab}	13.76^{a}	0.176	0.021
Feed conversion ratio[1], %	0.63^{b}	0.85^{a}	0.83^{a}	0.030	0.043

$^{a-b}$Different lowercase letters in row represent significant differences ($P < 0.05$).
[1]Feed conversion ratio (%) = 3.5% FCM/dry matter intake; 3.5% FCM = [0.515 × milk yield (kg)] + [13.86 × milk fat yield (kg)].
Means within a row without a common superscript lowercase letter differ significantly ($P < 0.05$, $n = 10$).

TABLE 3 Effects of biochanin A on rumen fermentation of dairy goats.

Items	Treatments			SEM	*P*-value
	Control	BL	BH		
pH	6.71^{a}	6.23^{b}	6.38^{b}	0.065	0.011
Total volatile fatty acids, mmol/L	68.7^{b}	96.2^{a}	91.3^{a}	3.05	<0.001
Acetate, mmol/L	44.6^{b}	64.6^{a}	58.7^{a}	2.07	<0.001
Propionate, mmol/L	14.6^{b}	20.1^{a}	18.6^{ab}	0.867	0.037
Butyrate, mmol/L	7.34^{b}	9.34^{ab}	10.92^{a}	0.505	0.009
Isobutyrate	0.60^{ab}	0.45^{b}	0.73^{a}	0.040	0.008
Valerate	0.94^{b}	1.08^{b}	1.42^{a}	0.076	0.026
Isovalerate	0.71^{b}	0.64^{b}	0.95^{a}	0.045	0.006
NH_3-N, mg/dL	20.4^{a}	17.1^{b}	16.7^{b}	0.484	0.004

Means within a row without a common superscript lowercase letter differ significantly ($P < 0.05$, $n = 10$).

TABLE 4 Effects of biochanin A on antioxidant capacity of plasma.

Items	Treatments			SEM	*P*-value
	Control	BL	BH		
Superoxide dismutase, U/mL	20.71	22.46	19.23	0.649	0.184
Glutathione peroxidase, U/mL	577^{b}	662^{ab}	836^{a}	37.9	0.020
Catalase, U/mL	2.22	2.34	1.86	0.177	0.269
Total antioxidant capacity, mmol/L	0.55^{ab}	0.53^{b}	0.59^{a}	0.011	0.035

Means within a row without a common superscript lowercase letter differ significantly ($P < 0.05$, $n = 10$).

Discussion

Currently, few studies have investigated the influence of BCA on the growth and production performance of dairy goats and cows. Supplementation of steers with BCA (6.3 g/d) exhibited an increase in the average daily gain. The reduction in ammonia concentration was attributed to BCA, which inhibited HAB fermentation of AA deamination (Harlow et al., 2017c). Steers supplemented with 0.91 kg of red clover hay (equivalent to 0.9–1.4 g BCA) and 1.51 kg DDG also exhibited daily gain (Harlow et al., 2020). When the grass silage was replaced with red and white clover silage, the DMI was increased by 1.2 and 1.3 kg, respectively, and the milk yield was increased by 1.5 and 2.2 kg/d, respectively (Steinshamn, 2010). In this study, milk yield and composition were increased when dairy goats were fed BCA. Similarly, supplementation with flavonoid-rich plant extracts improved the milk yield of dairy cows; however, flavonoids from different sources have different effects (Gessner et al., 2015; Zhan et al., 2017). Alfalfa flavonoid extract increased milk lactose synthesis and secretion by reducing the milk fat and protein content (Zhan et al., 2017). However, soy isoflavone supplementation reduced milk production (Kasparovska et al., 2016). These varying results could be caused by extracts from different sources containing different types and amounts of flavonoids. The expression of milk protein and lactose synthesis-related genes were inhibited by BCA and genistein, however, they were upregulated by formononetin and daidzein, and the metabolite of BCA, *p*-ethylphenol (Tsugami et al., 2017).

TABLE 5 Effects of biochanin A on the changes of plasma biochemical parameters.

Items	Treatments			SEM	*P*-value
	Control	BL	BH		
Total protein, g/L	84.60	84.32	81.86	0.799	0.343
Albumin, g/L	33.40	32.08	33.59	0.490	0.433
Globulin, g/L	51.19	52.25	48.26	0.96	0.232
Alanine aminotransferase, U/L	15.76	16.47	13.36	1.06	0.477
Aspartate aminotransferase, U/L	112	126	89.5	6.96	0.081
Uric acid, μmol/L	0.90	2.69	2.85	0.473	0.127
Creatinine, μmol/L	36.33	33.66	36.40	0.95	0.360
β-hydroxybutyric acid, mmol/L	0.32	0.37	0.34	0.016	0.552

TABLE 6 Effects of biochanin A on endocrine indexes of plasma.

Items	Treatments			SEM	*P*-value
	Control	BL	BH		
17β-estradiol, pg/mL	321^{ab}	282^{b}	381^{a}	14.51	0.003
Prolactin, μIU/mL	72.81	49.82	62.12	4.79	0.208
Growth hormone, ng/mL	1.02^{ab}	0.85^{b}	1.27^{a}	0.072	0.027
Insulin, μIU/mL	18.80	16.79	20.21	0.810	0.114
Insulin-like growth factor 1, ng/mL	140^{b}	180^{a}	178^{a}	6.61	0.020

Means within a row without a common superscript lowercase letter differ significantly ($P < 0.05$, $n = 10$).

The nitrogen intake of dairy goats was not significantly affected by BCA addition; however, urine secretion was increased with BCA addition. Urinary nitrogen excretion remained constant. BCA could delay the rate of urea hydrolysis in the rumen, thereby allowing for more urea to be used by rumen microorganisms for microbial protein synthesis. Furthermore, BCA reduced the degradation of proteins and AAs in the rumen by inhibiting the activity of proteolytic bacteria (Liu et al., 2020) and hyper-ammonia-producing bacteria (Harlow et al., 2017c). BCA supplementation increased milk yield, indicating that dietary N utilization efficiency was improved (Harlow et al., 2017c; Pereira et al., 2020). Fewer non-degradable rumen proteins increased protein utilization and produced more milk protein (Morales et al., 2010). In another study, the acetate, propionate, and total VFA contents increased, and pH was decreased by BCA supplementation, which affected cellulose fermentation by rumen cellulolytic bacteria (Harlow et al., 2018). Similar results were obtained when BCA was added to a corn diet fermented by amylolytic bacteria; BCA increased the activity of lactic acid-utilizing bacteria and reduced the starch degradation rate (Harlow et al., 2021). However, the VFA content remained unchanged when no dietary supplements were added to the artificial medium (Liu et al., 2020). In contrast to these studies, all the VFA contents were increased by BCA in this study, which may be caused by different fermentation substrates (Harlow et al., 2020, 2021). In addition, the total VFAs and propionate contents in the rumen were also increased by replacing corn silage with different proportions of red clover (Castro-Montoya et al., 2018). Thus, the increase in VFAs by BCA was dependent on the fermentation substrate.

In this study, rumen ammonia nitrogen concentration was linearly decreased with increasing BCA. The hydrolysis rate of urea was decreased, which ultimately resulted in the inhibition of AA degradation by BCA (Liu et al., 2020). The inhibition of AA degradation by BCA was mainly achieved by inhibiting the growth of hyper-ammonia-producing bacteria, which ferment AAs into ammonia in the rumen (Harlow et al., 2017c). Hyper-ammonia bacterial growth was reduced not inhibited by BCA through the enhancement, by rumen microorganisms, of antibacterial activity of bacteriocin, which reduced the ammonia nitrogen content rather than exerting an inhibitory effect, thereby indicating that BCA had synergistic antibacterial activity (Flythe et al., 2013). Crude red clover phenolic extracts also inhibited the growth of hyper-ammonia-producing bacteria (*Clostridium sticklandii SR*) (Kagan and Flythe, 2012), increased bypass protein, and promoted the by reducing protein nitrogen loss (Harlow et al., 2017c). Therefore, BCA promoted growth of steers rumen fermentation and increased microbial protein production. In addition, the SOD, GSH-Px, and catalase are the main antioxidant enzymes in animals. Serum GSH-Px activity reflected oxidative stress and contributed to oxidative defense in animals (Tuzun et al., 2002). The activities of SOD and GSH-Px were enhanced by BCA through inhibiting nicotinamide adenine dinucleotide phosphate and malondialdehyde production (Yu et al., 2019). In addition, BCA prevented oxidative stress by enhancing the total antioxidant status and the levels of SOD and catalase (Jalaludeen et al., 2016; Sadri et al., 2017). Isoflavone BCA is activating a novel nuclear factor erythroid 2-related factor 2-antioxidant response element activator and can protect against oxidative damage (Yu et al., 2019). Flavonoid extracts containing BCA had a similar effect on improving the antioxidant capacity of livestock and poultry. In chickens supplemented with soybean isoflavone, increased T-AOC and SOD levels in the plasma were

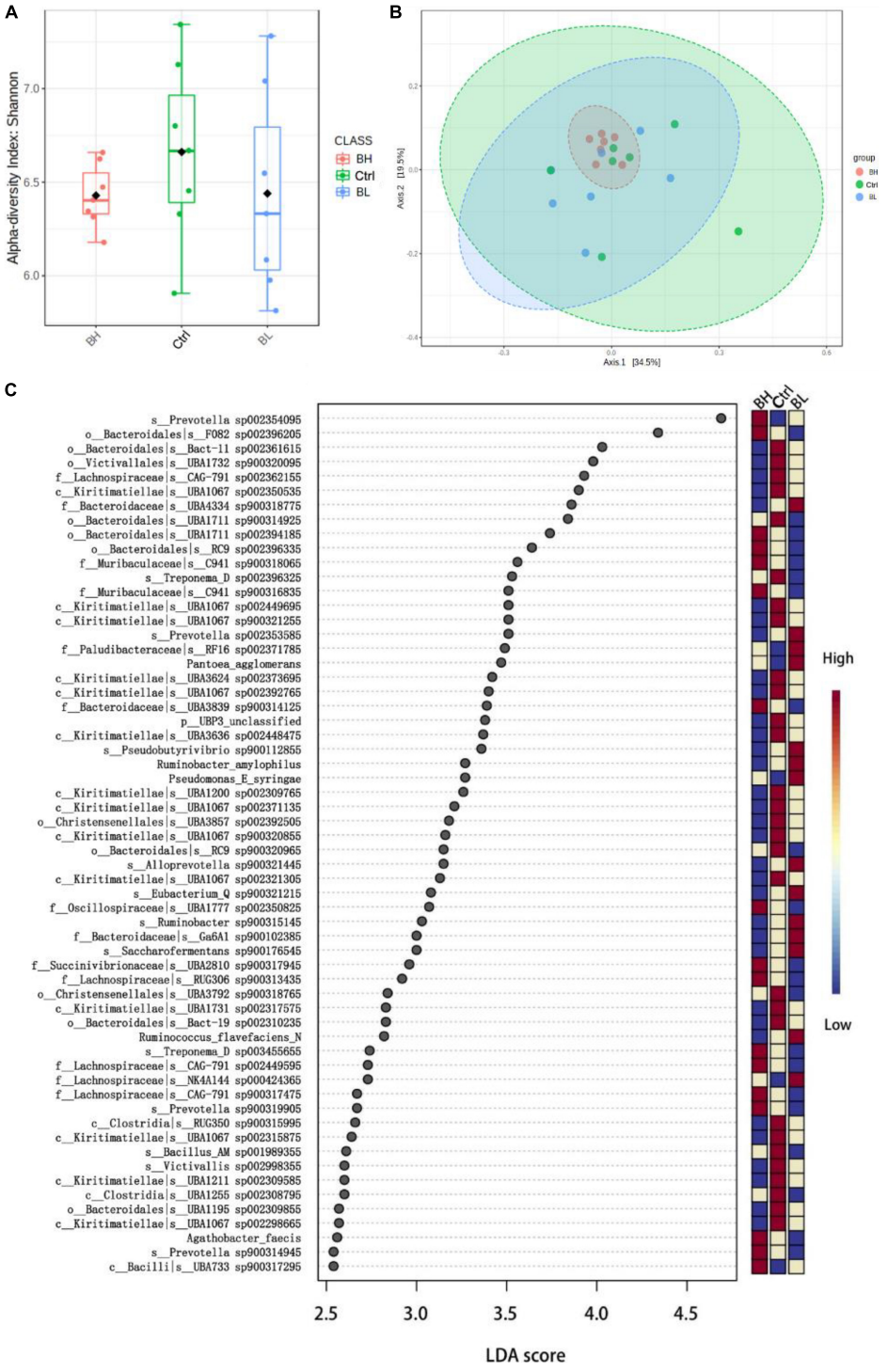

FIGURE 1
Effect of biochanin A (BCA) on the structure of rumen microbiome of dairy goats (n = 7, mean ± SEM). **(A)** Diversity (Shannon index) analysis of rumen microbiome. **(B)** Principal component analysis of rumen microbiome. **(C)** Linear discriminant analysis (LDA) Effect Size analysis of rumen microbial differential flora in genus level. Ctrl, control group; BL, low level BCA group; BH, high level BCA group.

reported (Jiang et al., 2007). Dietary supplementation with flavonoids from *Scutellaria baicalensis Georgi* enhanced the antioxidative ability of broilers (Liao et al., 2018). The activities of SOD and GSH-Px were also increased by feeding alfalfa flavone extract to dairy cows (Zhan et al., 2017). The GSH-Px activity was increased with an increase in BCA supplementation, indicating that BCA can improve the antioxidant performance of dairy goats by enhancing T-AOC and the activities of antioxidant enzymes.

Plasma biochemical parameters reflected the metabolism of nutrients and health conditions of the animal body (Wang et al., 2011). No differences were observed in the total protein, albumin, globulin, uric acid, creatinine, and β-hydroxybutyric acid contents, suggesting that BCA had no effect on the health of dairy goats.

Alanine and aspartate aminotransferase are vital indices of liver function, reflecting the permeability and metabolic function of liver cells (Li et al., 2017). Increases in alanine and aspartate aminotransferase activities were caused by damage to the integrity of the hepatic membrane architecture (Kotyzova et al., 2013). In this study, although there is no significant difference, aspartate aminotransferase activity had a decreased trend in the BH group, indicating that a high dose of BCA may have a hepatoprotective effect in dairy goats, and this trend was also observed in a previous study in rats (Breikaa et al., 2013). PUN and NH_3-N were the final protein metabolites that reflected AA metabolism (Li et al., 2017). PUN concentrations reached a peak approximately 2 h after feeding, then gradually decreased, reverting to normal levels at 6 h (Sinclair et al., 2012). A similar trend was observed in the NH_3-N concentrations, which may have resulted from the decreased hepatic clearance of NH_3-N (Ludden et al., 2000). Soybean isoflavone, an isoflavone phytoestrogen, is a physiological regulator of the reproductive and nutritional processes of the body through the neuroendocrine system to improve the production performance of animals (Jiang et al., 2007). Isoflavone promoted the development of mammary glands, increased milk yield, and accelerated animal growth by inducing changes in endogenous hormone levels in blood, such as prolactin, growth hormone, and IGF-1 (Tsugami et al., 2017). In this study, BCA treatment significantly increased the levels of 17β-estradiol, growth hormone, and IGF-1. Similarly, the levels of 17β-estradiol, growth hormone, and prolactin in the plasma of mid-lactation dairy cows were also increased by soybean isoflavone daidzein (Tsugami et al., 2017). These hormones can regulate the lactation of dairy goats and the structural development of mammary gland, and estradiol and IGF-1 can regulate mammary epithelial cells by binding to estrogen receptor and maintaining its expression (Tsugami et al., 2017). However, there are limited reports on the effects of BCA on the endocrine system of animals; therefore, further studies are required to explore the underlying mechanisms.

In this study, the flora structure of the rumen microbiome was modulated by BCA supplementation, which promoted nitrogen metabolic efficiency in the rumen. Similarly, in another study, the abundance of Bacteroidetes was increased by isoflavone-enriched feed, resulting in a high nitrogen metabolic efficiency in the rumen (Kasparovska et al., 2016). Rumen microbial diversity was not significantly affected, whereas the abundance of rumen microbiota was significantly affected by red clover isoflavones (Melchior et al., 2018). These results indicated that the nitrogen metabolic efficiency in the rumen was improved by BCA treatment by modulating the structure of the rumen microbiome. In this study, the flora of nitrogen metabolism and cellulolytic bacteria in rumen was regulated by BCA, and these rumen microbiota can promote nitrogen metabolic efficiency of dairy goats, resulting in enhancing feed conversion and increasing VFA production. As we know, acetate and butyrate are the precursors of milk fat, and propionate is the precursor of lactose in milk. Therefore, the increased VFA led to the improvement in milk production. In addition, reduced ammonia can be used by rumen microbes, and the increase of microbial protein can increase the milk protein, also leading to the increased feed conversion and VFA production. Additionally, in health dairy goats, the improvement in antioxidative capacity can enhance their resistance of pathogen and diseases, which is beneficial to the health of goats. Moreover, unchanged plasma biochemical parameters indicated that BCA had no side effects on the immune and health of dairy goats. The altered endocrine indexes indicated BCA can increased the production performance of dairy goat through hormone to some extent, including 17β-estradiol, GH, and IGF-1.

Conclusion

Milk production performance, nitrogen metabolism, and the feed conversion ratio of dairy goats were improved by BCA supplementation. Rumen AA degradation were inhibited, whereas VFAs were increased with BCA supplementation, indicating that rumen fermentation was promoted. Antioxidant performance (GSH-Px activity and T-AOC) and endocrine hormones levels (prolactin, growth hormone, and IGF-1) were also increased by BCA supplementation. The relative abundance of rumen microbiota were also affected by BCA supplementation, such as decreased *Verrucomicrobiota* and increased nitrogen metabolism and cellulolytic bacteria, including *Prevotella sp*., *Treponema sp*., *Ruminococcus flavefaciens*, and *Ruminobacter amylophilus*. Thus, dairy goat supplemented with BCA at a concentration of 6 g/day per goat can improve its performance and health status.

Data availability statement

The original contributions presented in this study are publicly available. This data can be found here: https://nmdc.cn/resource/genomics/project/detail/NMDC10018266.

Ethics statement

This animal study was reviewed and approved by Animal Care and Use Committee of the Institute of Animal Science of the Chinese Academy of Agricultural Sciences (approval no. IAS2020-97).

Author contributions

YL, JW, and SZ conceived and designed the experiments. QX and YL wrote and prepared the original draft. QX, WD, NZ, JW, and SZ edited the manuscript. QX, JW, and SZ critically reviewed the manuscript. All authors reviewed and approved the final manuscript.

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Conflict of interest

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

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References

Breikaa, R., Algandaby, M., El-Demerdash, E., and Abdel-Naim, A. (2013). Biochanin a protects against acute carbon tetrachloride-induced hepatotoxicity in rats. *Biosci. Biotechnol. Biochem.* 77, 909–916. doi: 10.1271/bbb.120675

Butkute, B., Dagilyte, A., Benetis, R., Padarauskas, A., Ceseviciene, J., Olsauskaite, V., et al. (2018). Mineral and phytochemical profiles and antioxidant activity of herbal material from two temperate *Astragalus* species. *Biomed Res. Int.* 2018:6318630. doi: 10.1155/2018/6318630

Castro-Montoya, J., Witzig, M., Rahman, M., Westreicher-Kristen, E., and Dickhoefer, U. (2018). In vitro rumen fermentation, microbial protein synthesis and composition of microbial community of total mixed rations replacing maize silage with red clover silage. *J. Anim. Physiol. Anim. Nutr.* 102, 1450–1463. doi: 10.1111/jpn.12970

Chen, X., and Gomes, M. (1992). *Estimation of microbial protein supply to sheep and cattle based on urinary excretion of purine derivatives – an overview of technical details*. Aberdeen: Rowett Research lnstitute.

Chong, J., Liu, P., Zhou, G., and Xia, J. (2020). Using microbiomeanalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nat. Protoc.* 15, 799–821. doi: 10.1038/s41596-019-0264-1

Chukwumah, Y., Walker, L., Verghese, M., and Ogutu, S. (2009). Effect of frequency and duration of ultrasonication on the extraction efficiency of selected isoflavones and trans-resveratrol from peanuts (*Arachis hypogaea*). *Ultrason. Sonochem.* 16, 293–299. doi: 10.1016/j.ultsonch.2008.07.007

Flythe, M., and Kagan, I. (2010). Antimicrobial effect of red clover (*Trifolium pratense*) phenolic extract on the ruminal hyper ammonia-producing bacterium, *Clostridium sticklandii*. *Curr. Microbiol.* 61, 125–131. doi: 10.1007/s00284-010-9586-5

Flythe, M., Harrison, B., Kagan, I., Klotz, J., and Aiken, G. (2013). Antimicrobial activity of red clover (*Trifolium pratense* L.) extract on caprine hyper-ammonia-producing bacteria. *Agric. Food Anal. Bacteriol.* 3, 176–185.

Galal, A., Mohamed, A., Khater, S., and Metwally, M. (2018). Beneficial role of biochanin A on cutaneous and renal tissues of ovariectomized rats treated with anastrozole. *Life Sci.* 201, 9–16. doi: 10.1016/j.lfs.2018.03.037

Gao, Y., Yao, Y., Zhu, Y., and Ren, G. (2015). Isoflavone content and composition in chickpea (*Cicer arietinum* L.) sprouts germinated under different conditions. *J. Agric. Food Chem.* 63, 2701–2707. doi: 10.1021/jf5057524

Gessner, D., Koch, C., Romberg, F., Winkler, A., Dusel, G., Herzog, E., et al. (2015). The effect of grape seed and grape marc meal extract on milk performance and the expression of genes of endoplasmic reticulum stress and inflammation in the liver of dairy cows in early lactation. *J. Dairy Sci.* 98, 8856–8868. doi: 10.3168/jds.2015-9478

Harlow, B., Flythe, M., and Aiken, G. (2017b). Effect of biochanin A on corn grain (Zea mays) fermentation by bovine rumen amylolytic bacteria. *J. Appl. Microbiol.* 122, 870–880. doi: 10.1111/jam.13397

Harlow, B., Flythe, M., Kagan, I., and Aiken, G. (2017c). Biochanin A (an isoflavone produced by red clover) promotes weight gain of steers grazed in mixed grass pastures and fed dried-distillers', Grains. *Crop Sci.* 57, 506–514. doi: 10.2135/cropsci2016.07.0590

Harlow, B., Aiken, G., Klotz, J., and Flythe, M. (2017a). Biochanin A mitigates rumen microbial changes associated with a sub-acute ruminal acidosis challenge. *J. Anim. Sci.* 95:263. doi: 10.2527/asasann.2017.536

Harlow, B., Flythe, M., and Aiken, G. (2018). Biochanin A improves fibre fermentation by cellulolytic bacteria. *J. Appl. Microbiol.* 124, 58–66. doi: 10.1111/jam.13632

Harlow, B., Flythe, M., Kagan, I., Goodman, J., Klotz, J., and Aiken, G. (2020). Isoflavone supplementation, via red clover hay, alters the rumen microbial community and promotes weight gain of steers grazing mixed grass pastures. *PLoS One* 15:e0229200. doi: 10.1371/journal.pone.0229200

Harlow, B., Flythe, M., Klotz, J., Harmon, D., and Aiken, G. (2021). Effect of biochanin A on the rumen microbial community of Holstein steers consuming a high fiber diet and subjected to a subacute acidosis challenge. *PLoS One* 16:e0253754. doi: 10.1371/journal.pone.0253754

Hu, K., Shi, X., Xu, D., Laborda, P., Wu, G., Liu, F., et al. (2021). Antibacterial mechanism of Biochanin A and its efficacy for the control of *Xanthomonas axonopodis* pv. glycines in soybean. *Pest Manag. Sci.* 77, 1668–1673. doi: 10.1002/ps.6186

Jalaludeen, A., Ha, W., Lee, R., Kim, J., Do, J., Park, C., et al. (2016). Biochanin a ameliorates arsenic-induced hepatoand hematotoxicity in rats. *Molecules* 21:69. doi: 10.3390/molecules21010069

Jiang, Z., Jiang, S., Lin, Y., Xi, P., Yu, D., and Wu, T. (2007). Effects of soybean isoflavone on growth performance, meat quality, and antioxidation in male broilers. *Poult. Sci.* 86, 1356–1362. doi: 10.1093/ps/86.7.1356

Kagan, I., and Flythe, M. (2012). Factors affecting the separation and bioactivity of red clover (*Trifolium pratense*) extracts assayed against *Clostridium sticklandii*, a ruminal hyper ammonia-producing bacterium. *Nat. Prod. Commun.* 7, 1605–1608. doi: 10.1177/1934578X1200701217

Kagan, I., Goff, B., and Flythe, M. (2015). Soluble phenolic compounds in different cultivars of red clover and alfalfa, and their implication for protection against proteolysis and ammonia production in ruminants. *Nat. Prod. Commun.* 10, 1263–1267. doi: 10.1177/1934578X1501000732

Kasparovska, J., Pecinkova, M., Dadakova, K., Krizova, L., Hadrova, S., Lexa, M., et al. (2016). Effects of isoflavone-enriched feed on the rumen microbiota in dairy cows. *PLoS One* 11:e0154642. doi: 10.1371/journal.pone.0154642

Kopylova, E., Noé, L., and Touzet, H. (2012). SortMeRNA: Fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. *Bioinformatics* 28, 3211–3217. doi: 10.1093/bioinformatics/bts611

Kotyzova, D., Bludovska, M., and Eybl, V. (2013). Differential influences of various arsenic compounds on antioxidant defense system in liver and kidney of rats. *Environ. Toxicol. Pharmacol.* 36, 1015–1021. doi: 10.1016/j.etap.2013.09.003

Křížová, L., Dadáková, K., Kašparovská, J., and Kašparovský, T. (2019). Isoflavones. *Molecules* 24:1076. doi: 10.3390/molecules24061076

Lee, K., and Choi, E. (2005). Biochanin A stimulates osteoblastic differentiation and inhibits hydrogen peroxide-induced production of inflammatory mediators in MC3T3-E1 cells. *Biol. Pharm. Bull.* 28, 1948–1953. doi: 10.1248/bpb.28.1948

Lemeziene, N., Padarauskas, A., Butkute, B., Ceseviciene, J., Taujenis, L., Norkeviciene, E., et al. (2015). The concentration of isoflavones in red clover (*Trifolium pratense* L.) at flowering stage. *Zemdirbyste* 102, 443–448. doi: 10.13080/z-a.2015.102.057

Li, Y., Wang, Z., Yang, H., Xu, L., Xie, Y., Jin, S., et al. (2017). Effects of dietary fiber on growth performance, slaughter performance, serum biochemical parameters, and nutrient utilization in geese. *Poult. Sci.* 96, 1250–1256. doi: 10.3382/ps/pew385

Liao, X., Wen, Q., Zhang, L., Lu, L., Zhang, L., and Luo, X. (2018). Effect of dietary supplementation with flavonoid from *Scutellaria baicalensis* Georgi on growth performance, meat quality and antioxidative ability of broilers. *J. Integr. Agric.* 17, 1165–1170. doi: 10.1016/S2095-3119(17)61803-3

Liu, S., Zhang, Z., Hailemariam, S., Zheng, N., Wang, M., Zhao, S., et al. (2020). Biochanin a inhibits ruminal nitrogen-metabolizing bacteria and alleviates the decomposition of amino acids and urea in vitro. *Animals* 10:368. doi: 10.3390/ani10030368

Ludden, P., Harmon, D., Huntington, G., Larson, B., and Axe, D. (2000). Influence of the novel urease inhibitor N-(n-butyl) thiophosphoric triamide on ruminant nitrogen metabolism: II. Ruminal nitrogen metabolism, diet digestibility, and nitrogen balance in lambs. *J. Anim. Sci.* 78, 188–198. doi: 10.2527/2000.781188x

Mehdipour, P., Marhon, S., Ettayebi, I., Chakravarthy, A., Hosseini, A., Wang, Y., et al. (2020). Epigenetic therapy induces transcription of inverted SINEs and ADAR1 dependency. *Nature* 588:169. doi: 10.1038/s41586-020-2844-1

Melchior, E., Smith, J., Schneider, L., Mulliniks, J., Bates, G., McFarlane, Z., et al. (2018). Effects of red clover isoflavones on tall fescue seed fermentation and microbial populations in vitro. *PLoS One* 13:e0201866. doi: 10.1371/journal.pone.0201866

Mendowski, S., Chapoutot, P., Chesneau, G., Ferlay, A., Enjalbert, F., Cantalapiedra-Hijar, G., et al. (2020). Effects of pretreatment with reducing sugars or an enzymatic cocktail before extrusion of fava bean on nitrogen metabolism and performance of dairy cows. *J. Dairy Sci.* 103, 396–409. doi: 10.3168/jds.2019-17286

Morales, E., Adarve, G., Alcaide, E., and Sampelayo, M. (2010). Nitrogen and energy utilization in lactating dairy goats fed diets with different legume seeds. *J. Anim. Physiol. Anim. Nutr.* 94, 659–664. doi: 10.1111/j.1439-0396.2009.00953.x

Pereira, A., Moura, D., Whitehouse, N., and Brito, A. (2020). Production and nitrogen metabolism in lactating dairy cows fed finely ground field pea plus soybean meal or canola meal with or without rumen-protected methionine supplementation. *J. Dairy Sci.* 103, 3161–3176. doi: 10.3168/jds.2019-17186

Sadri, H., Goodarzi, M., Salemi, Z., and Seifi, M. (2017). Antioxidant effects of biochanin a in streptozotocin induced diabetic rats. *Braz. Arch. Biol. Technol.* 60, 1–10. doi: 10.1590/1678-4324-2017160741

Sears, A., Gonzalez, O., Alberto, A., Young, A., de Souza, J., Relling, A., et al. (2020). Effect of feeding a palmitic acid-enriched supplement on production responses and nitrogen metabolism of mid-lactating Holstein and Jersey cows. *J. Dairy Sci.* 103, 8898–8909. doi: 10.3168/jds.2020-18232

Sinclair, L., Blake, C., Griffin, P., and Jones, G. (2012). The partial replacement of soyabean meal and rapeseed meal with feed grade urea or a slow-release urea and its effect on the performance, metabolism and digestibility in dairy cows. *Animal* 6, 920–927. doi: 10.1017/S1751731111002485

Steinshamn, H. (2010). Effect of forage legumes on feed intake, milk production and milk quality–a review. *Anim. Sci. Pap. Rep.* 28, 195–206.

Tsugami, Y., Matsunaga, K., Suzuki, T., Nishimura, T., and Kobayashi, K. (2017). Isoflavones and their metabolites influence the milk component synthesis ability of mammary epithelial cells through prolactin/STAT5 signaling. *Mol. Nutr. Food Res.* 61:1700156. doi: 10.1002/mnfr.201700156

Tuzun, A., Erdil, A., Inal, V., Aydin, A., Bagci, S., Yesilova, Z., et al. (2002). Oxidative stress and antioxidant capacity in patients with inflammatory bowel disease. *Clin. Biochem.* 35, 569–572. doi: 10.1016/S0009-9120(02)00361-2

Uritskiy, G., DiRuggiero, J., and Taylor, J. (2018). MetaWRAP-a flexible pipeline for genome-resolved metagenomic data analysis. *Microbiome* 6:158. doi: 10.1186/s40168-018-0541-1

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

Wang, J., Wu, C., and Feng, J. (2011). Effect of dietary antibacterial peptide and zinc-methionine on performance and serum biochemical parameters in piglets. *Czech J. Anim. Sci.* 56, 30–36. doi: 10.17221/341/2009-CJAS

Waschulin, V., Borsetto, C., James, R., Newsham, K., Donadio, S., Corre, C., et al. (2022). Biosynthetic potential of uncultured Antarctic soil bacteria revealed through long-read metagenomic sequencing. *ISME J.* 16, 101–111. doi: 10.1038/s41396-021-01052-3

Wenjin, S., Yangdong, Z., Xiang, L., Yufeng, D., and Qingbiao, X. (2021). Understanding the functional activity of polyphenols using omics-based approaches. *Nutrients* 13:3953. doi: 10.3390/nu13113953

Yu, C., Zhang, P., Lou, L., and Wang, Y. (2019). Perspectives regarding the role of biochanin a in humans. *Front. Pharmacol.* 10:793. doi: 10.3389/fphar.2019.00793

Yu, X., Chen, J., Deng, W., Xu, X., Liu, Q., Shi, M., et al. (2020). Biochanin a mitigates atherosclerosis by inhibiting lipid accumulation and inflammatory response. *Oxid. Med. Cell. Longev.* 2020:8965047. doi: 10.1155/2020/8965047

Zhan, J., Liu, M., Su, X., Zhan, K., Zhang, C., and Zhao, G. (2017). Effects of alfalfa flavonoids on the production performance, immune system, and ruminal fermentation of dairy cows. *Asian-Australas. J. Anim. Sci.* 30, 1416–1424. doi: 10.5713/ajas.16.0579

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Fermented cottonseed and rapeseed meals outperform soybean meal in improving performance, rumen fermentation, and bacterial composition in Hu sheep

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Background: This study examined the effects of substituting cottonseed meal (CSM) or rapeseed meal (RSM) for soybean meal (SBM) on Hu sheep performance, rumen fermentation, and bacterial composition. 51 four-month-old indigenous male Hu sheep with starting body weights of 22.51±2.84kg and similar origins were randomly assigned to three treatments; (1) non-fermented total mixed ration (TMR) with SBM (CK), (2) fermented TMR containing CSM (F-CSM group), and (3) fermented TMR containing RSM (F-RSM group).

Results: The three groups' intake of dry matter differed significantly ($p<0.05$). In terms of average daily gain, the F-RSM group outperformed the CK and F-CSM groups ($p<0.05$). The pH of the rumen was substantially lower in the CK group than in the F-CSM and F-RSM groups ($p<0.05$), and the F-CSM group had greater amounts of volatile fatty acids (VFA) than the F-RSM and CK groups. In comparison to the CK group, the microbial crude protein yield was significantly higher in the F-CSM and F-RSM groups ($p<0.05$). The F-CSM group significantly outperformed the F-RSM group of pepsin and cellulose enzyme activity ($p<0.05$). The relative abundance of *Bacteroidetes* was greater in the CK and F-RSM groups compared to the F-CSM group ($p<0.05$). In comparison to the other groups, *Firmicutes* were less abundant in the CK group ($p<0.05$). *Prevotella* was present in a higher relative abundance in the F-CSM and F-RSM groups than in the CK group ($p<0.05$). *Prevotella* was greater in relative abundance in the F-CSM and F-RSM groups than in the CK group ($p<0.05$). The relative abundances of *Veillonellaceae_UCG-001* and *Lachnospiraceae_XPB1014* correlated with rumen butyric acid content and NH_3-N content ($p<0.05$). Gene function prediction revealed that replacing SBM with F-CSM or F-RSM in the diet of Hu sheep can promote glycan biosynthesis and metabolism.

Conclusion: The replacement of F-CSM and F-RSM for SBM has an influence on the richness and diversity of rumen bacteria at the phylum and genus levels. Replacement of SBM with F-CSM increased VFA yield and further promoted the performance of Hu sheep.

KEYWORDS

fermented total mixed ration, cottonseed meal, rapeseed meal, Hu sheep, rumen microbiota

1. Introduction

The worldwide population is forecast to spread 9.7 billion (Olimid and Olimid, 2019), while universal need for meat and milk is expected to grow by 57 and 48%, respectively (Alexandratos and Bruinsma, 2012). As an outcome, it is estimated that livestock production will rise by 21% between 2010 and 2025 (Mottet et al., 2017). The universal need for livestock protein for human nourishment is increasing exponentially, increasing the price of animal feed concentrates (Tufarelli et al., 2018). To overcome the problem, it is vital to adopt different sources of feed and strategies using of alternative feeding methods. By-products are the sole viable option for making low-cost feed ingredients; they are generated during food processing and production and thus are not appropriate for human consumption (Xu et al., 2007). In ruminant diets, CSM and RSM may be appropriate alternatives to SBM as they are less expensive and relatively more widely accessible regionally (Zeng, 2020).

China has a large supply of CSM and has long been measured as a reasonable alternative to SBM (Wang et al., 2017). As a by-product of cottonseed oil manufacturing, CSM exhibits a crude protein content of 34–40%, crude fibre content of 11%, vitamin B, and organic phosphorus, among other nutrients (Zhang et al., 2018). However, due of the presence of Free gossypol (FG), a poisonous that may significantly influence animal development and reproductive and digestive development, as well as cause anomalies in internal organs, the use of CSM in animal diets is restricted (Francis et al., 2001; Robinson et al., 2001). Cotton-rich feed is a common source of protein for ruminants, particularly in cotton-growing regions such as India, China, and the United States, where it is used to replace soybean meal (SBM) (Heuze et al., 2015). However, CSM is now a cost-effective source of high protein (Nie et al., 2015). RSM, a by-product of the rapeseed oil industry, has a high protein concentration and a well-balanced amino acid composition (Wanasundara et al., 2017; Ashayerizadeh et al., 2018), making RSM appropriate for use in livestock feed. Nonetheless, because of the presence of glucosinolates (Huisman and Tolman, 1992; Tripathi and Mishra, 2007) and other anti-nutritional factors, RSM can poison animals and impair their development (Nagalakshmi et al., 2003). As a result, there is a limited usage of CSM and RSM in livestock diets.

Several approaches had been used to decrease the anti-nutritiona factors of CSM, such as calcium hydroxide (Nagalakshmi et al., 2003), chemical treatment with ferrous sulfate (Barrett et al., 1997), and microbial fermentation (Weng and Sun, 2006). To reduce the anti-nutritional factors of RSM, methods such as inactivation of myrosinase, solvent extraction, steam removal, and liquidation have been applied. Still, such methods also have some disadvantages including loss of protein, high expense, commercial relative unimportance, and environmental pollution (Pal and Walia, 2001). Among the different methods, microbial fermentation can help in increasing crude protein and other nutritional content, reduce toxin content, and maintain stomach microbial stability of the plants (McSweeney et al., 2001; Xu et al., 2020). Furthermore, our preliminary study showed that the nutritious value of CSM and RSM can be increased by microbial fermentation, while the anti-nutritional factors (FG and glucosinolate) in them can be reduced.

The diverse and adaptable rumen microbiota, which includes bacteria, archaea, fungus, protozoa, and viruses, allows for the utilization of a variety of food materials, including recalcitrant plant wall elements, and the production of assimilable energy and nutrients needed by ruminants (Kolver et al., 2001). Undoubtedly, the rumen microbiome also allows for nutritional manipulations. In fact, functions of the rumen microbiota and a number of the production phenotypes of ruminants are linked (Zhang et al., 2017; Li et al., 2019; Zhang Y. et al., 2020). VFAs, which supply 70% of the required energy, can be produced by ruminants from solid diet. The microbes in the rumen enable this (Anantasook et al., 2013; Khan et al., 2016). The variations in feed efficiency among individual cattle can be primarily attributed to rumen bacteria and VFAs (Liang et al., 2017; Zhou et al., 2018; Zhang Y. X. et al., 2020). In a study by Zhang et al. (2018), the amount of FG and total gossypol in fermented CSM fell by 78 and 49%, respectively, under the best fermentation conditions in comparison to the control. These results indicate that rumen bacteria have considerable capacity to digest gossypol, which helps to explain why ruminants have a high tolerance for gossypol (Tang et al., 2018). However, the microbes involved in such rumen fermentation are not known. Moreover, the impact of CSM and RSM on rumen fermentation and rumen microorganisms has only been studied by a few researchers. Therefore, this study's objective was to determine whether replacement of SBM with F-CSM or F-RSM affected growth performance, rumen fermentation and bacterial composition in Hu sheep.

2. Methods and materials

2.1. Ethics committee approval

The experiment was conducted in compliance with the Guidelines for Experimental Animals (2006) established by the Ministry of Science and Technology (Beijing, China), and it was given the go-ahead by the Chinese Academy of Agricultural Sciences' Animal Ethics Committee (AEC-CAAS-20190517).

2.2. Animals, diets, and experimental design

51 four-month-old native male Hu sheep with ancestries that were identical and an initial weight of 22.51 ± 2.84 kg were acquired from China's Inner Mongolia autonomous region. The Hu sheep were divided into three groups (17 Hu sheep each) using a single-factor randomized block design: CK group, F-CSM group, and F-RSM

TABLE 1 Ingredients and chemical composition of the TMRs.

Items	Groups[1]		
	CK[1]	F-CSM	F-RSM
Ingredients (% of DM)			
Corn	34	33.55	33.48
Wheat bran	12	12	12
Soybean meal	10	0	0
Cottonseed meal	0	10	0
Rapeseed meal	0	0	10
Fat powder	0	0.3	0.3
Urea	0	0.15	0.22
Whole corn silage	20	20	20
Corn stalk	20	20	20
Premix[2]	4	4	4
Total	100	100	100
Chemical compositions (% of DM)[3]			
Dry matter (fresh basis)	55.85	56.86	56.9
Crude protein	13.98	14.66	13.82
Eether extract	3.07	3.43	3.53
Neutral detergent fiber	27.66	26.15	27.74
Acid detergent fiber	18.04	17.28	15.74
Ash	7.99	8.07	8.65
Calcium	1.07	1.11	1.15
Phosphours	0.46	0.52	0.46

[1]CK, total mixed ration with soyabean meal; FTMR-CSM, fermented total mixed ration with cottonseed meal; FTMR-RSM, fermented total mixed ration with rapeseed meal.
[2]Premix provide following per kg to the total mixed rations: Vitamins A, 10000 IU; Vitamin D3,2,500 IU; Vitamin E,50 IU; Fe, 90 mg; Cu, 12.5 mg; Mn, 30 mg; Zn, 100 mg; Se, 0.3 mg; I, 1.0 mg; Co, 0.5 mg.
[3]All the value are measured.

group. Subsequently, they were fed the total mixed ration (TMR). A stall (3 m × 1.5 m) equipped with individual feed and water buckets was utilized to maintain the Hu sheep. Unrestricted access to TMR and clean water was given to the Hu sheep.

Along with SBM, CSM, or RSM meals, wheat bran, maize, and corn stalks were used in the TMR formulation. Additionally, urea, premixed vitamins, and fat powder were added. The dietary feed was formulated according to the guidelines for meeting the nutrient requirements of sheep and allowing them to gain 300 g per day (NRC, 2007). Table 1 is a list of the TMRs' components and chemical composition. Based on our previous studies (Yusuf et al., 2021), F-CSM was prepared with a 50% moisture content and a 1:5 mixture of microbial strains (1.0×10^9 CFU/kg DM (dry matter) of *Bacillus clausii* and 5.0×10^9 CFU/kg DM of *Saccharomyces cariocanus*). F-RSM was also prepared with a 50% moisture content and a mixture of microbial strains (1.0×10^{10} CFU/kg DM of *B. clausii* and 5.0×10^{10} CFU/kg DM of *S. cariocanus*). In a 500 kg fermenter machine, the mixture was fermented (Model SSJX-WH-3.0; Shengshun Machinery Manufacturing Co., Ltd., Shenyang, China) for 60 h at 32°C (F-CSM group) or 28°C (F-RSM group). Following fermentation, the mixture was uniformly mixed with whole corn silage before being bagged in plastic. Every 3 days, TMR (CK group) without fermentation was mixed.

During July to October of 2021, the experiment was conducted at the Chinese Academy of Agricultural Sciences' Nankou Experimental Pilot Test Base (China's Beijing). The study lasted for 97 days, including 10 days for adaptation, 80 days for the feeding trial, and 7 days for the apparent digestion trial. At 07:00 and 17:00 each day, *ad libitum* feedings were given, with the delivered amount adjusted considering a nearly 10% refusal rate.

2.3. Sample collection and analyze

2.3.1. Growth performance

During all feeding periods, the diets and ort samples of individual Hu sheep were harvested daily to calculate the nutrient intake. The initial and final body weights were measured before the morning feed on the start and finish days. Afterwards, the dry matter intake (DMI), average daily gain (ADG) and feed conversion ratio (FCR) were calculated (Hassan et al., 2022a).

2.3.2. Rumen fermentation and enzyme activity

At the end of the experiment, 2 h after the morning feeding, using an oral stomach tube, rumen fluid was taken (Anscitech Co., Wuhan, Hubei, China). A sample of rumen fluid (1.0 ml) and 0.3 ml of 25% metaphosphoric acid were mixed and stored at-20°C.till the VFA, microbial crude protein (MCP) and ammonia nitrogen (NH_3-N) concentrations were investigated. The pH of rumen fluid was quickly determined using a portable pH detector (Testo-206-pH, Testo Co., Germany). The VFA concentration was determined using an Agilent Technology-78-90A gas chromatograph (Agilent Technologies, Waldbronn, Germany) coupled to an attached silica vessel column (30 mm × 0.25 mm × 0.25 μm film thickness; SP-3420A; Beifenrili Analyzer Associates, Beijing, China). A modified colorimetric technique was used to measure the NH_3-N content (Chaney and Marbach, 1962). The microbial protein component of the rumen fluid was identified using an updated colourimetric technique at 595 nm (Verdouw et al., 1978). Utilizing materials acquired from the Chinese company Nanjing Jiancheng Institute of Biological Engineering Co., we determined the activities of amylase ($CNPG_3$ method), pepsin (A080-1), and cellulase (A) in rumen fluid. The main instruments used were an HWS-12 water bath (Shanghai Blue Pard Instruments Co., Ltd.), a 721 spectrophotometer, a thermostat, and a PL-6906 microplate meter (Shanghai Youke Instrument Co., Ltd).

2.3.3. DNA extraction and 16S rRNA pyrosequencing

The V3-V4 region of the 16S rDNA was amplified using specific primers with a barcode; the primer sequences were 338F: ACTCCTACGGGAGGCAGCAG; and 806R: GGACTACHVGGGTWTCTAAT. DNA was extracted using the kit (Omega Biotek, Norcross, GA, United States). By using 1% agarose gel electrophoresis to identify PCR products, they were then purified using the Agencourt AMPure XP nucleic acid purification kit. Equal

TABLE 2 Effects of the diets on growth performance of Hu sheep.

Items	Groups			SEM	*p* value
	CK[1]	F-CSM	F-RSM		
Initial weight (kg)	22.7	22.3	22.4	0.40	0.83
Final weight (kg)	39.7[b]	42.0[ab]	43.2[a]	0.67	0.40
Average daily gain (g/d)	192.1[b]	210.1[ab]	226.0[a]	0.03	0.03
Dry matter intake (g/d)	970.1[c]	1140.0[a]	1110.1[b]	0.04	<0.01
Feed conversion rate, feed/gain	5.4[a]	5.1[a]	4.9[b]	0.08	0.02

[1]CK, total mixed ration with soyabean meal; F-CSM, fermented total mixed ration with cottonseed meal; F-RSM, Fermented total mixed ration with rapeseed meal.
[a,b,c]Values with different superscripts within the same row differ ($p < 0.05$).

quantities of the purified amplified products were mixed, and the "Y" shaped connection was connected. Magnetic bead screening was used to remove the connector's self-connecting parts. The library template was enriched by PCR amplification to generate single-stranded DNA fragments, and MiSeq was used to construct and sequence the library.

2.3.4. Sequence analysis

As stated previously, sequencing data was analyzed using the Quantitative Insights into Microbial Ecology (QIIME, v1.8.0) pipeline (Caporaso et al., 2010). In brief, raw sequencing reads that exactly matched the barcodes on the samples tested were assigned to them and identified as valid sequences. Low-quality sequences were detected by the following parameters: length of 150 bp, average Phred scores of 20, ambiguous bases, and > 8 bp of mononucleotide repetitions. FLASH was used to assemble paired-end reads (Magoc and Salzberg, 2011). Following chimera identification, UCLUST was used to group the remaining high-quality sequences into operational taxonomic units (OTUs) with 97% sequence identity. Using the default parameters, we selected a representative sample sequence from each OTU. By comparing the representative sequence set to the Greengenes Database and selecting the best hit, BLAST was used to classify OTUs. Each OTU's abundance and taxonomy were then recorded in an OTU table for each sample. OTUs with less than 0.001% sequence content across all samples were discarded. A typical, rounded rarefied OTU table to even out sample-to-sample sequencing depth differences. We calculated richness estimates and diversity indices using the QIIME v1.8, including Shannon, Simpson, Chao 1, Observed species, Goods coverage, and PD entire tree. We obtained the species classification information corresponding to each OTU by annotating specific information for the communities at various levels and comparing OTU representative sequences using BLAST (2.6.0+). We used linear discriminant analysis (LDA) effect size analysis to identify species with significant differences in abundance among groups (Langille et al., 2013). TThe first method for identifying species with statistically significant differences in abundance among groups was the analysis of variance (ANOVA) test. The Wilcoxon rank-sum method was then used to investigate group differences. For the purpose of data reduction and an investigation of the significance of significant phenotypic differences between species, the algebraic linear discriminant analysis score was set to 3.0. To compare all samples, we used Principal Coordinates Analysis (PCoA) based on weighted UniFrac distances, and a distance-based matrices analysis was performed to identify sample differences.

2.3.5. Microbial function prediction

To estimate the functions of the rumen microbial community, we used the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt v2.0.0[1]). The abundance of each functional category was determined using data on the functional orthologues, pathways, and enzyme commission numbers collected in accordance with the analysis module of the Kyoto Encyclopedia of Genes and Genomes (Langille et al., 2013).

2.3.6. Statistical analysis

All data were represented using the mean standard deviation. A randomized complete block design with repeated measurements was utilized to analyze the data on rumen fermentation parameters. The Kruskal-Wallis technique in the R program was used to analyze the differences in alpha diversity, relative abundance at the phylum, family, and genus levels, and microbiota function among the three groups (v 4.0.3). The "ape" and "ggplot2" packages in R (v 4.0.3) were used to display PCoA, Venn, column, and linear discriminant analysis effect size (LEfSe) data, respectively. Using R (v 4.0.3), package "corrplot," we investigated the relationship between the top 20 genera in all data (based on Spearman's coefficient). The "igraph" package of the R (v 4.0.3) language was used to show the network of the top 20 genera. At P 0.05, all results related to intergroup differences were statistically significant.

3. Results

3.1. Effect on feed intake and performance

The Hu sheep in the CK, F-CSM, and F-RSM groups exhibited ADG of 192.10 g/d, 210.12 g/d, and 226.03 g/d, respectively; Compared to the CK group, Hu sheep ADG in F-RSM group significantly higher than CK and F-CSM group ($p < 0.05$). The F-CSM group had significantly higher DMI than the F-RSM and CK groups ($p < 0.05$). Additionally, the difference between both the FCR in the CK and F-CSM groups and the F-RSM group was statistically significant ($p < 0.05$; Table 2).

3.2. Effect on rumen fermentation

In comparison to the CK group, the pH of the rumen fluid was considerably lower in the F-CSM and F-RSM groups ($p < 0.05$; Table 3). Total VFA, acetate, propionate, and butyrate concentrations differed ($p < 0.05$) among the groups, with the F-CSM group having higher concentrations than the F-RSM and CK groups. However, the

1 http://galaxy.morganlangille.com

TABLE 3 Effects of the diets on ruminal fermentation parameters of Hu sheep.

Items	Groups			SEM	*p* value
	CK[1]	F-CSM	F-RSM		
pH	6.4[a]	5.9[b]	6.0[b]	0.08	0.03
Total VFA (mmol/L)	79.6[b]	116.9[a]	62.9[b]	6.10	0.01
Acetate (%)	70.5[b]	70.7[b]	71.8[a]	4.34	0.01
Propionate (%)	15.9[b]	18.3[a]	16.5[b]	1.35	0.01
Butyrate (%)	13.6[ab]	11.0[a]	11.7[b]	0.82	0.02
A/P	4.4[a]	3.9[b]	4.3[a]	0.22	0.02
NH_3-N (mmol/L)	4.9[b]	7.1[a]	7.1[a]	0.5	0.05
MCP (mg/100 ml)	3.1[b]	4.7[ab]	4.8[a]	0.4	0.04

VFA: volatile fatty acid; NH_3-N: Ammonia nitrogen; MCP: microbial crude protein; A/P: a ratio of acetate to propionate.
[1]CK, total mixed ration with soyabean meal; F-CSM, fermented total mixed ration with cottonseed meal; F-RSM, fermented total mixed ration with rapeseed meal.
[a,b,c]Values with different superscripts within the same row differ ($p < 0.05$).

TABLE 4 Effects of the diets on ruminal enzyme activity of Hu sheep.

Items	Groups			SEM	*p* value
	CK[1]	F-CSM	F-RSM		
Alpha-Amylase(U/g)	38.6	40.8	35.3	0.97	0.06
Pepsin (U/mg)	14.4[ab]	15.5[a]	13.2[b]	0.39	0.04
Cellulose (U/mg)	2.3[ab]	2.6[a]	1.9[b]	0.12	0.04

[1]CK, total mixed ration with soyabean meal; F-CSM, fermented total mixed ration with cottonseed meal; F-RSM, Fermented total mixed ration with rapeseed meal.
[a,b,c]Values with different superscripts within the same row differ ($p < 0.05$).

F-CSM group exhibited a higher proportion of propionate than that exhibited by the CK and F-RSM groups ($p < 0.05$). When compared to the CK group, the F-CSM and F-RSM groups had substantially greater NH_3-N contents and MCP concentrations, respectively ($p < 0.05$).

3.3. Effect on enzyme activity

The enzyme activities in each group are shown in Table 4. Adding different proteins to the TMR did not affect rumen α-amylase activity ($p > 0.05$). Pepsin and cellulose activities in the F-CSM group were substantially greater than those in the F-RSM group ($p < 0.05$). There was no significant difference between the CK and F-CSM groups ($p > 0.05$).

3.4. Effect on the composition of microbial community OTU

The OUT rank curve graphic gradually stabilized, indicating that the test samples' high coverage (Figure 1). A total of 1,831,937 high-quality reads were obtained, for a sample size of 76,330 reads, composed the rumen microbiota. Nucleotide sequence identification (97%) resulted in a total of 2,285 OTUs (Figure 1A). Among these, 1,448 OTUs (63.37%) were shared among samples from different groups. There were no dietary effects observed, and PCoA analysis based on Weighted UniFrac or Bray–Curtis dissimilarity revealed that the rumen liquid fraction was not completely differentiated from other liquids (Figure 1B).

3.5. Effect on ruminal bacterial alpha diversity

Analysis of alpha diversity (Figure 2) showed that the F-CSM group had numerically higher Shannon and community richness (Chao) indices than those in the CK and F-RSM groups; however, the differences were not significantly different ($p > 0.05$). The detected species were not substantially different among groups ($p > 0.05$). The Simpson index showed no discernible differences between the three groups ($p > 0.05$).

3.6. Effect on ruminal bacteria at phylum and genus levels

Seventeen bacterial phyla were found among all treatments, with *Bacteroidota*, *Firmicutes*, and *Proteobacteria* being the three most predominant. In comparison to that in the F-CSM group (58.72%), in the CK and F-RSM groups, the relative abundance of *Bacteroidetes* was greater (71.41 and 79.89%, respectively; $p < 0.05$). *Firmicutes* showed lower abundance in the CK group (18.30%) than in the F-CSM and F-RSM groups ($p < 0.05$). There were 195 diverse microbial genera found among all groups, but only 14 of them were determined to be the "major genera" since they each accounted for more than 0.5% of the total sequences through at least one treatment (Figure 3). Except for the genera *Prevotella* and *Christensenellaceae_R-7* ($p < 0.05$), no interaction ($p > 0.05$) was found for any bacterial abundance at the genus level. When compared to the CK group, *Prevotella* was more abundant in the F-CSM and F-RSM groups ($p < 0.05$), but the TMR type had no influence on their relative abundance (Figures 4, 5).

3.7. Effect on inferred functional pathways

In Table 5, the top 20 metabolic pathways are listed. The TMR type had a significant effect on 8 out of the 20 pathways. F-CSM and F-RSM exhibited higher ($p < 0.05$) relative abundance of the functions related to terpenoid, polyketide, lipid, and folding and transcription-associated metabolisms, despite the fact that there was no discernible difference between the two groups ($p > 0.05$).

3.8. Relationship between VFA and rumen microbial diversity

The relationship between the fermentation-related parameters and the relative abundance of the top 20 bacterial genera is shown in Figure 6. The relative abundances of *Veillonellaceae_UCG-001* and

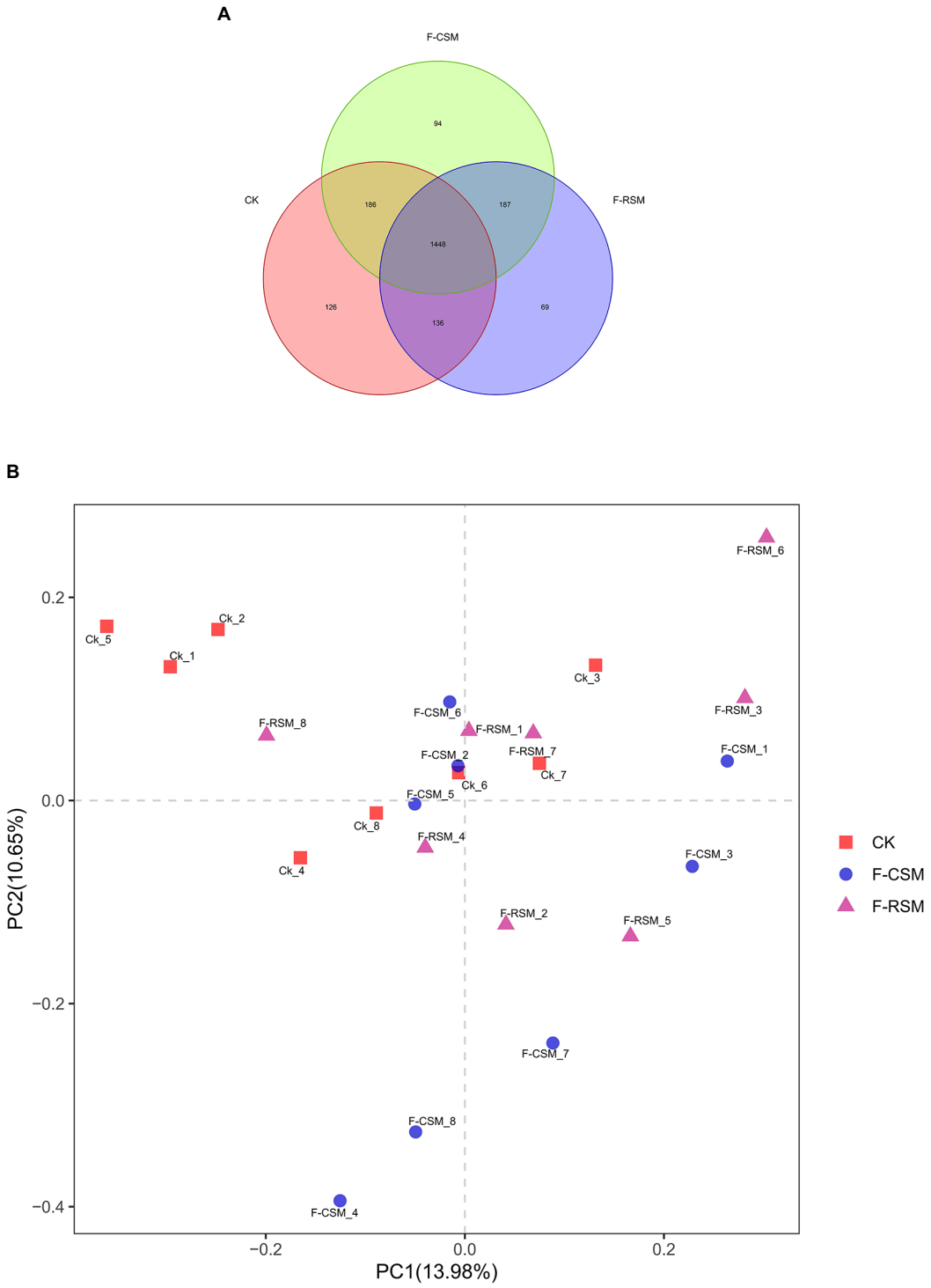

FIGURE 1
Effects of the diets on rumen microbiota of Hu sheep **(A)** Composition of rumen microbiota from Hu sheep fed three diets (OTU-level analysis). **(B)** PCoA analysis of rumen microbiota from Hu sheep fed different diets (%); CK, non-fermented group; F-CSM, fermented cotton seed meal TMR; F-RSM, fermented rapeseed meal TMR.

Lachnospiraceae_XPB1014 correlated with rumen fermentation and NH_3-N content ($p < 0.05$). While the relative abundance of *Lachnospiraceae_XPB1014* negatively correlated with NH_3-N concentration ($r = 0.53$), the relative abundance of *Veillonellaceae_UCG-001* positively correlated with butyric acid content ($r = 0.72$).

4. Discussion

Due to the presence of anti-nutritional factors and the characteristics of the available substrates, diet composition has a significant impact on nutrient utilization and gastrointestinal

physiology (Kiaie et al., 2014; Kim et al., 2017). Although it is generally recognized that TMR fermentation with microorganisms improves feed quality, there is an energy cost and a dry matter loss (Liang et al., 2017). Microbial fermentation can significantly reduce anti-nutritional factor content while improving palatability and nutritional value. CSM contains FG, while RSM contains glucosinolates, which affect the palatability and nutritional value of feed, reduce the production performance of animals, and also harm physical organs such as the liver and thyroid, greatly limiting its popularization and application in production. Hu et al. (2010) found that replacing SBM with 10% fermented CSM significantly increased the weight gain of sheep and reduced meat production costs. In a study by Xu et al. (2020), fermented feed partly breaks down the protein into the minor peptides & free amino acids, that imparts a pleasant acidic aroma to the feeds, enhances palatability as well as increases diet digestibility, which finally changes growth performance (Xu et al., 2020). However, our study showed that the F-CSM or F-RSM diet increased the overall body weight and daily weight gain in Hu sheep.

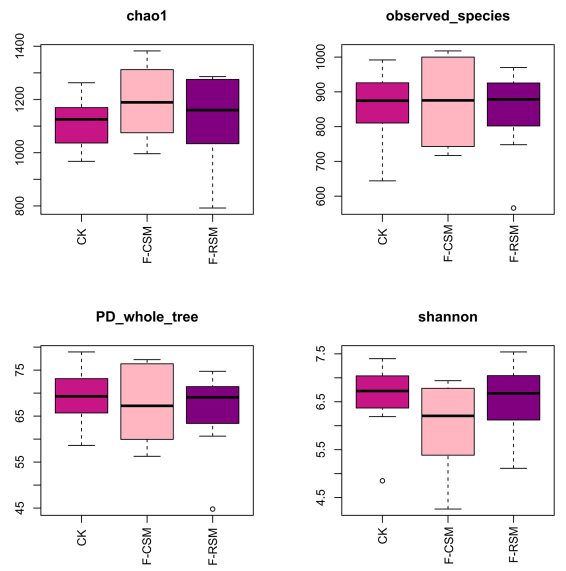

FIGURE 2
Effects of the diets on rumen alpha diversity of Hu sheep. CK, non-fermented group; F-CSM, fermented cotton seed meal TMR; F-RSM, fermented rapeseed meal TMR.

Rumen pH is the result of the interaction between VFAs in rumen digesta and buffer salts in saliva, absorption of VFAs by the rumen epithelium, and excretion of rumen digesta (Segata et al., 2011). It has an important impact on the rumen microbiota, rumen fermentation products, and rumen function, and is the most direct manifestation of rumen physiological conditions (Feng, 2004). Rumen pH is closely related to dietary composition and nutrient composition, and can comprehensively reflect the production, absorption, discharge, and neutralization of rumen microorganisms, metabolites, and organic acids (Nagaraja and Titgemeyer, 2007). The ruminal fluid pH varied from 5.93 to 6.39, which was within the normal range (Gilani et al., 2005), and among the groups, there was no significant different. These results indicate that various beneficial components contained in the fermented mixed meals were conducive to maintaining the stability of rumen pH, which could partially or completely replace SBM in the diet.

The VFAs produced during fermentation in the rumen are used as a source of energy in ruminants. VFAs produced by rumen fermentation, can provide up to 80% of the body's energy and serve as the primary source of carbon for the proliferation of rumen microbes (Zhou et al., 2018). Acetic acid is the main precursor for milk fat synthesis, whereas propionic acid synthesizes the glucose required by the body through gluconeogenesis. As a result, propionic acid fermentation can provide the body more energy, which is beneficial for improving animal productivity and feed utilization (Spears et al., 2004). Studies have shown that microbial fermented feed can regulate rumen fermentation mode (Guo, 2016). Zhang Z. (2017) found that microbial fermented feed could

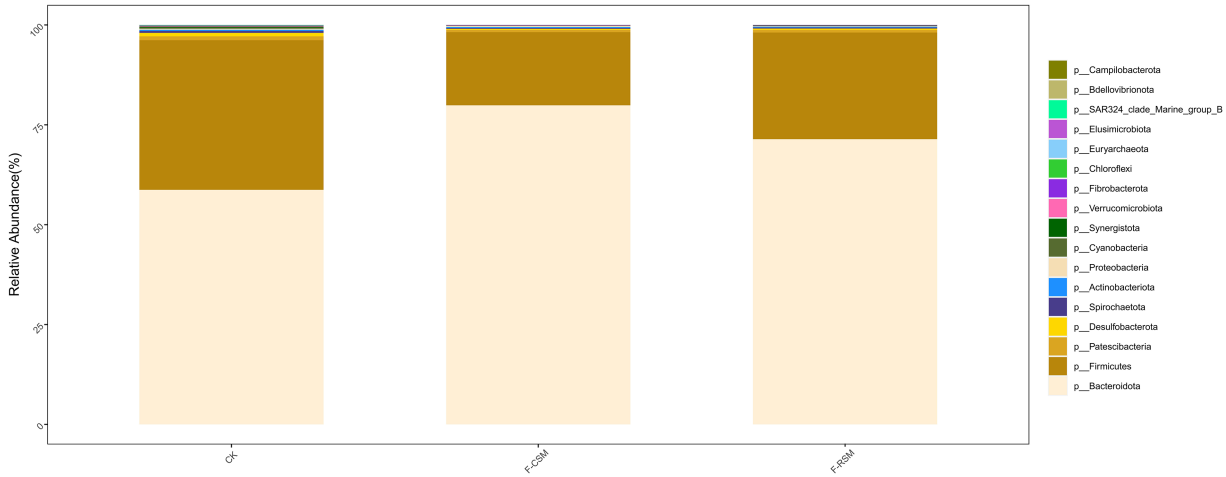

FIGURE 3
Effects of the diets on compositional profiles of rumen microbiota of Hu sheep (Phylum level; %). CK, non-fermented group; F-CSM, fermented cotton seed meal TMR; F-RSM, fermented rapeseed meal TMR.

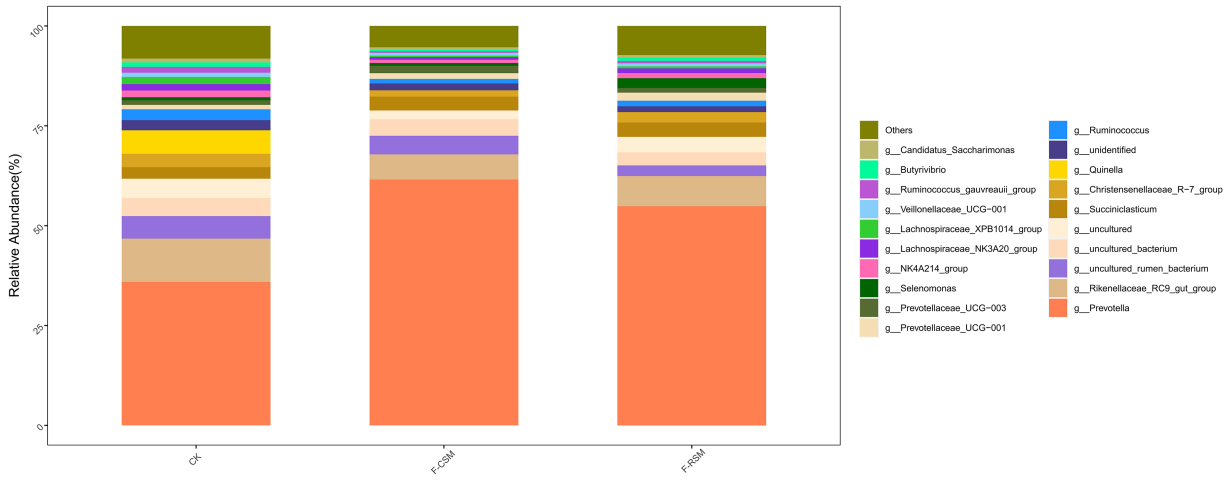

FIGURE 4
Effects of the diets on compositional profiles of rumen microbiota of Hu sheep (Genus level; %); CK, non-fermented group; F-CSM, fermented Cotton seed meal TMR; F-RSM, fermented Rapeseed meal TMR.

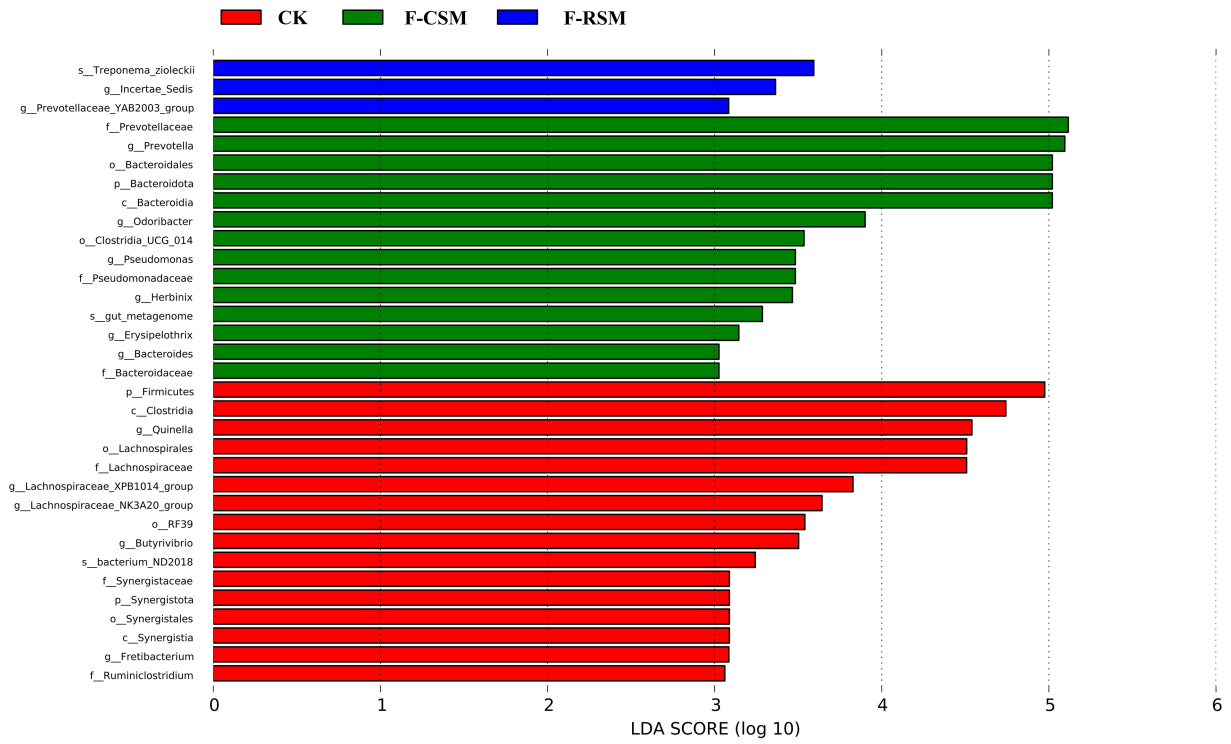

FIGURE 5
Effect of potential biomarkers was defined by LEfSe at Hu sheep rumen liquite (log_{10}); CK, non-fermented group; F-CSM, fermented Cotton seed meal TMR; F-RSM, fermented Rapeseed meal TMR.

significantly increase the contents of T-VFA, acetic acid and propionic acid and the ratio of acetate to propionate in the rumen of dairy cows through *in vitro* experiments, Guo (2016) also obtained similar results, and Chen et al. (2015) reported that fermented feed could increase the contents of rumen VFA, acetic acid and propionic acid. However, it had no significant effect on the ratio of acetate to propionate. Meanwhile, Li Z. et al. (2015) found that fermented feed could increase the content of rumen VFA of sheep and reduce the ratio of acetate to propionate. The present results show that rumen VFA concentration was the highest and the acetate/propionate ratio was the lowest in the animals fed with F-CSM. The F-CSM group showed lower VFA concentration than the F-RSM and CK groups. These results indicate that the substitution of fermented TMR contain CSM for SBM in Hu sheep

TABLE 5 Effect of feeding different diets on predominant predicted gene pathways in the different ruminal ecological niches of growing Hu sheep.

Indices	Groups			SEM	*p* value
	CK[1]	F-CSM	F-RSM		
Carbohydrate metabolism	14.3	14.4	14.5	0.08	0.85
Metabolism of cofactors and vitamins	14.1	14.8	14.7	0.13	0.11
Amino acid metabolism	13.2	12.9	13.1	0.05	0.14
Metabolism of terpenoids and polyketides	8.9[a]	8.1[b]	8.3[b]	0.12	0.03
Metabolism of other amino acids	6.9	6.9	6.8	0.07	0.86
Replication and repair	6.3	6.4	6.4	0.03	0.67
Glycan biosynthesis and metabolism	5.7[b]	6.7[a]	6.3[a]	0.07	0.04
Energy metabolism	5.9	5.9	5.9	0.03	0.27
Lipid metabolism	4.2[a]	3.7[b]	3.9[b]	0.08	0.01
Translation	3.5	3.4	3.5	0.02	0.29
Folding, sorting and degradation	3.0[a]	2.8[b]	2.9[b]	0.03	0.01
Biosynthesis of other secondary metabolites	2.6	2.9	2.9	0.05	0.07
Nucleotide metabolism	2.2	2.2	2.2	0.02	0.17
Cell motility	2.0[a]	1.0[b]	1.5[ab]	0.15	0.02
Cell growth and death	1.7	1.8	1.8	0.14	0.08
Xenobiotics biodegradation and metabolism	1.5	2.1	1.7	0.19	0.24
Membrane transport	1.4[a]	1.3[b]	1.3[b]	0.03	0.05
Transcription	1.2[b]	1.3[a]	1.2[a]	0.01	0.05
Transport and catabolism	0.3	0.4	0.3	0.01	0.06
Signal transduction	0.3[a]	0.2[b]	0.2[b]	0.01	0.02

[a,b]Means with different superscripts within a same row differ ($p < 0.05$). [1]CK, total mixed ration with soyabean meal; F-CSM, fermented total mixed ration with cottonseed meal; F-RSM, Fermented total mixed ration with rapeseed meal.

diet is beneficial to the transformation of rumen fermentation type into propionic acid fermentation type and improve energy utilization efficiency.

The NH_3-N concentration reflects the rate of decomposition of nitrogen-containing substances to produce NH_3 and the uptake and utilization of NH_3 by rumen microorganisms. A maximum concentration over 5 mg/dl can satisfy the needs of rumen microbial development. The NH_3-N concentration in the rumen is essential for protein degradation and microbial protein synthesis (Li M. S. et al., 2015). In this experiment the replacement of SBM with F-CSM or F-RSM had a significant effect on rumen NH_3-N content; however, the rumen NH_3-N content was 7.09 mmol/l and 7.11 mmol/l, respectively, in the F-CSM and F-RSM groups, which is within the normal physiological range of rumen NH_3-N (6–30 mg/d L; Yang et al., 2001). In our study, the diversity and richness of rumen bacteria did not significantly change when SBM was replaced with F-CSM or F-RSM in the diet of Hu sheep.

Rumen microorganisms decompose and utilize various secreted enzymes. Enzyme activity directly reflects the decomposition efficiency of feed nutrients in the rumen and determines the utilization efficiency of feed nutrients by animals. In this study, the activities of pepsin and cellulose in rumen fluid increased after replacing SBM with F-CSM. This may be because fermented TMR changes the pH of rumen fluid and provides favourable conditions for the reaction of digestive enzymes, thus improving the activity of digestive enzymes (Bowman and Asplund, 1988). In the F-RSM group, enzyme activity decreased. This could be the result of the anti-nutritional factors in RSM (Hassan et al., 2022b).

Researchers (Diao et al., 2013; Zhang et al., 2016; Zhou et al., 2017; Bi et al., 2018; Guo et al., 2021; Liu et al., 2021; Lu et al., 2021) have shown that the two dominant phyla in ruminants are *Bacteroidetes* and *Firmicutes*. The two most frequent taxa discovered in this study were *Bacteroidetes* and *Firmicutes*; the findings are consistent with other studies. In this study, we found that *Prevotella* and *Christensenellaceae R-7* were successively dominant at the genus level. Studies have shown that *Bacteroides* plays an important role in the degradation of non-fibrous substances, and *Firmicutes* mainly participate in the decomposition of fibrous substances (Evans et al., 2011), while non-fibrous substances produce propionic acid after rumen fermentation, while cellulose substances mainly produce acetic acid after degradation (Li et al., 2017). Our study the relative abundances of *Bacteroidetes* and *Firmicutes* in the F-CSM and F-RSM groups were greater than those in the CK group. These results indicate that fermented TMR can promote the growth and proliferation of *Bacteroidetes* in rumen, accelerate the degradation of non-fiber substances in diet, produce more propionic acid, provide more energy for the body, and promote the growth of animals.

The most abundant genus of Bacteroidetes is *Prevotella* (Zou et al., 2019). It participates in the metabolism of various microorganisms, has a high hemicellulose degradation ability, and can adapt to different dietary structures (Wolff et al., 2017). *Prevotella* is important in the degradation of crude protein, starch, xylan, and pectin (Li Z. et al., 2015; Jami et al., 2013). *Prevotella* relative abundance in the rumen was significantly greater in the F-CSM and F-RSM groups in this study. This suggests that F-CSM and F-RSM can replace SBM and help Hu sheep in improved nutrient absorption and digestion, thereby improving feed conversion rate. *Veillonellaceae* belongs to the phylum

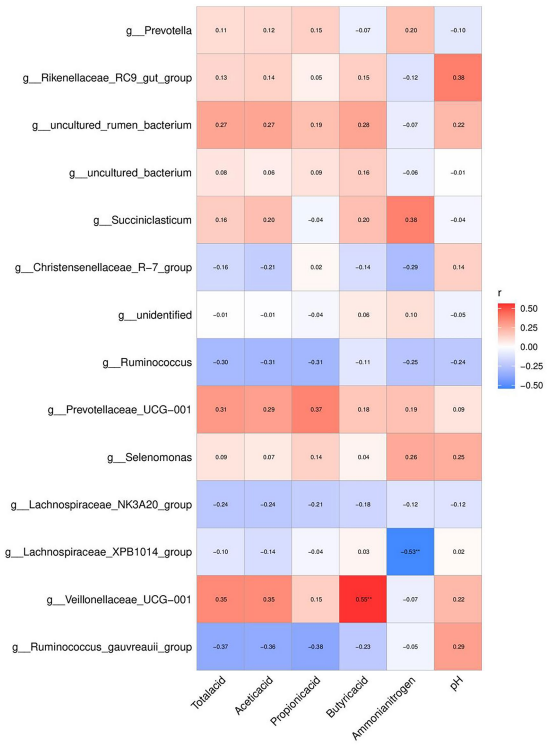

FIGURE 6
Effects of the diets on correlation between the top 20 relative abundances of genera, rumen VFA and enzyme activity mpositional profifiles of Hu sheep. *p<0.05; **p<0.01.

Firmicutes and can degrade and utilize cellulose (Long et al., 2022). We also found that the proportion of butyric acid was significantly positively correlated with the relative abundance of *Veillonellaceae UCG-001*. This indicated that *Veillonellaceae_UCG-001* was related to rumen VFA metabolism, which is consistent with the results of Thoetkiattikul et al. (2013). These results indicate that F-CSM and F-RSM can promote the growth of microorganisms, maintain intestinal structure and function, and regulate immunity in Hu sheep. *Lachnospiraceae_XPB1014*'s relative abundance and NH_3-N content showed a negative correlation. These findings suggest a relationship between NH_3-N concentrations and rumen bacteria in Hu sheep. Therefore, replacing SBM with F-CSM or F-RSM likely influenced the VFA and NH_3-N contents, and ultimately impacted the rumen microbiota. Microbiota functional properties affect host-microbiome interaction (Lin et al., 2019; Zeng, 2020). The gene function prediction results showed that F-CSM and F-RSM can replace SBM in the diet of Hu sheep and have an important influence on their metabolism. Particularly, it promoted glycan biosynthesis and metabolism, indicating that F-CSM and F-RSM contributed to the maturation and stability of Hu sheep rumen microflora.

5. Conclusion

Substituting SBM with F-CSM or F-RSM in the diet of Hu sheep is beneficial for the conversion of microbiota from rumen fermentation type to propionic acid fermentation type and improving energy utilization efficiency. No negative effects were observed on rumen microbiota, and substituting SBM with F-CSM resulted in the best effect.

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 reviewed and approved by Committee of IFRCAAS (AEC-IFR-CAAS, Beijing, China). Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

HR and HY: animal trial, data collection and evaluation, laboratory and statistical analysis, and writing. TM: critical manuscript review. QD: data evaluation and manuscript review. LxK and LyK: animal trial, laboratory analysis, and data collection. YT: study design, feed formulation, data evaluation, and critical manuscript review. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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References

Alexandratos, N., and Bruinsma, J. (2012). *World agriculture towards 2030/2050: The 2012 Revision. ESA Working Paper No. 12-03*. Rome: Food and Agriculture Organization.

Anantasook, N., Wanapat, M., Cherdthong, A., and Gunun, P. (2013). 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.* 26, 1583–1591. doi: 10.5713/ajas.2013.13182

Ashayerizadeh, A., Dastar, B., Shargh, M. S., Mahoonak, A. S., and Zerehdaran, S. (2018). Effects of feeding fermented rapeseed meal on growth performance, gastrointestinal microflora population, blood metabolites, meat quality, and lipid metabolism in broiler chickens. *Livest. Sci.* 216, 183–190. doi: 10.1016/j.livsci.2018.08.012

Barrett, J. E., Klopfenstein, C. F., and Leipold, H. W. (1997). Detoxification of rapeseed meal by extrusion with an added basic salt. *Cereal Chem.* 74, 168–170. doi: 10.1094/CCHEM.1997.74.2.168

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

Bowman, J. G. P., and Asplund, J. M. (1988). Nitrogen utilization, ruminal fermentation and abomasal nitrogen flow in sheep fed Caucasian bluestem hay supplemented with lucerne or urea. *Anim. Feed Sci. Technol.* 20, 33–44. doi: 10.1016/0377-8401(88)90125-3

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

Chaney, A. L., and Marbach, E. P. (1962). Modified Reagents for Determination of Urea and Ammonia. *Clin. Chem.* 8, 130–132.

Chen, G. J., Peng, Z. L., Song, S. D., Wang, B. X., Guo, C. H., Zhang, Z. F., et al. (2015). Effects of the fermented distillers' on performance, apparent nutrient digestibility, ruminal fermentation and serum biochemical indices in house-fed yaks. *Chin. J. Anim. Nutr.* 27, 2920–2927. doi: 10.3969/j.issn.1006-267x.2015.09.032

Diao, H., Zheng, P., Yu, B., He, J., Mao, X. B., Yu, J., et al. (2013). Effects of benzoic acid on growth performance, serum biochemical parameters, nutrient digestibility and digestive enzyme activities of jejunal digesta in weaner piglets. *Chin. J. Anim. Nutr.* 25, 768–777. doi: 10.3969/j.issn.1006-267x.2013.04.014

Evans, N. J., Brown, J. M., Murray, R. D., Getty, B., Birtles, R. J., Hart, C. A., et al. (2011). Characterization of novel bovine gastrointestinal tract treponema isolates and comparison with bovine digital dermatitis treponemes. *Appl. Environ. Microbiol.* 77, 138–147. doi: 10.1128/AEM.00993-10

Feng, Y. L. (2004). *Ruminant Nutrition*, Beijing: Science Press.

Francis, G., Makkar, H. P. S., and Becker, K. (2001). Anti nutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture* 199, 197–227. doi: 10.1016/S0044-8486(01)00526-9

Gilani, G. S., Cockell, K. A., and Sepehr, E. (2005). Effects of anti nutritional factors on protein digestibility and amino acid availability in foods. *J. AOAC Int.* 88, 967–987. doi: 10.1093/jaoac/88.3.967

Guo, P., (2016). *Study on Microbial Fermentation Feed Formula of Dairy Cows and the Matched Application Technique*. Inner Mongolia Agricultural University, Hohhot, China.

Guo, N., Wu, Q., Shi, F., Niu, J., Zhang, T., Degen, A. A., et al. (2021). Seasonal dynamics of diet-gut microbiota interaction in adaptation of yaks to life at high altitude. *NPJ Biofifilms Microbiomes* 7:38. doi: 10.1038/s41522-021-00207-6

Hassan, A. Y., Halidai, R., Tao, M., Min, Y. P., Rui, Y. H., and Yan, T. (2022a). Fermented Total mixed ration with cottonseed meal or rapeseed meal improved growth performance and meat quality of Hu lamb compared to Total mixed ration with soybean meal. *Fermentation* 8:576. doi: 10.3390/fermentation8110576

Hassan, A. Y., Halidai, R., Tao, M., Min, Y. P., Rui, Y. H., and Yan, T. (2022b). Effect of lactic acid bacteria and yeast supplementation on anti-nutritional factors and chemical composition of fermented total mixed ration containing cottonseed meal or rapeseed meal. *Anim. Biosci.* 35, 556–566. doi: 10.5713/ab.21.0270

Heuze, V., Tran, G., Noziere, P., Lessire, M., and Lebas, F. (2015). *Linseed Meal. Feedipedia, a Programme by INRA, CIRAD, AFZ and FAO*. Rome, Italy: FAO.

Hu, P. F., Wu, Z. H., S, T., and Y, G. Q. (2010). Effect of microbial treatment of cottonseed meal on fattening effect of lambs. *Chin. Heilongjiang Anim. Sci. Vet.* 24, 105–106.

Huisman, J., and Tolman, G. (1992). "Anti nutritional factors in the plant proteins of diets for non-ruminants" in *Recent Developments in Pig Nutrition*. eds. P. C. Garnsworthy and J. Wiseman, vol. *68* (Nottingham: Nottingham University Press), 101–110.

Jami, E., Israel, A., Kotser, A., and Mizrahi, I. (2013). Exploring the bovine rumen bacterial community from birth to adulthood. *ISME J.* 7, 1069–1079. doi: 10.1038/ismej.2013.2

Khan, M. A., Bach, A., Weary, D. M., and Keyserlingk, M. A. G. (2016). Invited review: transitioning from milk to solid feed in dairy heifers. *J. Dairy Sci.* 99, 885–902. doi: 10.3168/jds.2015-9975

Kiaie, E., Romero, L. F., and Ravindran, V. (2014). Growth performance, nutrient utilization, and digesta characteristics in broiler chickens fed corn or wheat diets without or with supplemental xylanase. *Poult. Sci.* 93, 1186–1196. doi: 10.3382/ps.2013-03715

Kim, E., Leung, H., Akhtar, N., Li, J., Barta, J. R., and Wang, Y. (2017). Growth performance and gastrointestinal responses of broiler chickens fed corn-soybean meal diet without or with exogenous epidermal growth factor upon challenge with *Eimeria*. *Poult. Sci.* 96, 3676–3686. doi: 10.3382/ps/pex192

Kolver, E. S., Roche, J. R., DMiller, D., and Densley, R. (2001). Maize Silage for Dairy Cows. In: *Proceedings of the Conference-New Zealand Grassland Association*. pp. 195–201.

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

Li, L. J., Cheng, S. R., Diao, Q. Y., Fu, T., Bi, Y. L., Wang, A. S., et al. (2017). Effects of diets ith different NFC/NDF levels on the rumen fermentation parameters and bacterial community in male calves. *Chin. J. Acta Veterinaria et Zootechnica Sinica*. 48. 2347–2357. doi: 10.11843/j.issn.0.66-6964.2017.12.014

Li, F. H., Ding, Z. T., Ke, W. C., Xu, D. M., Zhang, P., and Bai, J. (2019). Ferulic acid esterase producing lactic acid bacteria and cellulase pretreatments of corn stalk silage at two different temperatures: ensiling characteristics, carbohydrates composition and enzymatic saccharification. *Bioresour. Technol.* 282, 211–221. doi: 10.1016/j.biortech.2019.03.022

Li, Z., Wright, A. D., Liu, H., Fan, Z., Yang, F., Zhang, Z., et al. (2015). Response of the rumen microbiota of sika deer (*Cervus nippon*) fed different concentrations of tannin rich plants. *PLoS One* 10:e0123481. doi: 10.1371/journal.pone.0123481

Li, M. S., Xue, S. Y., Wang, C., Wang, L. M., Guo, T. L., and Jin, H. (2015). Effects of *Salix* mixed fermented feed on ruminal environmental parameters by in vitro gas production method. *Chin. J. Anim. Nutr.* 27, 1943–1953. doi: 10. 3969/j.issn.1006-267x.2015. 02. 039

Liang, Y. S., Li, G. Z., Li, X. Y., Lv, J. Y., Li, F. D., and Tang, D. F. (2017). Growth performance, rumen fermentation, bacteria composition, and gene expressions involved in intracellular pH regulation of rumen epithelium in finishing hu lambs differing in residual feed intake phenotype. *J. Anim. Sci.* 95, 1727–1738. doi: 10.2527/jas.2016.1134

Lin, L., Xie, F., Sun, D. M., Liu, J. H., Zhu, W. Y., and Mao, S. Y. (2019). Ruminal microbiome-host crosstalk stimulates the development of the ruminal epithelium in a lamb model. *Microbiome* 7:83. doi: 10.1186/s40168-019-0701-y

Liu, W. W., Wang, Q., Song, J. J., Xin, J. W., Zhang, S. S., Lei, Y. H., et al. (2021). Comparison of gut microbiota of yaks from different geographical regions. *Front. Microbiol.* 12:666940. doi: 10.3389/fmicb.2021.666940

Long, P. L., Lei, Q., and Tuo, L. (2022). Supplemental dietary selenohomolanthionine affects growth and rumen bacterial population of Shaanbei white cashmere wether goats. *Front. Microbiol.* 13:942848. doi: 10.3389/fmicb.2022.942848

Lu, L. H., Wan, C. X., Han, W. C., Xiao, Y. C., Fa, S. Q., Tao, M., et al. (2021). Comparison of rumen fermentation parameters and microbiota of yaks from different altitude regions in Tibet, China. *Front. Virol.* 12, 1–12. doi: 10.3389/fmicb.2021.807512

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

McSweeney, C. S., Palmer, B., McNeill, D. M., and Krause, D. O. (2001). Microbial interactions with tannins: nutritional consequences for ruminants. *Anim. Feed Sci. Technol.* 91, 83–93. doi: 10.1016/S0377-8401(01)00232-2

Mottet, A., Dehaan, C., Falcucci, A., Tempio, G., Opio, C., and Gerber, P. (2017). Livestock: on our plates or eating at our table? A new analysis of the feed/food debate. *Glob. Food Sec.* 14, 1–8. doi: 10.1016/j.gfs.2017.01.001

Nagalakshmi, D., Sastry, V. R. B., and Pawde, A. (2003). Rumen fermentation patterns and nutrient digestion in lambs fed cottonseed meal supplemental diets. *Anim. Feed Sci. Technol.* 103, 1–14. doi: 10.1016/S0377-8401(02)00140-2

Nagaraja, T. G., and Titgemeyer, E. C. (2007). Ruminal acidosis in beef cattle: the current microbiological and nutritional outlook. *J. Dairy Sci.* 90, E17–E38. doi: 10.3168/jds.2006-478

Nie, C., Zhang, W., Ge, W., Liu, Y., Wang, Y., and Liu, J. (2015). Effect of cottonseed meal fermented with yeast on the lipid-related gene expression in broiler chickens. *Braz. J. Poult. Sci.* 17, 57–64. doi: 10.1590/1516-635XSPECIALISSUENutrition-PoultryFeedingAdditives057-064

NRC. (2007). *Nutrient Requirements of Small Ruminants*. Ottawa: National Research Council.

Olimid, A. P., and Olimid, D. A. (2019). Societal challenges, population trends and human security: evidence from the public governance within the United Nations publications (2015-2019). *Rev. Stiint. Polit.* 64, 53–64.

Pal, V. A., and Walia, A. (2001). Beneficial effects of *Rhizopus oligosporus* fermentation on reduction of glucosinolates, fibre and phytic acid in rapeseed (*Brassica napus*) meal. *Bioresour. Technol.* 78, 309–312. doi: 10.1016/S0960-8524(01)00030-X

Robinson, P. H., Getachew, G., De, P. E. J., and Calhoun, M. C. (2001). Influence of variety and storage for up to 22 days on nutrient composition and gossypol level of Pima

cottonseed (*Gossypium* spp.). *Anim. Feed Sci. Technol.* 91, 149–156. doi: 10.1016/S0377-8401(01)00202-4

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., and Garrett, W. S. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Spears, J. W., Schlegel, P., Seal, M. C., and Lloyda, K. E. (2004). Bioavailability of zinc from zinc sulfate and different organic zinc sources and their effects on ruminal volatile fatty acid proportions. *Livest. Prod. Sci.* 90, 211–217. doi: 10.1016/j.livprodsci.2004.05.001

Tang, C. H., Liu, J., Zhao, Q. Y., and Zhang, J. M. (2018). Pharmacokinetic comparison of gossypol isomers in cattle: transfer from diet to plasma and degradation by rumen microbes. *J. Zhejiang Univ. Sci. B* 19, 471–480. doi: 10.1631/jzus.B1700289

Thoetkiattikul, H., Mhuantong, W., Laothanachareon, T., Tangphatsornruang, S., Pattarajinda, V., Eurwilaichitr, L., et al. (2013). Comparative analysis of microbial profiles in cow rumen fed with different dietary fiber by tagged 16S r RNA gene pyrosequencing. *Curr. Microbiol.* 67, 130–137. doi: 10.1007/s00284-013-0336-3

Tripathi, M. K., and Mishra, A. S. (2007). Glucosinolates in animal nutrition: a review. *Anim. Feed Sci. Technol.* 132, 1–27. doi: 10.1016/j.anifeedsci.2006.03.003

Tufarelli, V., Ragni, M., and Laudadio, V. (2018). Feeding forage in poultry: a promising alternative for the future of production systems. *Agriculture* 8:81. doi: 10.3390/agriculture8060081

Verdouw, H., Van Echteld, C. J. A., and Dekkers, E. M. J. (1978). Ammonia determination based on indophenol formation with sodium salicylate. *Water Res.* 12, 399–402. doi: 10.1016/0043-1354(78)90107-0

Wanasundara, J. P. D., Tan, S., Alashi, A. M., Pudel, F., and Blanchard, C. (2017). "Proteins from canola/rapeseed" in *Sustainable Protein Sources*. eds. S. R. Nadathur, J. P. D. Wanasundara and L. Scanlin (San Diego, CA, USA: Elsevier), 285–304.

Wang, D. Q., Song, D., Wang, W., Zhou, H., Wang, L., and Li, A. (2017). Effects of fermented cottonseed meal on growth performance, serum biochemical parameters, immune functions, antioxidative abilities, and cecal microflora in broilers. *Food Agric. Immunol.* 28, 725–738. doi: 10.1080/09540105.2017.1311308

Weng, X. Y., and Sun, J. Y. (2006). Biodegradation of free gossypol by a new strain of *Candida tropicalis* under solid state fermentation: effects of fermentation parameters. *Process Biochem.* 41, 1663–1668. doi: 10.1016/j.procbio.2006.03.015

Wolff, S. M., Ellison, M. J., Hao, Y., Cockrum, R. R., Austin, K. J., and Baraboo, M. (2017). Diet shifts provoke complex and variable changes in the metabolic networks of the ruminal microbiome. *Microbiome* 5:60. doi: 10.1186/s40168-017-0274-6

Xu, X., Ma, L., Fan, S., Ma, W., and Zhang, X. (2020). Effects of fermented *Caragana korshinskii* feed on meat quality characteristics in different muscles of Tan sheep. *Ital. J. Anim. Sci.* 19, 1036–1045. doi: 10.1080/1828051X.2020.1816505

Xu, C. Y., Moriya, N., and Ogawa, M. (2007). Nutritive value for ruminants of green tea grounds as a replacement of brewers' grains in totally mixed ration silage. *Anim. Feed Sci. Technol.* 138, 228–238. doi: 10.1016/j.anifeedsci.2006.11.014

Yang, W. Z., Beauchemin, K. A., and Rode, L. M. (2001). Effects of grain processing, forage to concentrate ratio, and forage particle size on rumen pH and digestion by dairy cows. *J. Dairy Sci.* 84, 2203–2216. doi: 10.3168/jds.s0022-0302(01)74667-x

Yusuf, H. A., Piao, M. Y., Ma, T., Huo, R. Y., and Tu, Y. (2021). Enhancing the quality of Total mixed ration containing cottonseed or rapeseed meal by optimization of fermentation conditions. *Fermentation* 7:234. doi: 10.3390/fermentation7040234

Zeng, Y. (2020). *Effects of Fermented Mixed Meals to Replace Soybean Meal and Small Peptide Products Supplementation in Diets on Growth Performance, Nutrient Digestion, Rumen Fermentation and Bacterial Composition of Beef Cattle*. Southwest Minzu University, Chengdu, China.

Zhang, Z. (2017). *Effects of Active Yeeast and its Fermented Feeds on the Rumen Fermentation and Nutrient Digestibility*. Inner Mongolia Agricultural University, Hohhot, China.

Zhang, Y. X., Ke, W. C., Bai, J., Li, F. H., Xu, D. M., and Ding, Z. T. (2020). The effect of *Pediococcus acidilactici* J17 with high-antioxidant activity on antioxidant, α-tocopherol, β-carotene, fatty acids, and fermentation profiles of alfalfa silage ensiled at two different dry matter contents. *Anim. Feed Sci. Technol.* 268:114614. doi: 10.1016/j.anifeedsci.2020.114614

Zhang, Y., Li, F., Chen, Y., Wu, H., Meng, Q., and Guan, L. L. (2020). Metatranscriptomic profifiling reveals the effect of breed on active rumen eukaryotic composition in beef cattle with varied feed efficiency. *Front. Microbiol.* 11:367. doi: 10.3389/fmicb.2020.00367

Zhang, L. Y., Ma, Q. G., Ma, S. S., Zhang, J. Y., Jia, R., and Ji, C. (2017). Ameliorating effects of *Bacillus subtilis* ANSB060 on growth performance, antioxidant functions, and aflatoxin residues in ducks fed diets contaminate with aflatoxins. *Toxins* 9:1. doi: 10.3390/toxins9010001

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

Zhang, Y., Zhang, Z., Dai, L., Liu, Y., Cheng, M., and Chen, L. (2018). Isolation and characterization of a novel gossypol-degrading bacteria Bacillus subtilis strain rumen Bacillus Subtilis. *Asian Australas. J. Anim. Sci.* 31, 63–70. doi: 10.5713/ajas.17.0018

Zhou, Z. M., Fang, L., Meng, Q. X., Li, S. L., Chai, S. T., Liu, S. J., et al. (2017). Assessment of ruminal bacterial and archaeal community structure in yak (*Bos grunniens*). *Front. Microbiol.* 8:179. doi: 10.3389/fmicb.2017.00179

Zhou, M., Peng, Y. J., Chen, Y. H., Klinger, C. M., Oba, M., and Liu, J. X. (2018). Assessment of microbiome changes after rumen transfaunation: implications on improving feed efficiency in beef cattle. *Microbiome* 6:62. doi: 10.1186/s40168-018-0447-y

Zou, H., Hu, R., Wang, Z., Shah, A. M., Zeng, S., and Peng, Q. (2019). Effects of nutritional deprivation and re-alimentation on the feed efficiency, blood biochemistry, and rumen microflflora in yaks (*Bos grunniens*). *Animals* 9:807. doi: 10.3390/ani9100807

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Comparative analysis of macroalgae supplementation on the rumen microbial community: *Asparagopsis taxiformis* inhibits major ruminal methanogenic, fibrolytic, and volatile fatty acid-producing microbes *in vitro*

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Seaweeds have received a great deal of attention recently for their potential as methane-suppressing feed additives in ruminants. To date, *Asparagopsis taxiformis* has proven a potent enteric methane inhibitor, but it is a priority to identify local seaweed varieties that hold similar properties. It is essential that any methane inhibitor does not compromise the function of the rumen microbiome. In this study, we conducted an *in vitro* experiment using the RUSITEC system to evaluate the impact of three red seaweeds, *A. taxiformis, Palmaria mollis,* and *Mazzaella japonica*, on rumen prokaryotic communities. 16S rRNA sequencing showed that *A. taxiformis* had a profound effect on the microbiome, particularly on methanogens. Weighted Unifrac distances showed significant separation of *A. taxiformis* samples from the control and other seaweeds ($p<0.05$). Neither *P. mollis* nor *M. japonica* had a substantial effect on the microbiome ($p>0.05$). *A. taxiformis* reduced the abundance of all major archaeal species ($p<0.05$), leading to an almost total disappearance of the methanogens. Prominent fiber-degrading and volatile fatty acid (VFA)-producing bacteria including *Fibrobacter* and *Ruminococcus* were also inhibited by *A. taxiformis* ($p<0.05$), as were other genera involved in propionate production. The relative abundance of several other bacteria including *Prevotella*, *Bifidobacterium, Succinivibrio, Ruminobacter*, and unclassified *Lachnospiraceae* were increased by *A. taxiformis* suggesting that the rumen microbiome adapted to an initial perturbation. Our study provides baseline knowledge of microbial dynamics in response to seaweed feeding over an extended period and suggests that feeding *A. taxiformis* to cattle to reduce methane may directly, or indirectly, inhibit important fiber-degrading and VFA-producing bacteria.

KEYWORDS

methane, rumen, *Asparagopsis*, seaweed, livestock, greenhouse gas, microbiome

1. Introduction

To combat the ongoing climate change crisis, international legislative agreements have mandated limits on global warming to 1.5°C and 2°C by 2030 and 2050, respectively (Arndt et al., 2022), targets which are unlikely to be met if global food systems continue business as usual practices (Clark et al., 2020). Food production industries are significant sources of anthropogenic carbon, contributing around 30% of total greenhouse gas (GHG) emissions annually, with livestock responsible for ~30% of this total (Arndt et al., 2022). Methane (CH_4) is produced as a normal by-product of microbial feed digestion in the rumen and is the principal source of carbon emissions from livestock (IPCC, 2018). CH_4 has a 100-year global warming potential at least 28-times greater than that of CO_2, and enteric methanogenesis contributes around 40% of all livestock GHG emissions (Gerber et al., 2013). In addition to the environmental impacts, ruminal methanogenesis reduces the dietary energy available to the animal by up to 12%; developing effective CH_4 mitigation strategies is attractive from both environmental and economic perspectives (Johnson and Johnson, 1995).

Vaccination, selective breeding, dietary manipulation, and improvements in feed efficiency have all been examined for their efficacy in CH_4 mitigation, with varying results (Gerber et al., 2013). The addition of certain seaweed species and their by-products to the diets of ruminant livestock has been extensively investigated and presents a promising and renewable approach to mitigate enteric CH_4 emissions (Abbott et al., 2020). Several species of red and brown macroalgae are known to inhibit microbial methanogenesis when included as a feed additive in the diet of ruminants (Machado et al., 2014, 2016a, 2018; Roque et al., 2019). Among them, the bromoform containing red seaweed *Asparagopsis taxiformis* has been shown to have anti-methanogenic properties and can reduce CH_4 production by over 95% *in vitro* and *in vivo* (Machado et al., 2016b; Roque et al., 2019, 2021; Terry et al., 2022). *A. taxiformis* is native to warm temperate waters and considered invasive elsewhere; therefore, the identification of local varieties of seaweeds that can favorably impact rumen function is a great priority. In addition to the potential role of seaweeds to reduce enteric CH_4 emissions, there is also evidence that other bioactive compounds present in seaweeds may confer other benefits such as improved gut health and pathogen suppression (Belanche et al., 2016; Zhou et al., 2018).

Recently we examined the effects of three red seaweeds, *A. taxiformis, Mazzaella japonica*, and *Palmaria mollis*, on microbial fermentation and CH_4 production in a simulated rumen environment over 21 days (Terry et al., 2022). In this experiment, rapid changes in gas production and fermentation parameters were observed during the first four days that seaweed was supplemented as the system adapted to the additive (days 8–11). This period was followed by an intermediate phase (days 12–16) in which small changes in the system were observed, and a stable phase (days 17–21) when the system had stabilized (Terry et al., 2022). The inclusion of *A. taxiformis*, resulted in an 84.0% reduction of CH_4 production within the first 48 h, continuing to drop throughout the course of the experiment resulting in a 95.1% reduction in CH_4 compared to controls during the stable phase (Terry et al., 2022). Accompanying the decrease in CH_4 production, *A. taxiformis* significantly reduced acetate, propionate and total VFA production throughout the course of the experiment. *A. taxiformis* was found to reduce organic matter (OM) and neutral detergent fiber (NDF) digestibility compared to controls during the adaptation phase. Interestingly, digestibility recovered in the stable phase suggesting that adaptation to *A. taxiformis* occurred (Terry et al., 2022). In contrast, *M. japonica* and *P. mollis* did not have a measurable effect on *in vitro* fermentation or digestibility.

It is not clear how the inclusion of *A. taxiformis, M. japonica*, and *P. mollis* in diets influences the rumen microbiome and understanding of the microbial mechanisms underpinning ruminal adaptation to seaweed supplementation is generally lacking. To address these gaps, we employed 16S rRNA amplicon sequencing to assess the temporal dynamics of the rumen microbiome in response to supplementation with *A. taxiformis, M. japonica*, and *P. mollis* over a 13-day period using a RUSITEC apparatus. These data provide insight into rumen microbial adaptation to seaweed feeding, but also highlight potentially negative impacts on key rumen functions concurrent to CH_4 inhibition.

2. Materials and methods

2.1. RUSITEC system

Details of seaweed procurement, RUSITEC design, sampling protocols have been published elsewhere (Terry et al., 2022). Donor cows used in this experiment were cared for in accordance with the guidelines of the Canadian Council on Animal Care (Canadian Council on Animal Care, 2009) under an approved animal ethics protocol ACC1830. This experiment was performed in duplicate using two identical RUSITEC apparatus equipped with eight fermentation vessels each. The experiment was divided into four phases: baseline (days 0–7), adaptation (days 8–11), intermediate (days 12–16) and stable (days 17–21). Each fermenter was inoculated with rumen fluid collected from 3 ruminally canulated beef heifers. The basal substrate (10 g dry matter (DM) 50:50 barley silage:barley straw) was added daily to the fermenters. The three seaweeds, *Asparagopsis taxiformis, M. japonica*, and *P. mollis* were added to the basal substrate on a 2% DM basis from day 8 onward. The control vessels were treated in an identical manner with no seaweed added. Beginning on day 8, A 1.5 mL sample of digester fluid was collected daily from each vessel for microbial community analysis. The sample was centrifuged at 21,000 RCF for 5 min to pellet the microbial cells. The supernatant was discarded, and the cell pellet was re-suspended in DNA/RNA Shield (Zymo Research), snap frozen in liquid nitrogen and stored at −80°C, pending molecular analysis.

2.2. Bromoform content analysis

The concentration of bromoform in each of the three seaweeds was determined using a Shimadzu QP2010 Ultra GC/MS system at a commercial laboratory (Bigelow Analytical Services, United States). Bromoform concentration was recorded as mg/g dry weight.

2.3. DNA isolation

Microbial DNA was isolated from all samples using the ZymoBIOMICS DNA Miniprep Kit, following the manufacturer's

instructions with some modifications; the cell pellet was re-suspended in 1,000 µL of DNA/RNA Shield (Zymo Research) and 250 µL of this cell suspension was added to 250 µL of lysis buffer in ZR BashingBead™ lysis tubes (Zymo Research) containing 0.1 mm and 0.5 mm beads. All samples underwent 5 rounds of bead beating using a MP FastPrep 24 (MP Bio, United States) for 20 s at 6.0 m/s with 1 min breaks between rounds. Following bead beating, samples were incubated at 70°C for 10 min to increase the effectiveness of DNA isolation. The remaining purification steps followed the manufacturers protocol, and all samples were eluted in 100 µL of distilled H_2O. DNA quantity and quality was assessed with a Nanodrop 2,100 spectrophotometer and visualization in a 1% agarose gel.

2.4. PCR amplification, library construction, and sequencing

Sequencing was performed at the Genome Quebec Innovation Center (Montreal, Canada) using the Illumina MiSeq Reagent Kit v2 (500 cycle) following the manufacturer's guidelines. The primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) targeting the V4 region of the 16S rRNA gene were used to examine both bacterial and archaeal diversity (Caporaso et al., 2012). A 33 cycle PCR using 1 µl of a 1 in 10 dilution of genomic DNA and the Fast Start High Fidelity PCR System (Roche, Montreal, PQ) was conducted with the following conditions: 94°C for 2 min, followed by 33 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, with a final elongation step at 72°C for 7 min. Fluidigm Corporation (San Francisco, CA) barcodes were incorporated in a second PCR reaction using the following conditions: 95°C for 10 min, followed by 15 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 1 min, followed by a final elongation step at 72°C for 3 min. After amplification, PCR products were assessed in a 2% agarose gel to confirm adequate amplification. All samples were quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, Carlsbad, CA) and were pooled in equal proportions. Pooled samples were then purified using calibrated Ampure XP beads (Beckman Coulter, Mississauga, ON). The pooled samples (library) were quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, Carlsbad, CA) and the Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (Kapa Biosystems, Wilmington, MA). Average fragment size was determined using a LabChip GX (PerkinElmer, Waltham, MA, United States) instrument.

2.5. Sequence data analysis

Raw reads were imported in .fastq format to a local server for analysis. Read quality was evaluated using FASTQC (Andrews, 2010) and MultiQC (Ewels et al., 2016). Data were processed using the QIIME2 software package (Bolyen et al., 2019). FIGARO (Sasada et al., 2020) was used to identify optimum truncation positions for read merging. Reads were denoised into amplicon sequencing variants (ASVs) using the DADA2 (Callahan et al., 2016) plugin for QIIME2. A phylogenetic tree was generated using MAFFT (Katoh and Standley, 2013). ASVs were taxonomically classified using a Native Bayes classifier trained on the V4 region of the 16S rRNA gene using the Sci-Kit plugin in QIIME2. The SILVA SSU (v.138.1) (Quast et al., 2013) database was used to classify bacterial sequences while the Rumen and Intestinal Methanogens (RIM) database (v.14.7) (Seedorf et al., 2014) was used for archaeal reads. Initial analysis (described in Supplementary Figures S3A–C) indicated that increasing the confidence threshold to 0.85 (from a default of 0.7) prevented spurious ASV classification using the RIM database.

2.6. Diversity and correlation analysis

QIIME2 objects (ASV frequency table, taxonomy table, and phylogenetic tree) were imported into R as a Phyloseq (McMurdie and Holmes, 2013) object using the qiime2R package (Bisanz, 2018). Analysis was performed separately for bacterial and archaeal datasets. In-house R scripts were used to calculate summary statistics of read counts and distributions. ASVs that were not assigned to at least a microbial phylum were discarded. Rarefaction curves were generated to identify suitable subsampling thresholds for diversity analyses. Rarified data was used for α- (Chao1 and Shannon) and β-diversity (Weighted Unifrac) calculations. α-Diversity data was summarized according to phase for comparison using a two-way analysis of variance (ANOVA) with Tukeys post-hoc test. Statistically significant differences were declared at Bonferroni-adjusted $p < 0.05$. Permutational Analysis of Variance (PERMANOVA) tests were performed using the Vegan (Oksanen et al., 2022) R package, with pairwise tests performed using the RVaideMomoire R package (Herve, 2021). Homogeneity of dispersion (β-dispersion) tests were performed using Vegan. Canonical correspondence analysis (CCA) between rarified ASV count data and environmental data collected from the RUSITEC system was also performed using Vegan. Statistically significant differences were declared at a threshold of $p < 0.05$, and all figures were generated using ggplot2 in R (Wickham, 2009). Core rumen taxa were declared as those present in more than 50% of the samples and represented by at least 100 sequencing reads. Correlation analysis was performed between selected fermentation metrics and (i) the core microbiome and (ii) differentially abundant taxa using log10 transformed count data and Spearman correlations in R.

2.7. Differential abundance testing

Differentially abundant (DA) bacterial taxa between seaweed and control samples were identified using ANCOM-BC (Lin and Peddada, 2020) and ALDEx2 (Fernandes et al., 2014). Sequence data were arranged according to experimental phase to identify DA taxa between seaweed treatments within the adaptation (days 8–11); intermediate (days 12–16) and stable (days 17–21) phases. Prior to DA testing, low-prevalence features - those present in <10% of the samples – were discarded as recommended for microbiome data (Nearing et al., 2022). Non-rarefied data were used as input for all DA tests. For ANCOM-BC, the phyloseq object was passed to the 'ancombc' function and run at a maximum of 1,000 iterations. Structural zeros - taxa present in one group but absent in another – were declared as DA where present. A pseudocount of 1 was added to all observations to facilitate log transformation. Significant features were those with a Benjamini-Hochberg-adjusted $p < 0.05$. For ALDEx2, count data and

metadata were passed to the 'aldex' command which employs a Dirichlet-multinomial model to infer abundance from counts. *p*-values generated from a Wilcoxon Rank Sum test were FDR-corrected using the Benjamini-Hochberg procedure and significant differences according to treatment group were declared at corrected $p < 0.05$. The significantly different taxa identified by both tools were compared and consensus taxa (i.e., those identified using both methods) declared as DA for each respective comparison.

3. Results

3.1. Bromoform analysis

Only *A. taxiformis* had detectable amounts of bromoform, at 0.517 mg/g^{-1} DM. Any bromoform present in *P. mollis* and *M. japonica* was below the limit of detection for the instrument.

3.2. Sequencing data characteristics

Sequencing of 16S rRNA V4 amplicons from 272 digesta samples generated a total of 12,456,461 reads (range: 2,663–78,773) with an average of 45,795 ± 10,306 (mean ± SD) per sample. Denoising with DADA2 retained 72.6% of the reads and identified a total of 18,925 ASVs. Rarefaction curves for both bacterial and archaeal annotation data reached a plateau (Supplementary Figures S1A,B) indicating that our sampling depth was sufficient. When the total number of reads successfully denoised into archaeal ASVs were plotted over time, there was a clear temporal decline of archaea in the *A. taxiformis* samples with only 30 archaeal reads successfully denoised on D21 among the four samples (Figure 1A). A similar trend was not evident for the other seaweed treatments. The number of successfully denoised bacterial reads did not appear to differ dramatically across treatments throughout the experimental period though there were some temporal patterns evident (Figure 1B).

3.3. Microbial data analysis

3.3.1. Alpha and beta diversity

Principal coordinate (Figures 2A,B) and PERMANOVA (Table 1) analyses based on weighted Unifrac distances showed separation of both bacterial and archaeal microbial profiles according to treatment and phase ($p < 0.05$), with the *A. taxiformis* samples clearly separating from the other groups (Figures 2A,B). The separation increased throughout the experiment and was greatest in the stable phase. The R^2 value was greater for treatment than experimental phase (0.30 vs. 0.04) in the bacterial data, indicating this was the largest factor contributing to compositional differences. Experimental phase and treatment made similar (0.17 vs. 0.18) contributions to dissimilarity of the archaeal data. Canonical correspondence analysis (Figures 2C,D) using the fermentation data reported by Terry et al. (2022) showed that the separation of the *A. taxiformis* samples from the other groups could be explained by several environmental measurements including: the decline in CH_4, the concordant increase in H_2, and the lower molar proportions of propionate and acetate (Terry et al., 2022). The environmental parameters (Supplementary Table S1) that drove the separation of samples was similar for both the bacterial and archaeal datasets (Figures 2C,D).

ANOVA analysis indicated that Chao1 and Shannon indices were influenced by treatment ($p < 0.05$), phase ($p < 0.05$), and the interaction of the two ($p < 0.05$). Only *A. taxiformis* exhibited major differences in alpha-diversity when compared to the controls (Figure 3), while differences between the controls and the other two seaweeds were minor. Results of all alpha-diversity comparisons are presented in Supplementary Tables S2, S3. *A. taxiformis* samples had lower Chao1 and Shannon index values in the intermediate phase compared to the controls ($p < 0.05$), while the *P. mollis, M. japonica* and Control samples had similar values. Treatment-wise differences declined for all treatments by the stable phase with few statistically significant differences evident (Supplementary Tables S2, S3).

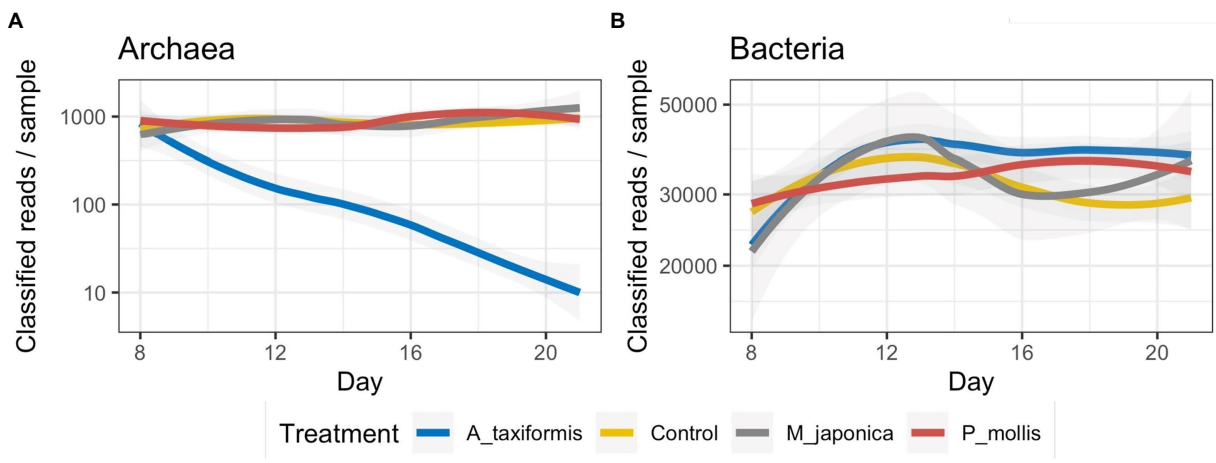

FIGURE 1
Number of reads denoised into **(A)** archaeal and **(B)** bacterial ASVs. Archaeal data is presented on a logarithmic scale for clarity.

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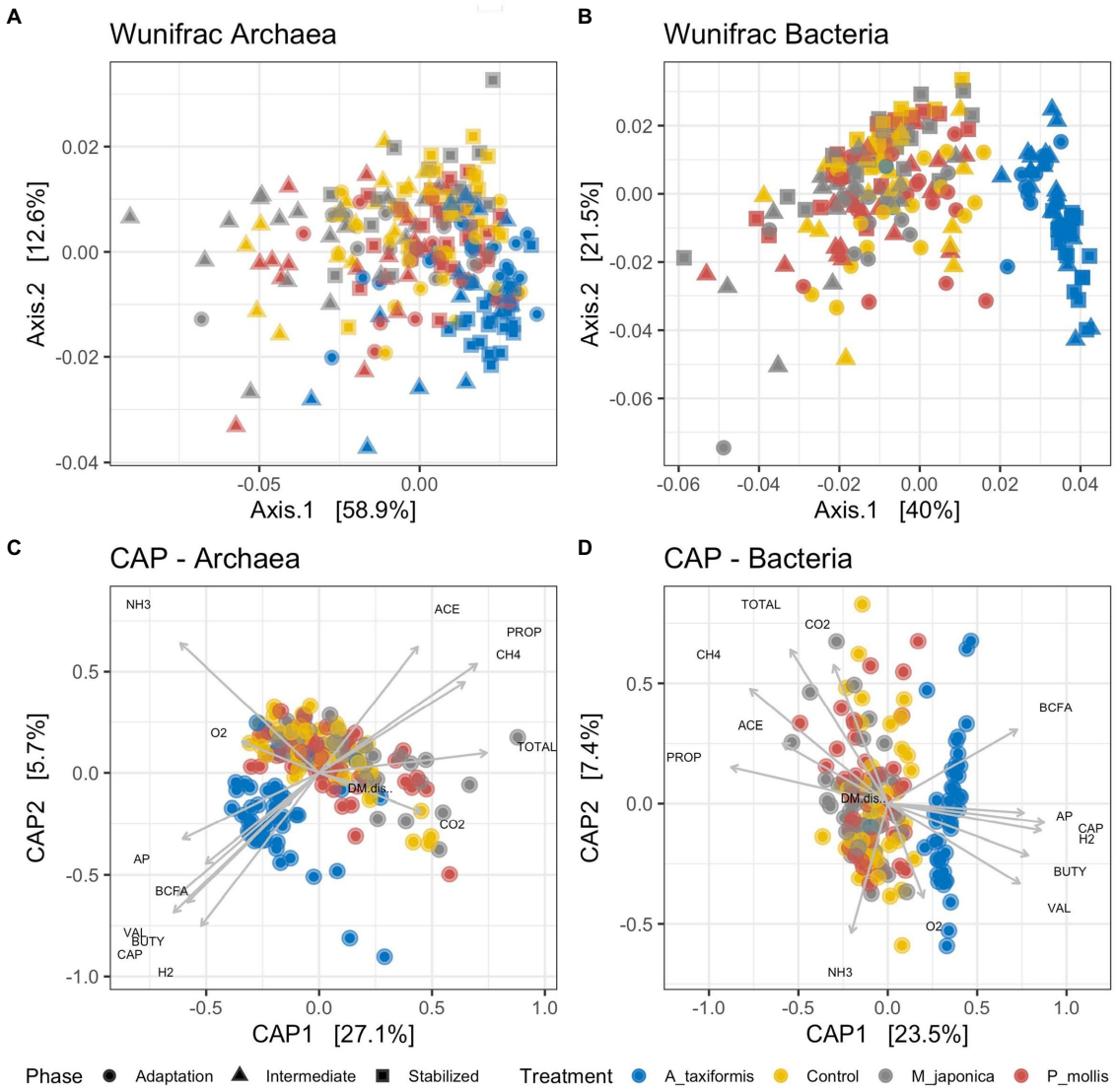

FIGURE 2
Unconstrained principal coordinate analysis (PCoA) plots based on rarefied Weighted Unifrac distance matrices for **(A)** archaeal and **(B)** bacterial communities. Constrained canonical analysis of principal coordinates (CAP) plots for **(C)** archaeal and **(D)** bacterial communities and environmental variables. The vectors indicate the axis that is best explained by each variable. Data was generated using Weighted Unifrac distance matrices. ACE, acetate; PROP, propionate; CH4, methane; TOTAL, total VFA; CO2, carbon dioxide; BCFA, branched-chain fatty acids; AP, acetate: propionate ratio; CAP, Caproate; H2, Hydrogen; BUTY, butyrate; VAL, valerate; O2, oxygen; NH3, ammonia. Individual sample values for all measurements are provided in the Supplementary material.

TABLE 1 Results of PERMANOVA analysis on bacterial and archaeal community dissimilarities.

Variable	*R^2	†F-value	p-value	R^2	F-value	p-value
	Bacteria			Archaea		
Treatment	0.30	35.63	0.001	0.18	20.02	0.001
Phase	0.05	8.41	0.001	0.17	28.40	0.001
Day	0.01	2.18	0.065	0.01	2.60	0.064
Treatment:Day	0.07	7.75	0.001	0.02	2.25	0.025
Treatment:Phase	0.02	1.03	0.416	0.03	1.44	0.124
Residual	0.56	–	–	0.60	–	–
Total	1.00	–	–	1.00	–	–

Tests conducted using a weighted Unifrac dissimilarity matrix. *R2: Proportion of variance explained by each variable; †F-value, estimate of effect size.

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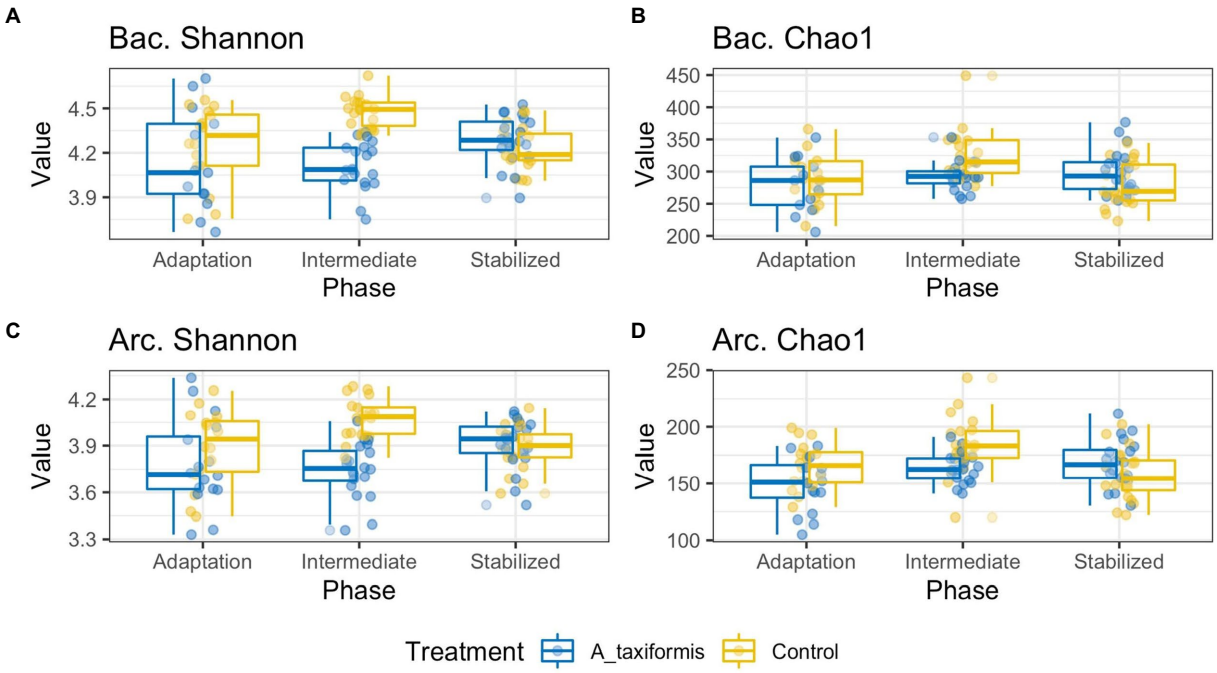

FIGURE 3
Alpha diversity boxplots of bacterial (Bac) and archaeal (Arc) sequencing data. Samples are grouped according to phase. All data was rarefied to an even depth prior to metric calculation. Panels **(A–D)** depict Shannon Evenness and Chao1 Richness metrics for *A. taxiformis* and control samples.

3.3.2. Microbial community composition and response to seaweed addition

Following the removal of sparse ASVs, 1 archaeal and 16 bacterial phyla were identified across all samples. Irrespective of treatment or phase, the microbiomes were dominated by Bacteroidota and Firmicutes throughout, with Proteobacteria and Actinobacteria also present at high proportions. The mean relative abundances for all genera identified in the adaptation, intermediate and stable phase are provided in Supplementary Table S4. The most abundant rumen bacteria included *Prevotella*, *Streptococcus*, *Megasphaera*, *Lactobacillus*, and *Fibrobacter* (Figure 4). The "core" bacteriome was calculated across control and *A. taxiformis* samples and consisted of 32 genera drawn from 8 phyla. The divergence in the core microbial groups between control and *A. taxiformis* samples increased across phases (Supplementary Figure S2) indicating that at least some of these taxa were influenced by *A. taxiformis* supplementation in the RUSITEC. As expected, major ruminal bacteria including *Fibrobacter, Prevotella, Succinivibrio, Megasphaera*, and *Butyrivibrio* were part of the core microbiome. The methanogen community was dominated by *Methanobrevibacter ruminantium, Methanobrevibacter gottschalkii*, and several poorly characterized species belonging to *Methanomassiliicoccaceae* (Figure 5). *Methanomicrobium mobile, Methanimicrococcus blatticola*, and *Methanosphaera* sp. were among the less abundant methanogens in the RUSITEC. When raw read counts were visualized, there was a clear decline in the number of sequences attributed to methanogenic species over time in the *A. taxiformis* samples (Figure 5A). Compared to control samples, there was a 86 and 97% decrease in sequences from methanogens in *A. taxiformis* samples in the intermediate and stable phases, respectively. Raw and relative abundances of all microbial taxa are presented in Supplementary Tables S4–S8.

The microbial response underpinning the dramatic reduction in CH_4 by *A. taxiformis* reported by Terry et al. (2022) was investigated in more detail *via* correlation analysis and differential abundance testing. To examine if bioactives present in the non-methanogenic seaweeds had an impact on the microbiome, testing was also conducted between the control and *M. japonica* samples. Differentially abundant (DA) features were those identified using both tools (see methods). *M. japonica* did not have a major effect on the bacterial community, with just 2 genera responding to treatment during the adaptation phase; *Oribacterium* was more abundant in the seaweed samples ($p < 0.05$), while *Pirellulaceae P1088_a5_gut_group* was more abundant in the controls ($p < 0.05$).

The bacterial community exhibited a progressive response to *A. taxiformis*, with 10, 76, and 92 DA bacterial genera identified during the adaptation, intermediate, and stable phases, respectively ($p < 0.05$). ANCOM-BC identified more DA genera throughout the experiment than Aldex2 (Figure 6A). Eight genus-level features were affected by *A. taxiformis* during all 3 phases. Among them, the abundance of *Fibrobacter* was 2.8 fold lower in *A. taxiformis* samples compared to controls in the adaptation phase and 19.5 fold lower in the stable phase ($p < 0.05$). The abundance of *Schwartzia*, was reduced by *A. taxiformis* throughout the experiment by 2 fold in the adaptation phase, 10.8 fold in the intermediate phase, and 17.8 fold in the stable phase compared to the control samples ($p < 0.05$). Reads from *Papillibacter* were no longer detected in the stable phase in *A. taxiformis* samples. *Sutterella* was the only genus with an abundance that was consistently higher in *A. taxiformis* samples (0.05–0.27%) compared to the controls throughout the experiment ($p < 0.05$) whereas, reads assigned to this genus comprised less than 0.01% of the total abundance in control samples (Figures 6B,C, 7). There was a large degree of overlap

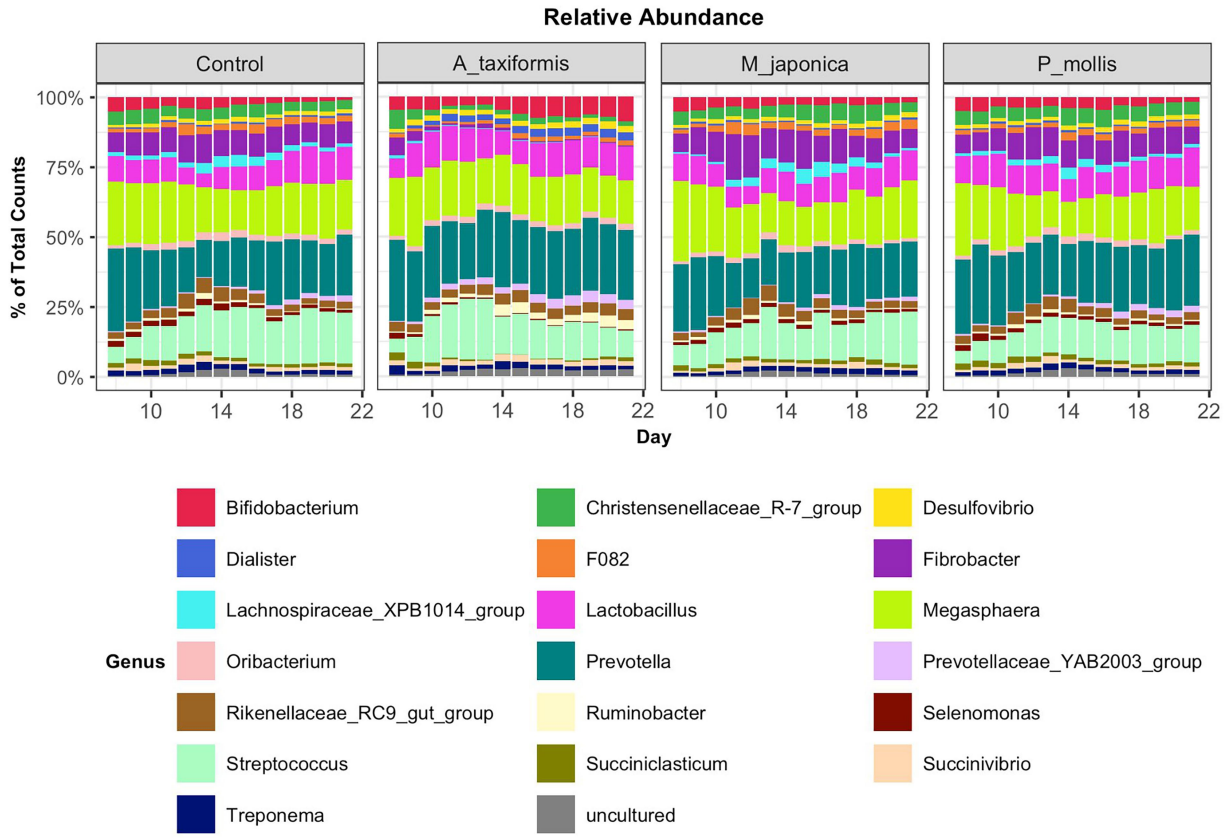

FIGURE 4
Relative abundances of the 20 most abundant genus-level features across all treatment groups. Abundances were scaled to 1 for ease of presentation.

between the DA taxa in the intermediate and stable phases, with 54 showing the same response to seaweed supplementation in both phases (Figures 6C, 7). Many major rumen bacteria and members of the core microbiome were influenced by *A. taxiformis* in the intermediate and stable phases of the experiment, including increased abundance of *Prevotella*, *Dialister*, *Succinivibrio* and *Ruminobacter* ($p < 0.05$). Members of *Clostridium sensu stricto 1, Roseburia,* and *Ruminiclostridium* were among the genera more abundant in the control samples ($p < 0.05$) in the intermediate and stable phases. Moreover, several minor members of the assemblage responded to *A. taxiformis* in the intermediate and stable phases, including lowered abundances of *Endomicrobium, Denitrobacterium, and Angelakisella* ($p < 0.05$). There were also shifts in the abundances of many unclassified and/or poorly annotated genera belonging to *Prevotellaceae* and *Lachnospiraceae* ($p < 0.05$), as well as other families (Supplementary Table S4).

When low-prevalence features were removed, there were 8 archaeal species-level taxa left in our dataset. No archaeal species exhibited a statistically significant response to *M. japonica* supplementation throughout the experiment ($p > 0.05$). The number of archaeal reads recovered from the *A. taxiformis* samples declined drastically in the latter stages of the experiment and sequences from many of the archaea present at the start of the experiment were not detected in *A. taxiformis* samples during the stable phase (Figure 5A). Interestingly, rumen methanogens appeared to show different levels of sensitivity to *A. taxiformis*, with the abundances of *M. mobile* and *Methanomassiliococcaceae* species declining almost immediately, while *Methanobrevibacter* species took several days to drop significantly compared to control samples (Figure 8B). Reads from *M. gottschalkii* and *M. ruminantium* were 2.4 and 2.3 fold lower (respectively) in *A. taxiformis* samples compared to levels observed in the control in the intermediate phase ($p < 0.05$) (Figure 8). In the stable phase of the experiment *M. gottschalkii* and *M. ruminantium* reads were 15 and 7.8 fold lower (respectively) in *A. taxiformis* samples compared to controls. No reads from *Methanosphaera* sp. ISO3-F5, *M. mobile*, *M. blatticola* were identified in *A. taxiformis* samples in intermediate phase samples. A total of 50 reads from *Methanomassiliococcaceae* groups were found in *A. taxifor*mis samples in the intermediate phase. In contrast, reads from these taxa had increased by 1.5 fold in control samples compared to levels found at the start of the experiment. Only reads from *M. ruminantium, M. gottschalkii*, and *Methanomassiliococcaceae* Group 9 were detected in the stable phase samples and reads from these groups were reduced by 93, 87, and 99% compared to the control samples, respectively. The collapse in the methanogen community in *A. taxiformis* samples resulted in a highly sparse data set and complicated the application of widely used tools for identifying differentially abundant microbial taxa. In spite of this challenge, the 97.1% decrease in reads from methanogens in *A. taxiformis* samples compared to the control clearly demonstrates the inhibitory activity this seaweed has on rumen methanogens.

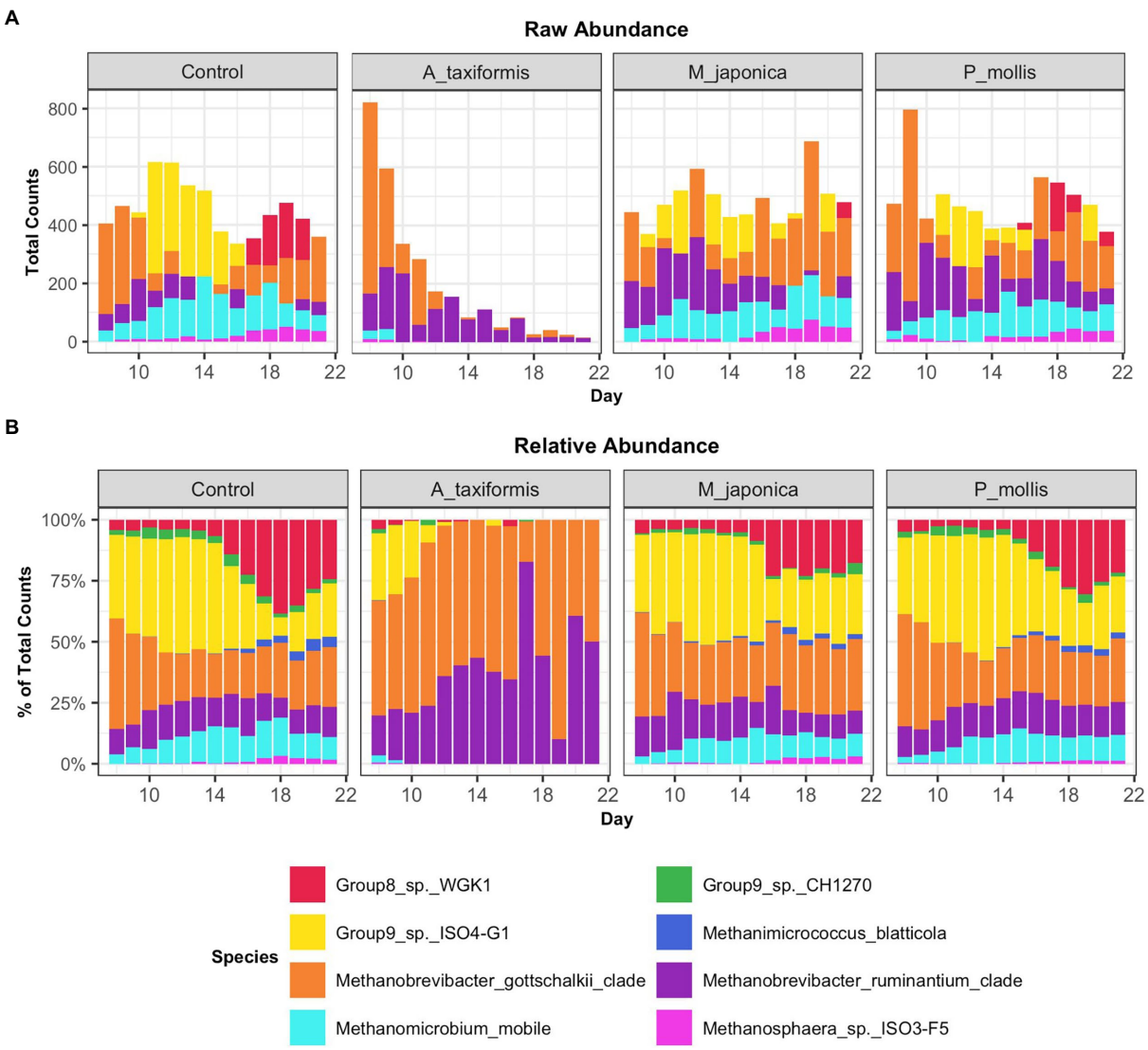

FIGURE 5
Raw **(A)** and relative **(B)** abundances of methanogenic species across all treatment groups. Values are the daily median across all samples.

3.3.3. Correlation between microbial communities and fermentation variables

Spearman correlation coefficients were used to determine the relationships between fermentation variables reported by Terry et al. (2022) and microbiome features. A relationship was considered strong at an absolute *R* value >0.5 and an adjusted *p*-value <0.05. The core microbes exhibited stronger relationships with fermentation variables in the *A. taxiformis* samples than in the controls. CH_4 concentration was strongly associated with 13 of the 32 core genera in the *A. taxiformis* samples; *Fibrobacter, Selenomonas, Schwartzia, NK4A214* (*Ruminococcaceae*), and *Lachnospiraceae XPB1014* were all positively correlated with CH_4 concentration ($p < 0.05$; Figure 9), while *Desulfovibrio*, *Ruminobacter*, *Erysipelotrichaceae* UCG-002 and *Dialister* were among those that exhibited negative relationships with CH_4 ($p < 0.05$). Conversely, only 2 genera correlated with CH_4 in the control samples, with *Prevotellaceae YAB2003* exhibiting a negative relationship and *Paraprevotella* a positive one ($p < 0.05$). Strong relationships between core taxon abundances and the molar proportions of individual VFA were also more evident in *A. taxiformis* than controls. *Schwartzia* and *Lachnospiraceae XPB1014* were positively correlated with acetate and propionate ($p < 0.05$), while *Erysipelotrichaceae UCG-002* was negatively correlated with these VFAs ($p < 0.05$). The molar proportion of propionate was also negatively correlated with the abundances of *Lactobacillus, Prevotellaceae YAB3003 group*, and *F082* ($p < 0.05$), while *Dialister* and *Prevotellaceae UCG-003* had a negative relationship with acetate proportion ($p < 0.05$). Butyrate was positively associated with *Streptococcus, Megasphaera* and *Oribacterium* in the *A. taxiformis* samples ($p < 0.05$). Total VFA concentration was also negatively correlated with *Desulfovibrio* and *Lactobacillus* in the *A. taxiformis* samples ($p < 0.05$). The molar proportion of propionate was positively correlated with *Lachnospiraceae XPB1014* in the control samples ($p < 0.05$). Total VFA level was positively correlated with *WCHB1-41* and *Paraprevotella* in the controls ($p < 0.05$), while *Erysipelotrichaceae UCG-002* and *Prevotellaceae YAB2003* group exhibited the opposite relationship. Correlation coefficients and *p*-values for the core genera

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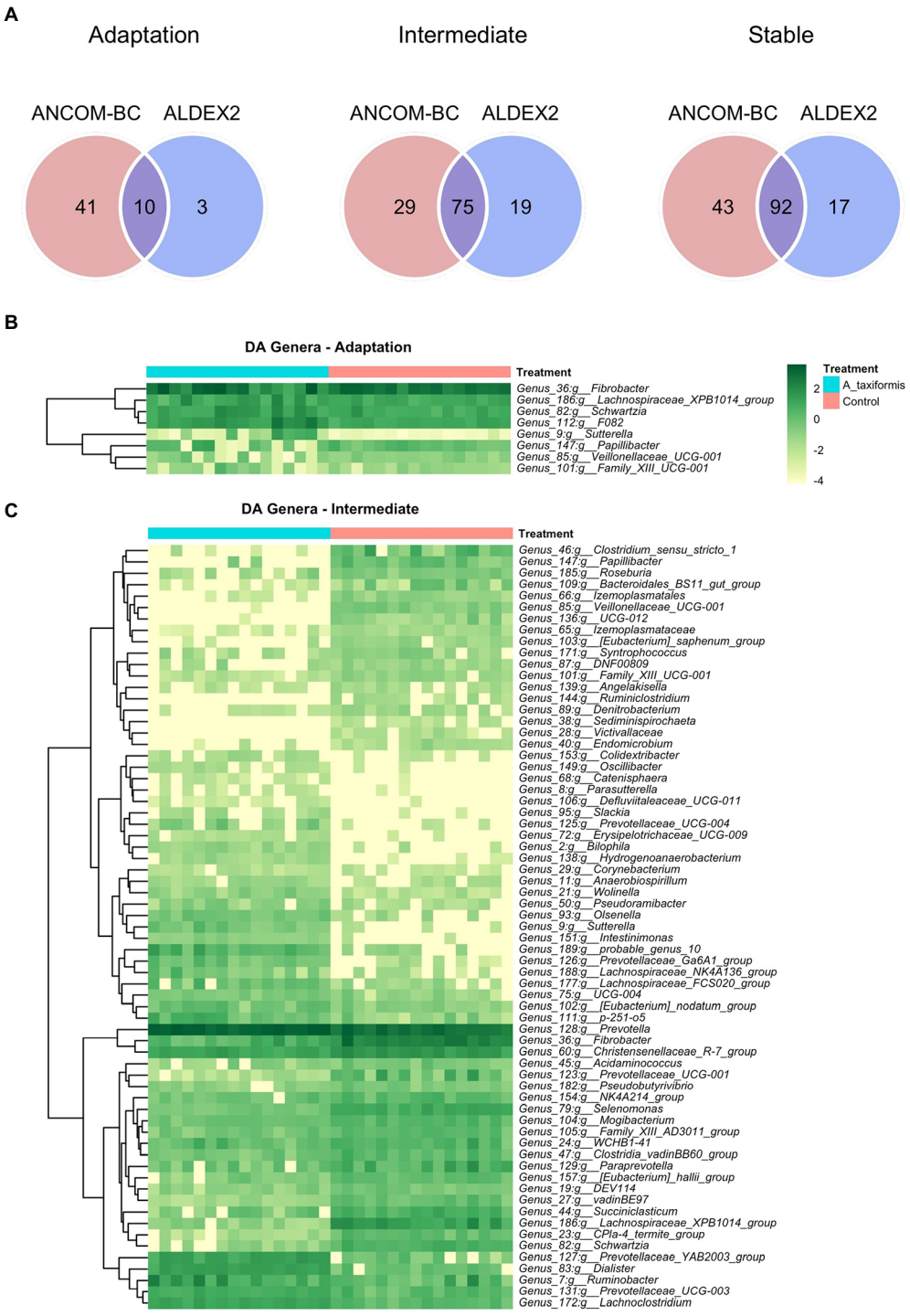

FIGURE 6
Differential abundance (DA) analysis of bacterial genera. **(A)** Venn diagrams showing the overlap between ANCOM-BC and Aldex2 results. Heatmaps of DA genera for the **(B)** adaptation and **(C)** intermediate phases are shown. Raw count data was log transformed for plotting. Genera denoted as uncultured or unknown are excluded from the heatmaps.

are provided in the Supplementary Table S9 and presented graphically in Figure 9.

Strong relationships were evident between the DA genera (*A. taxiformis* vs. control) and fermentation variables throughout the experiment and largely reflected the taxa identified as differentially abundant between these treatments (Figure 10). In the adaptation phase, CH_4 and propionate were positively correlated with multiple DA genera including *Fibrobacter, Schwartzia, Veillonellaceae* UCG-001 and *Lachnospiraceae XPB1014 group* ($p < 0.05$), while *Sutterella* had inverse relationships with both ($p < 0.05$). Conversely, caproate was negatively correlated with 5 of the DA genera, and positively correlated only with *Sutterella* ($p < 0.05$) (Figure 10A). In the latter phases of the

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FIGURE 7
Differential abundance (DA) analysis of bacterial genera. Heatmaps of DA genera for the stabilized phase. Raw count data was log transformed for plotting. Genera denoted as uncultured or unknown are excluded.

experiment, almost all the differentially abundant taxa were significantly correlated with CH_4 concentration and the molar proportions of VFAs ($p < 0.05$) (Figures 10B,C). Total VFA, H_2 concentration, and the acetate:propionate ratio also exhibited strong relationships with DA taxa in the intermediate and adaptation phases ($p < 0.05$). All correlation coefficients and *p*-values are presented in Supplementary Table S10.

4. Discussion

The effectiveness of the red seaweed *Asparagopsis taxiformis* in suppressing enteric methanogenesis in ruminants has been demonstrated both *in vitro* and *in vivo* (Kinley et al., 2016; Machado et al., 2016a; Roque et al., 2019, 2021). Our recent study examining the effects of three red seaweeds, *A. taxiformis*, *M. japonica*, and *P. mollis*, on *in vitro* rumen fermentation and gas production confirmed previous observations of the potency of *A. taxiformis* in reducing methanogenesis, with CH_4 concentrations declining by 95.1% compared to the control treatment (Terry et al., 2022). This mitigation effect was accompanied by reductions in fiber degradation and VFA production which could have negative impacts on animal performance. In contrast, there was no measurable impact of either *M. japonica* or *P. mollis* on methanogenesis or microbial fermentation. There is limited data concerning the microbial response to seaweed supplementation in ruminants. This study presented an opportunity

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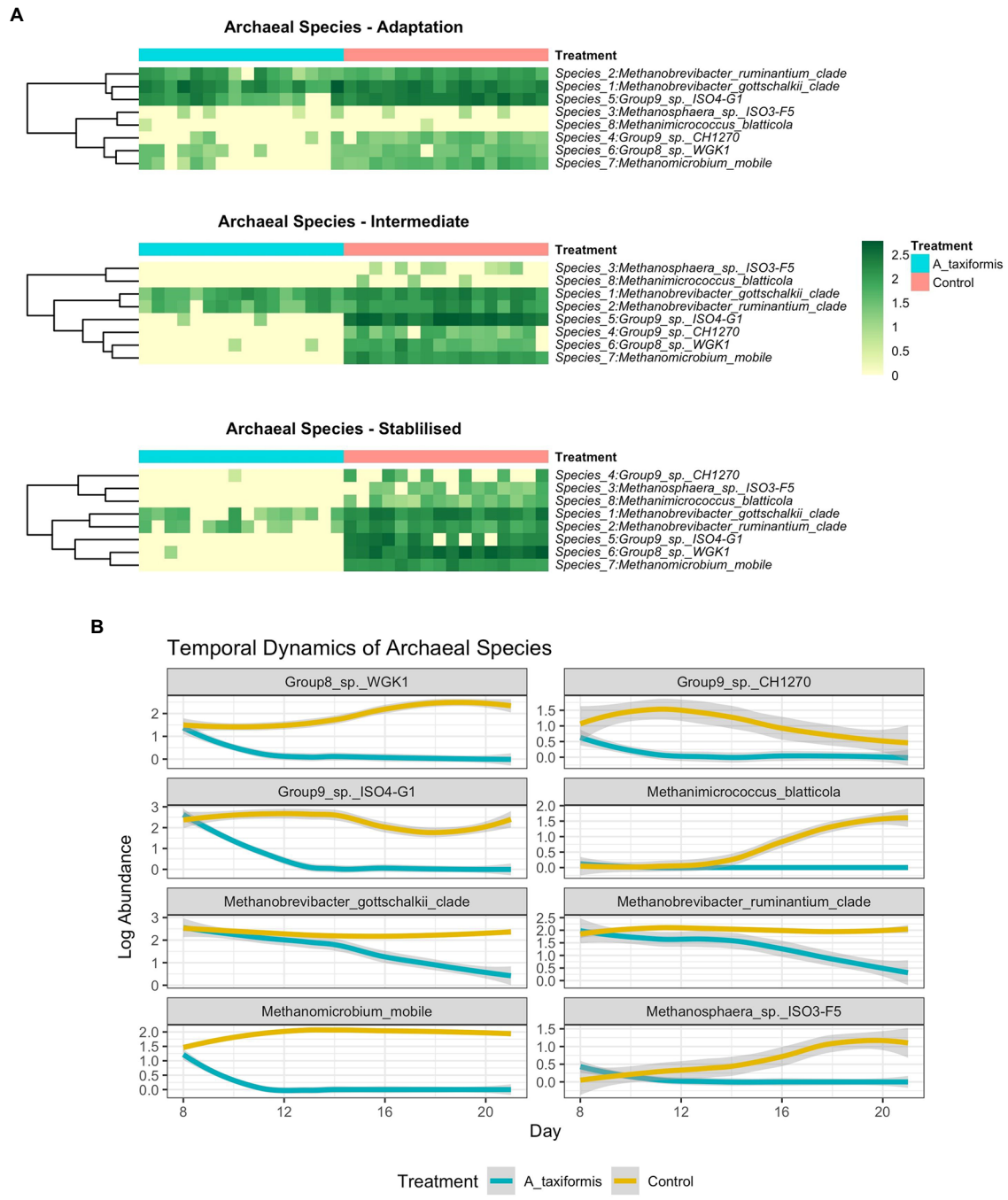

FIGURE 8
(A) Heatmaps depicting archaeal community composition in each experimental phase and **(B)** line plots showing temporal species dynamics throughout the experiment. Raw abundances were log-transformed prior to plotting.

to examine the impact of seaweed supplementation over an extended period on rumen microbes, and to assess if the rumen microbiome shows any evidence of adaptation to seaweeds which might lessen their anti-methanogenic effects over time as has been previously documented (Knight et al., 2011).

The near-total collapse of the methanogen community in the *A. taxiformis* samples (Figures 1A, 5A) was striking and cannot be attributed to temporal shifts in community composition commonly associated with RUSITEC fermenter apparatus (Mateos et al., 2017) or a decline in protozoa associated methanogens (Roque et al., 2019), as the archaeome of the control samples remained relatively stable throughout. We observed a 97.1% reduction in the number of reads from methanogenic archaea in *A. taxiformis* samples compared to controls during of the stable phase of the experiment. There was no suggestion of niche transition among the methanogen species following *A. taxiformis* addition, with the abundances of all major archaea declining throughout the experiment. CCA analysis suggested a strong relationship between the CH_4 and H_2 concentrations reported

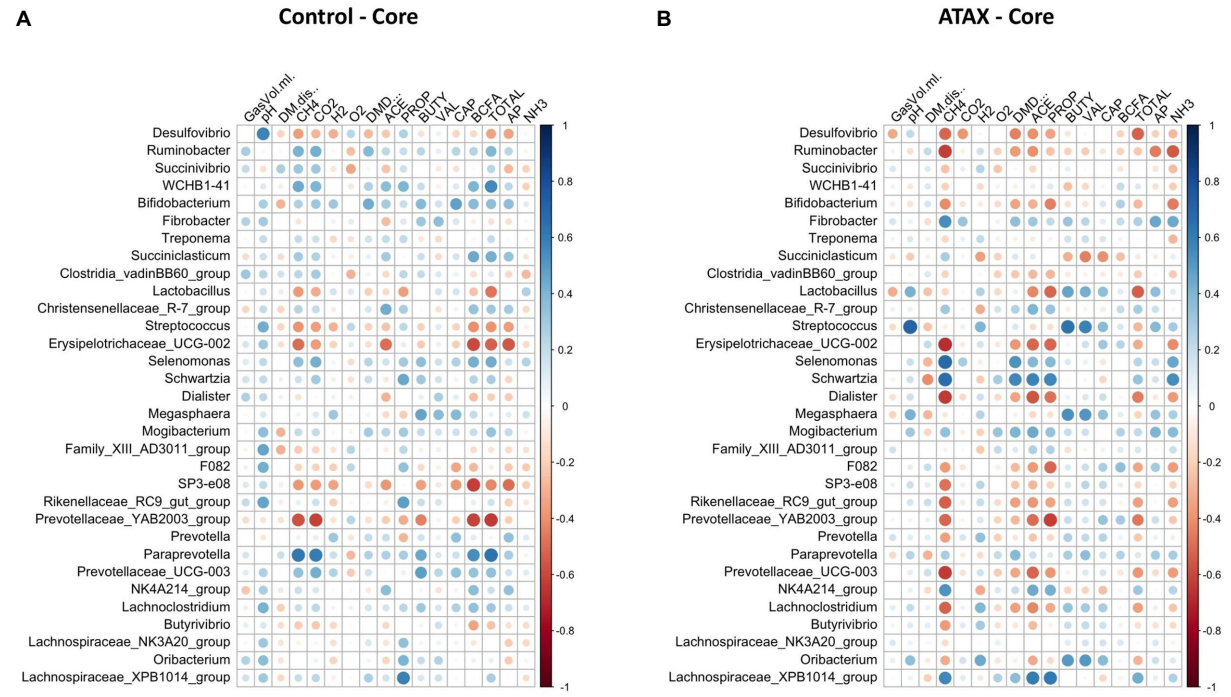

FIGURE 9
Spearman correlation analysis plots between the core bacteriome of **(A)** control samples and **(B)** *A taxiformis* samples and fermentation variables. Raw bacterial abundances were log-transformed prior to correlation. Only data from control and *A taxiformis* reactors was analyzed. GasVol.ml, total gas volume in mL; DM.dis., dry matter disappearance; CH4, methane; CO2, carbon dioxide; H2, hydrogen; O2, oxygen; DMD, dry matter digestibility; ACE, acetate; PROP, propionate; BUTY, butyrate; VAL, valerate; CAP, caproate; BCFA, branched-chain fatty acids; TOTAL, total VFA; AP, acetate: propionate ratio; NH3, ammonia. Individual sample values for all measurements are provided in the Supplementary material.

previously (Terry et al., 2022) and methanogen community dynamics. The extent of the decline in methanogen abundance observed here has not, to our knowledge, been previously documented. A recent *in vitro* study supplemented *A. taxiformis* at 5% (w/v) and achieved a similar reduction in CH_4 production to that observed in this work; however, while *A. taxiformis* decreased the abundance of methanogenic groups over a 96 h period, the reduction was not as dramatic as we observed (Roque et al., 2019). The concentration of bromoform in the supplement used by Roque et al. (2019) was not reported so it is unclear how this compares to the level that is present in the *A. taxiformis* used in our experiment. Our results show that *Methanomassiliicoccaceae* species declined in abundance almost immediately (within 24 h) following *A. taxiformis* addition in our study, while it took several days before the *Methanobrevibacter* species were inhibited (Figure 8B). This suggests greater resilience of *Methanobrevibacter* spp. to the seaweed-induced changes in the fermenter microenvironment compared to *Methanomassiliicoccaceae,* and would explain the comparatively modest reductions in methanogen abundance reported previously over shorter experimental periods (Roque et al., 2019). *Methanomassiliicoccaceae* spp. (formerly Rumen Cluster C) produce CH_4 *via* the reduction of methyl groups (Poulsen et al., 2013; Lang et al., 2015) rather than *via* the hydrogenotrophic pathway employed by *Methanobrevibacter gottschalkii* and *ruminantium* clades, indicating that this pathway is inhibited to a greater extent by bioactives found in *A. taxiformis*. The disparity in response to seaweed supplementation among methanogen species is likely multifaceted. Bromoform is the principal anti-methanogenic metabolite found in *Asparagopsis* species (Paul et al., 2006) and blocks the transfer of methyl groups as well as serving as an alternative electron accepter (Patra et al., 2017), which perhaps most readily explains the rapid decline of methylotrophic species observed here. Further, changes in bacterial composition would result in shifts in the substrate profile available to methanogens, which could indirectly influence archaeal metabolism to a different extent within the various methanogens found in the rumen. Seaweeds possess a multitude of other secondary metabolites (e.g., phlorotannins) which are known to impact microbial communities (Ku-Vera et al., 2020) and these may have directly or indirectly contributed to methanogen dynamics in the present study. Individual methanogens could also vary in their resistance to inhibitory compounds, and our data may simply reflect a greater resilience of the *Methanobrevibacter* species to the deleterious effects of seaweed bioactives (Ungerfeld et al., 2004). Protozoa-associated methanogens contribute up to 25% of ruminal CH_4 (Newbold et al., 1995), and numbers of protozoa typically decline over time in the RUSITEC system regardless of treatment (Lengowski et al., 2016; Mateos et al., 2017). While we did not assess the protozoan community in this study, it is likely that a decline in protozoan abundance over time would have at least partially contributed to the reduction in overall methanogen abundance observed here. 16S rRNA data offer limited mechanistic insight, and future studies using shotgun metagenomics or metatranscriptomics may provide clarity as to the mechanisms underpinning these observations.

The reduction in methanogenesis and inhibition of the methanogen community was accompanied by a general depression in microbial activity measured by a decline in VFA production and fiber degradation (Terry et al., 2022). This was mirrored in a substantial

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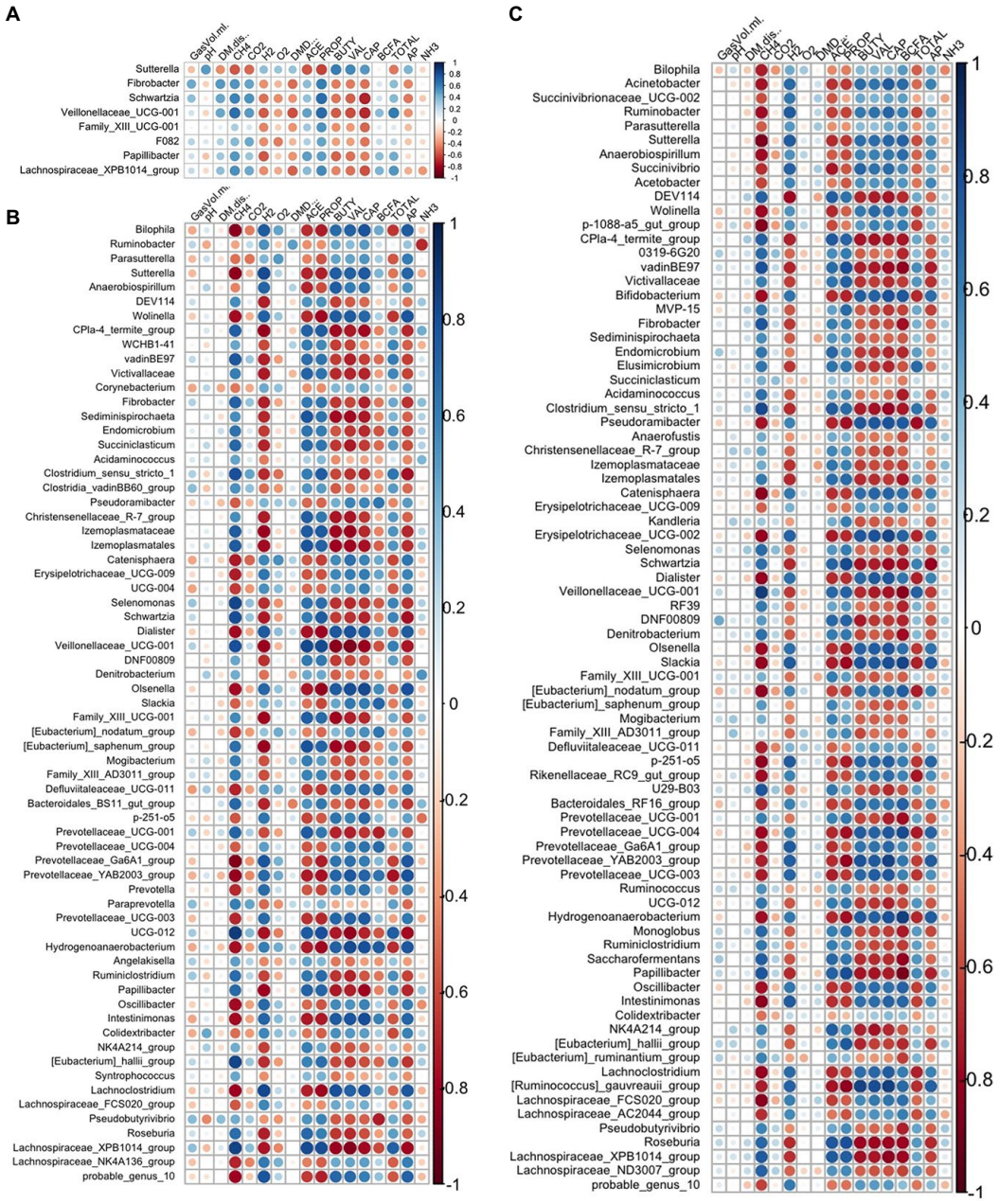

FIGURE 10
Spearman correlation analysis plots between the differentially abundant genera and fermentation variables during the **(A)** adaptation, **(B)** intermediate, and **(C)** stabilized phases. Raw bacterial abundances were log-transformed prior to correlation. GasVol.ml, total gas volume in mL; DM.dis., dry matter disappearance; CH4, methane; CO2, carbon dioxide; H2, hydrogen; O2, oxygen; DMD, dry matter digestibility; ACE, acetate; PROP, propionate; BUTY, butyrate; VAL, valerate; CAP, caproate; BCFA, branched-chain fatty acids; TOTAL, total VFA; AP, acetate: propionate ratio; NH3, ammonia. Individual sample values for all measurements are provided in the Supplementary material.

change in the bacteriome composition, as evidenced by diversity and differential abundance analyses presented here. We observed strong relationships between core bacterial genera and fermentation variables for the *A. taxiformis* samples. 19 of the 32 core bacterial genera were DA between *A. taxiformis* and control samples during at least one phase of the study. The core microbiome encompasses the most ecologically and functionally important taxa in an environment under given sampling conditions, and treatments that disrupt the core microbiome may have negative implications for the ecosystem as a whole (Neu et al., 2021). The observation that *A. taxiformis* significantly altered majority of the core microbiome members including prominent fiber degraders and VFA producers throughout the experiment suggests that inclusion of *A. taxiformis* in feed could have negative impacts on animal performance.

Prevotella is routinely reported as the most abundant rumen microbial genus, prominent in carbohydrate and nitrogen metabolism (Kim et al., 2017). *A. taxiformis* increased the abundance of *Prevotella* 1.6 fold during the intermediate phase of the experiment, while numerical differences in the stable phase did not reach significance. Several poorly characterized genera of *Prevotellaceae* were also

increased by *A. taxiformis*. It has been speculated that *Prevotella* spp. may redirect excess H_2 to propionate when CH_4 is inhibited in the rumen (Aguilar-Marin et al., 2020). Because propionate levels were reduced by *A. taxiformis* supplementation, the increases in *Prevotella* observed here more likely reflect niche transition of various *Prevotella* species as the abundances of several other established rumen microbes declined.

Acetate, propionate and butyrate are the major VFAs associated with ruminal metabolism (Bergman, 1990). In our companion study, *A. taxiformis* reduced total VFA throughout the experiment and the molar proportions of acetate and propionate in the intermediate and stable phases (Terry et al., 2022). While it is not reliable to directly extrapolate from *in vitro* findings, such changes may have negative impacts on animal performance given the importance of propionate and acetate to gluconeogenesis and fatty acid synthesis in the host, respectively (Bauman et al., 1970; Aschenbach et al., 2010). A major pathway for ruminal propionate production is *via* interactions between succinate-producing and utilizing species (Hobson and Stewart, 1997). *Selenomonas ruminantium* uses succinate in its role as a principal propionate producer in the rumen (Sawanon and Kobayashi, 2006) and A. *taxiformis* reduced the abundance of *Selenomonas* in both the intermediate and stable phases. The abundances of other succinate-utilizers were also significantly reduced, including *Schwartzia* (all phases) and *Succiniclasticum* (intermediate and stable phases) (van Gylswyk, 1995; van Gylswyk et al., 1997). The inhibition of several key succinate-utilizing bacteria could contribute to the reduction in propionate production when *A. taxiformis* is supplemented. Interestingly, *A. taxiformis* increased the abundance of genera containing known succinate producers including *Succinivibrio* (stable phase) and *Ruminobacter* (intermediate and stable phases), suggesting alternative roles for ruminal succinate beyond propionate production during *A. taxiformis* supplementation.

A primary role of the rumen bacteria is the degradation of recalcitrant lignocellulosic biomass by several specialized fiber-degrading bacteria, principally *Fibrobacter succinogenes* and *Ruminococcus* spp. (Ransom-Jones et al., 2012; Dassa et al., 2014). Terry et al. (2022) observed reduced OM and NDF degradation in the *A. taxiformis* samples during the adaptation phase, followed by a recovery of fiber digestion in the stable phase. The reduction in fiber degradation was reflected by the significantly lower abundance of *Fibrobacter*, *Ruminococcus*, and other less common cellulolytic genera like *Ruminiclostridium* in *A. taxiformis* samples compared to the controls (Ren et al., 2019; Karri et al., 2021). The abundance of *Fibrobacter* was also strongly positively correlated with CH_4 levels and negatively correlated with H_2 levels in the presence of *A. taxiformis*. Although fiber degradation was only impacted in the adaptation phase, the abundance of *Fibrobacter* was reduced throughout the experiment, and the abundance of *Ruminococcus* was reduced in the stable phase in *A. taxiformis* samples compared to controls. The recovery of OM and NDF digestion suggests that other microbial groups may have filled the niche for fiber degradation in the latter stages of the experiment, although we note that microbial abundance and activity do not necessarily correlate in complex microbial ecosystem (Hunt et al., 2013). Multiple members of the *Lachnospiraceae* family possess cellulolytic capabilities and are capable of butyrate synthesis (Meehan and Beiko, 2014). *Roseburia* is a prominent butyrate producer (Barcenilla et al., 2000) but was inhibited by *A. taxiformis* during the intermediate and stable phases despite butyrate being the only major VFA to increase in molar proportion during supplementation. We speculate that the increased abundance of several poorly described *Lachnospiraceae* genera associated with *A. taxiformis* (e.g., *Lachnospiraceae AC2004, Lachnospiraceae FCS020, Lachnospiraceae NK4A136*) may be responsible for maintaining fiber degradation in spite of the reduced abundance of cellulolytic bacteria and may have contributed to elevated butyrate levels (Terry et al., 2022). *Butyrivibrio* is the predominant butyrate producer in the rumen, and the observation that seaweed supplementation did not have any significant effects on the abundance of this bacterium also supports this hypothesis (Moon et al., 2008).

We also observed significantly higher abundances of *Bifidobacterium* in the *A. taxiformis* samples. *Bifidobacterium* is known for its probiotic properties and can adapt to a wide range of substrates (Pokusaeva et al., 2011). Its role in the rumen of adult cattle is less well defined, but elevated abundance is associated with improved feed efficiency (Abe et al., 1995; McLoughlin et al., 2020), and the increased abundance of *Bifidobacterium* may be an indicator of rumen microbial adaptation to *A. taxiformis*.

Terry et al. (2022) reported no impact of *P. mollis* or *M. japonica* on rumen fermentation profiles, so the observation that neither had a major impact on the microbiome was unsurprising in the context of this study. The anti-methanogenic effect of *Asparagopsis* species is attributed to their high bromoform content (Machado et al., 2018). Chemical analysis of the seaweeds examined in this work indicated that only *A. taxiformis* contained measurable amounts of bromoform, explaining the negligible impact that the other two algae had on methanogenesis. Although *P. mollis* or *M. japonica* did not reduce methane levels, some seaweeds without anti-methanogenic activity have proven to be potent modulators of rumen microbiomes both *in vivo* (Zhou et al., 2018) and in a RUSITEC system (Belanche et al., 2016; Zhou et al., 2018; Künzel et al., 2022) due to the wide range of bioactives present in algae. To our knowledge *M. japonica* has not previously been evaluated for its effect on microbial communities, while *P. mollis* has been found to modify the composition of the gut microbial community in mice (Mendez et al., 2020). While unlikely to have any potential as an anti-methanogenic feed additive, future studies may examine higher doses of *P. mollis* and *M. japonica* for potential prebiotic effects on the rumen and its microbes.

In summary, this study evaluated the impact of three red seaweeds on the bacterial and archaeal communities in a simulated rumen environment over a 13-day period. The red algae that did not have anti-methanogenic activity, *P. mollis* and *M. japonica,* had no measurable impact on the microbiome. In contrast, we found that the inhibition of methanogenesis following *A. taxiformis* supplementation reported in our companion study (Terry et al., 2022) was mirrored by substantial shifts in the microbiome. There was a near-total collapse of the rumen methanogen population following *A. taxiformis* supplementation, with few archaeal reads recovered during the stable phase. Similarly, the suppression in VFA synthesis by *A. taxiformis* was underpinned by inhibition of many taxa involved in acetate and propionate synthesis, and fiber degradation. *A. taxiformis* is receiving enormous attention for its role as an anti-methanogenic feed supplement in ruminants and has recently been commercialized. These data provide the first prolonged exploration of rumen microbial dynamics in response to *A. taxiformis* feeding. In-depth studies using shotgun metagenomics or metatranscriptomics may provide a more comprehensive understanding of ruminal microbial dynamics following seaweed feeding, particularly by allowing for the simultaneous evaluation of all prokaryotic and eukaryotic communities of the rumen.

Data availability statement

The data presented in the study are deposited in the Short Read Archive (SRA) repository, accession number PRJNA869720.

Ethics statement

The animal study was reviewed and approved by LeRDC animal care commitee protocol ACC1830.

Author contributions

TM, DA, KB, RG, and ST designed the experiment. PM, ST, and RG performed the laboratory work. EO'H and RG performed bioinformatics and data analysis. EO'H wrote the manuscript with revisions provided by all authors. RG and KB supervised the experiment and provided resources. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1104667/full#supplementary-material

References

Abbott, D. W., Aasen, I. M., Beauchemin, K. A., Grondahl, F., Gruninger, R., Hayes, M., et al. (2020). Seaweed and seaweed bioactives for mitigation of enteric methane: challenges and opportunities. *Animals* 10:E2432. doi: 10.3390/ani10122432

Abe, F., Ishibashi, N., and Shimamura, S. (1995). Effect of Administration of Bifidobacteria and Lactic Acid Bacteria to newborn calves and piglets. *J. Dairy Sci.* 78, 2838–2846. doi: 10.3168/jds.S0022-0302(95)76914-4

Aguilar-Marin, S. B., Betancur-Murillo, C. L., Isaza, G. A., Mesa, H., and Jovel, J. (2020). Lower methane emissions were associated with higher abundance of ruminal Prevotella in a cohort of Colombian buffalos. *BMC Microbiol.* 20, 364–313. doi: 10.1186/s12866-020-02037-6

Andrews, S. (2010). FastQC: a quality control tool for high throughput sequence data. Available at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (Accessed August 31, 2022).

Arndt, C., Hristov, A. N., Price, W. J., McClelland, S. C., Pelaez, A. M., Cueva, S. F., et al. (2022). Full adoption of the most effective strategies to mitigate methane emissions by ruminants can help meet the 1.5 °C target by 2030 but not 2050. *Proc. Natl. Acad. Sci. U. S. A.* 119:e2111294119. doi: 10.1073/pnas.2111294119

Aschenbach, J. R., Kristensen, N. B., Donkin, S. S., Hammon, H. M., and Penner, G. B. (2010). Gluconeogenesis in dairy cows: the secret of making sweet milk from sour dough. *IUBMB Life* 62, 869–877. doi: 10.1002/iub.400

Barcenilla, A., Pryde, S. E., Martin, J. C., Duncan, S. H., Stewart, C. S., Henderson, C., et al. (2000). Phylogenetic relationships of butyrate-producing bacteria from the human gut. *Appl. Environ. Microbiol.* 66, 1654–1661. doi: 10.1128/AEM.66.4.1654-1661.2000

Bauman, D. E., Brown, R. E., and Davis, C. L. (1970). Pathways of fatty acid synthesis and reducing equivalent generation in mammary gland of rat, sow, and cow. *Arch. Biochem. Biophys.* 140, 237–244. doi: 10.1016/0003-9861(70)90028-7

Belanche, A., Ramos-Morales, E., and Newbold, C. J. (2016). In vitro screening of natural feed additives from crustaceans, diatoms, seaweeds and plant extracts to manipulate rumen fermentation. *J. Sci. Food Agric.* 96, 3069–3078. doi: 10.1002/jsfa.7481

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

Bisanz, J. (2018) qiime2R: importing QIIME2 artifacts and associated data into R sessions. (unpublished). Available at: https://github.com/jbisanz/qiime2R (Accessed August 31, 2022).

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Canadian Council on Animal Care. (2009). *Guide to the Care and Use of Farm Animals in Research, Teaching and Testing*. CCAC: Ottawa

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* 6, 1621–1624. doi: 10.1038/ismej.2012.8

Clark, M. A., Domingo, N. G. G., Colgan, K., Thakrar, S. K., Tilman, D., Lynch, J., et al. (2020). Global food system emissions could preclude achieving the 1.5° and 2°C climate change targets. *Science* 370, 705–708. doi: 10.1126/science.aba7357

Dassa, B., Borovok, I., Ruimy-Israeli, V., Lamed, R., Flint, H. J., Duncan, S. H., et al. (2014). Rumen cellulosomics: divergent fiber-degrading strategies revealed by comparative genome-wide analysis of six ruminococcal strains. *PLoS ONE* 9:e99221. doi: 10.1371/journal.pone.0099221

Ewels, P., Magnusson, M., Lundin, S., and Käller, M. (2016). MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics* 32, 3047–3048. doi: 10.1093/bioinformatics/btw354

Fernandes, A. D., Reid, J. N., Macklaim, J. M., McMurrough, T. A., Edgell, D. R., and Gloor, G. B. (2014). Unifying the analysis of high-throughput sequencing datasets: characterizing RNA-seq, 16S rRNA gene sequencing and selective growth experiments by compositional data analysis. *Microbiome* 2:15. doi: 10.1186/2049-2618-2-15

Gerber, P. J., Hristov, A. N., Henderson, B., Makkar, H., Oh, J., Lee, C., et al. (2013). Technical options for the mitigation of direct methane and nitrous oxide emissions from livestock: a review. *Animal* 7, 220–234. doi: 10.1017/S1751731113000876

Herve, M. (2021). RVAideMemoire (0.9-80). Available at: https://cran.r-project.org/web/packages/RVAideMemoire/RVAideMemoire.pdf

Hobson, P. N., and Stewart, C. S. (1997) "*The Rumen Microbial Ecosystem*." Heidelberg: Springer Netherlands.

Hunt, D. E., Lin, Y., Church, M. J., Karl, D. M., Tringe, S. G., Izzo, L. K., et al. (2013). Relationship between abundance and specific activity of bacterioplankton in open ocean surface waters. *Appl. Environ. Microbiol.* 79, 177–184. doi: 10.1128/AEM.02155-12

IPCC (2018). "Global Warming of 1.5°C" in *An IPCC Special Report on the Impacts of Global Warming of 1.5°C Above Pre-Industrial Levels and Related Global Greenhouse Gas Emission Pathways, in the Context of Strengthening the Global Response to the Threat of Climate Change, Sustainable Development, and Efforts to Eradicate Poverty*. eds. V. Masson-Delmotte, P. Zhai, H.-O. Pörtner, D. Roberts, J. Skea and P. R. Shuklaet al. (Cambridge, UK and New York, NY: Cambridge University Press), 616.

Johnson, K. A., and Johnson, D. E. (1995). Methane emissions from cattle. *J. Anim. Sci.* 73, 2483–2492. doi: 10.2527/1995.7382483x

Karri, S., Vadela, M. B., and Gundi, V. A. K. B. (2021). "Chapter 15—fiber degradation strategies of bacteria in rumen ecosystem" in *Recent Developments in Applied Microbiology and Biochemistry*. ed. B. Viswanath (Cambridge, MA: Academic Press), 153–159.

Katoh, K., and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30, 772–780. doi: 10.1093/molbev/mst010

Kim, J. N., Mendez-Garcia, C., Geier, R. R., Iakiviak, M., Chang, J., Cann, I., et al. (2017). Metabolic networks for nitrogen utilization in *Prevotella ruminicola* 23. *Sci. Rep.* 7:7851. doi: 10.1038/s41598-017-08463-3

Kinley, R. D., Nys, R.De, Vucko, M. J., Machado, L., and Tomkins, N. W., (2016). The red macroalgae *Asparagopsis taxiformis* is a potent natural anti-methanogenic that reduces methane production during in vitro fermentation with rumen fluid. *Anim. Prod. Sci.* 56, 282–289. doi: 10.1071/AN15576

Knight, T., Ronimus, R. S., Dey, D., Tootill, C., Naylor, G., Evans, P., et al. (2011). Chloroform decreases rumen methanogenesis and methanogen populations without altering rumen function in cattle. *Anim. Feed Sci. Technol.* 166-167, 101–112. doi: 10.1016/j.anifeedsci.2011.04.059

Künzel, S., Yergaliyev, T., Wild, K. J., Philippi, H., Petursdottir, A. H., Gunnlaugsdottir, H., et al. (2022). Methane reduction potential of Brown seaweeds and their influence on nutrient degradation and microbiota composition in a rumen simulation technique. *Front. Microbiol.* 13:618. doi: 10.3389/fmicb.2022.889618

Ku-Vera, J. C., Jiménez-Ocampo, R., Valencia-Salazar, S. S., Montoya-Flores, M. D., Molina-Botero, I. C., Arango, J., et al. (2020). Role of secondary plant metabolites on enteric methane mitigation in ruminants. *Front Vet. Sci.* 7:584. doi: 10.3389/fvets.2020.00584

Lang, K., Schuldes, J., Klingl, A., Poehlein, A., Daniel, R., and Brune, A. (2015). New mode of energy metabolism in the seventh order of methanogens as revealed by comparative genome analysis of Candidatus *Methanoplasma termitum*. *Appl. Environ. Microbiol.* 81, 1338–1352. doi: 10.1128/AEM.03389-14

Lengowski, M. B., Zuber, K. H. R., Witzig, M., Möhring, J., Boguhn, J., and Rodehutscord, M. (2016). Changes in rumen microbial community composition during adaption to an in vitro system and the impact of different forages. *PLoS One* 11:e0150115. doi: 10.1371/journal.pone.0150115

Lin, H., and Peddada, S. D. (2020). Analysis of compositions of microbiomes with bias correction. *Nat. Commun.* 11:3514. doi: 10.1038/s41467-020-17041-7

Machado, L., Magnusson, M., Paul, N. A., de Nys, R., and Tomkins, N. (2014). Effects of marine and freshwater macroalgae on in vitro total gas and methane production. *PLoS One* 9:e85289. doi: 10.1371/journal.pone.0085289

Machado, L., Magnusson, M., Paul, N. A., Kinley, R., de Nys, R., and Tomkins, N. (2016a). Dose-response effects of *Asparagopsis taxiformis* and Oedogonium sp. on in vitro fermentation and methane production. *J. Appl. Phycol.* 28, 1443–1452. doi: 10.1007/s10811-015-0639-9

Machado, L., Magnusson, M., Paul, N. A., Kinley, R., de Nys, R., and Tomkins, N. (2016b). Identification of bioactives from the red seaweed *Asparagopsis taxiformis* that promote anti-methanogenic activity in vitro. *J. Appl. Phycol.* 28, 3117–3126. doi: 10.1007/s10811-016-0830-7

Machado, L., Tomkins, N., Magnusson, M., Midgley, D. J., de Nys, R., and Rosewarne, C. P. (2018). In vitro response of rumen microbiota to the anti-methanogenic red macroalga *Asparagopsis taxiformis*. *Microb. Ecol.* 75, 811–818. doi: 10.1007/s00248-017-1086-8

Mateos, I., Ranilla, M. J., Saro, C., and Carro, M. D. (2017). Shifts in microbial populations in Rusitec fermenters as affected by the type of diet and impact of the method for estimating microbial growth (15N v. microbial DNA). *Animal* 11, 1939–1948. doi: 10.1017/S1751731117000878

McLoughlin, S., Spillane, C., Claffey, N., Smith, P. E., O'Rourke, T., Diskin, M. G., et al. (2020). Rumen microbiome composition is altered in sheep divergent in feed efficiency. *Front. Microbiol.* 11:981. doi: 10.3389/fmicb.2020.01981

McMurdie, P. J., and Holmes, S. (2013). Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8:e61217. doi: 10.1371/journal.pone.0061217

Meehan, C. J., and Beiko, R. G. (2014). A phylogenomic view of ecological specialization in the Lachnospiraceae, a family of digestive tract-associated bacteria. *Genome Biol. Evol.* 6, 703–713. doi: 10.1093/gbe/evu050

Mendez, R. L., Miranda, C., Armour, C. R., Sharpton, T. J., Stevens, J. F., and Kwon, J. Y. (2020). Supplementation with sea vegetables *Palmaria mollis* and Undaria pinnatifida exerts metabolic benefits in diet-induced obesity in mice. *Curr. Dev. Nutr.* 4:4005006. doi: 10.1093/cdn/nzaa072

Moon, C. D., Pacheco, D. M., Kelly, W. J., Leahy, S. C., Li, D., Kopečný, J., et al. (2008). Reclassification of clostridium proteoclasticum as *Butyrivibrio proteoclasticus* comb. Nov., a butyrate-producing ruminal bacterium. *Int. J. Syst. Evol. Microbiol.* 58, 2041–2045. doi: 10.1099/ijs.0.65845-0

Nearing, J. T., Douglas, G. M., Hayes, M. G., MacDonald, J., Desai, D. K., Allward, N., et al. (2022). Microbiome differential abundance methods produce different results across 38 datasets. *Nat. Commun.* 13:342. doi: 10.1038/s41467-022-28034-z

Neu, A. T., Allen, E. E., and Roy, K. (2021). Defining and quantifying the core microbiome: challenges and prospects. *Proc. Natl. Acad. Sci. U. S. A.* 118:e2104429118. doi: 10.1073/pnas.2104429118

Newbold, C. J., Lassalas, B., and Jouany, J. P. (1995). The importance of methanogens associated with ciliate protozoa in ruminal methane production in vitro. *Lett. Appl. Microbiol.* 21, 230–234. doi: 10.1111/j.1472-765x.1995.tb01048.x

Oksanen, J., Blancet, G. F., Friendly, M., Kindt, R., Legandre, P., McGlinn, D., et al. (2022). Vegan-package: community ecology package: ordination, diversity and. In vegan: community ecology package (2.6-4). Available at: https://rdrr.io/cran/vegan/man/vegan-package.html (Accessed August 31, 2022).

Patra, A., Park, T., Kim, M., and Yu, Z. (2017). Rumen methanogens and mitigation of methane emission by anti-methanogenic compounds and substances. *J. Anim. Sci. Biotechnol.* 8:13. doi: 10.1186/s40104-017-0145-9

Paul, N. A., Nys, R.De, and Steinberg, P. D. (2006). Chemical defence against bacteria in the red alga *Asparagopsis armata*: linking structure with function. *Mar. Ecol. Prog. Ser.* 306, 87–101. doi: 10.3354/meps306087

Pokusaeva, K., Fitzgerald, G. F., and van Sinderen, D. (2011). Carbohydrate metabolism in Bifidobacteria. *Genes Nutr.* 6, 285–306. doi: 10.1007/s12263-010-0206-6

Poulsen, M., Schwab, C., Borg Jensen, B., Engberg, R. M., Spang, A., Canibe, N., et al. (2013). Methylotrophic methanogenic Thermoplasmata implicated in reduced methane emissions from bovine rumen. *Nat. Commun.* 4:1428. doi: 10.1038/ncomms2432

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Ransom-Jones, E., Jones, D. L., McCarthy, A. J., and McDonald, J. E. (2012). The Fibrobacteres: an important phylum of cellulose-degrading bacteria. *Microb. Ecol.* 63, 267–281. doi: 10.1007/s00248-011-9998-1

Ren, Z., You, W., Wu, S., Poetsch, A., and Xu, C. (2019). Secretomic analyses of *Ruminiclostridium papyrosolvens* reveal its enzymatic basis for lignocellulose degradation. *Biotechnol. Biofuels* 12:183. doi: 10.1186/s13068-019-1522-8

Roque, B. M., Brooke, C. G., Ladau, J., Polley, T., Marsh, L. J., Najafi, N., et al. (2019). Effect of the macroalgae *Asparagopsis taxiformis* on methane production and rumen microbiome assemblage. *Anim. Microbiome* 1:3. doi: 10.1186/s42523-019-0004-4

Roque, B. M., Venegas, M., Kinley, R. D., de Nys, R., Duarte, T. L., Yang, X., et al. (2021). Red seaweed (*Asparagopsis taxiformis*) supplementation reduces enteric methane by over 80 percent in beef steers. *PLoS One* 16:e0247820. doi: 10.1371/journal.pone.0247820

Sasada, R., Weinstein, M., Prem, A., Jin, M., and Bhasin, J. (2020). FIGARO: an efficient and objective tool for optimizing microbiome rRNA gene trimming parameters. *J. Biomol. Tech.* 31:S2.

Sawanon, S., and Kobayashi, Y. (2006). Synergistic fibrolysis in the rumen by cellulolytic *Ruminococcus flavefaciens* and non-cellulolytic *Selenomonas ruminantium*: evidence in defined cultures. *Anim. Sci. J.* 77, 208–214. doi: 10.1111/j.1740-0929.2006.00339.x

Seedorf, H., Kittelmann, S., Henderson, G., and Janssen, P. H. (2014). RIM-DB: a taxonomic framework for community structure analysis of methanogenic archaea from the rumen and other intestinal environments. *PeerJ* 2:e494. doi: 10.7717/peerj.494

Terry, S., Krüger, A., Lima, P. D. M. T., Gruninger, R., Abbott, W., and Beauchemin, K. (2022). Evaluation of rumen fermentation and microbial adaptation to three red seaweeds using the rumen simulation technique. Preprints 2022:2022080186. doi: 10.20944/preprints202208.0186.v1,

Ungerfeld, E. M., Rust, S. R., Boone, D. R., and Liu, Y. (2004). Effects of several inhibitors on pure cultures of ruminal methanogens. *J. Appl. Microbiol.* 97, 520–526. doi: 10.1111/j.1365-2672.2004.02330.x

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

van Gylswyk, N. O., Hippe, H., and Rainey, F. A. (1997). *Schwartzia succinivorans* gen. Nov., sp. Nov., another ruminal bacterium utilizing succinate as the sole energy source. *Int. J. Syst. Bacteriol.* 47, 155–159. doi: 10.1099/00207713-47-1-155

Wickham, H. (2009). *ggplot2: Elegant Graphics for Data Analysis*. Berlin: Springer-Verlag

Zhou, M., Hünerberg, M., Chen, Y., Reuter, T., McAllister, T. A., Evans, F., et al. (2018). Air-dried Brown seaweed, Ascophyllum nodosum, alters the rumen microbiome in a manner that changes rumen fermentation profiles and lowers the prevalence of foodborne pathogens. *MSphere* 3, e00017–e00018. doi: 10.1128/mSphere.00017-18

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Isolation and pan-genome analysis of *Enterobacter hormaechei* Z129, a ureolytic bacterium, from the rumen of dairy cow

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Introduction: Urea is an important non-protein nitrogen source for ruminants. In the rumen, ureolytic bacteria play critical roles in urea-nitrogen metabolism, however, a few ureolytic strains have been isolated and genomically sequenced. The purpose of this study was to isolate a novel ureolytic bacterial strain from cattle rumen and characterize its genome and function.

Methods: The ureolytic bacterium was isolated using an anaerobic medium with urea and phenol red as a screening indicator from the rumen fluid of dairy cattle. The genome of isolates was sequenced, assembled, annotated, and comparatively analyzed. The pan-genome analysis was performed using IPGA and the biochemical activity was also analyzed by test kits.

Results: A gram-positive ureolytic strain was isolated. Its genome had a length of 4.52 Mbp and predicted genes of 4223. The 16S rRNA gene and genome GTDB-Tk taxonomic annotation showed that it was a novel strain of *Enterobacter hormaechei*, and it was named *E. hormaechei* Z129. The pan-genome analysis showed that Z129 had the highest identity to *E. hormaechei* ATCC 49162 with a genome average nucleotide identity of 98.69% and possessed 238 unique genes. Strain Z129 was the first *E. hormaechei* strain isolated from the rumen as we know. The functional annotation of the Z129 genome showed genes related to urea metabolism, including urea transport (*urt*A-*urt*E), nickel ion transport (*ure*J, *ton*B, *nix*A, *exb*B, *exb*D, and *rcn*A), urease activation (*ure*A-ureG) and ammonia assimilation (*gdh*A, *gln*A, *gln*B, *gln*E, *gln*L, *gls*A, *glt*B, and *glt*D) were present. Genes involved in carbohydrate metabolism were also present, including starch hydrolysis (*amy*E), cellulose hydrolysis (*cel*B and *bgl*X), xylose transport (*xyl*F-*xyl*H) and glycolysis (*pgi*, *pgk*, *fba*A, *eno*, *pfk*A, *gap*, *pyk*, *gpm*L). Biochemical activity analysis showed that Z129 was positive for alkaline phosphatase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, β-glucosidase, and pyrrolidone arylaminase, and had the ability to use D-ribose, L-arabinose, and D-lactose. Urea-nitrogen hydrolysis rate of Z129 reached 55.37% at 48h of incubation.

Discussion: Therefore, the isolated novel ureolytic strain *E. hormaechei* Z129 had diverse nitrogen and carbon metabolisms, and is a preferred model to study the urea hydrolysis mechanism in the rumen.

KEYWORDS

rumen, ureolytic bacteria, isolation, urea metabolism, pan-genome analysis

1. Introduction

The protein level of feed is crucial in ruminant animal growth and production, and the lower economic cost of urea promotes its partial replacement of plant protein in feed (Cherdthong and Wanapat, 2010). Urea recycling is especially important for ruminants because it provides recycled endogenic nitrogen, which is combined with energy for the synthesis of microbial crude protein in the rumen (Reynolds and Kristensen, 2008; Calsamiglia et al., 2010). The microbial crude protein is easily digested, has balanced amino acids (AAs; Schwab and Broderick, 2017), and is also the major component of the metabolizable protein to meet the AA requirement of dairy cows (Owens et al., 2014). The utilization of urea relies on the activity of ureolytic bacteria, but more than 55% of ureolytic bacteria in the rumen are not matched to any known bacterial family (Jin et al., 2016), indicating that many ureolytic bacteria remain to be discovered and isolated. Up to now, 24 strains of rumen ureolytic bacteria have been isolated from various families including Lactobacillaceae, Oscillospiraceae, Staphylococcaceae, Enterobacteriaceae, Bacteroidaceae, and Succinivibrionaceae, Selenomonadaceae (Gibbons and Doetsch, 1958; Slyter et al., 1968; John et al., 1974; Vanwyk and Steyn, 1975; Cook, 1976; Wozny et al., 1977; Lauková and Koniarová, 1995; Cook et al., 2007; Kim et al., 2014; Hailemariam et al., 2020). Among them, only three strains were isolated after 2000 (Cook et al., 2007; Kim et al., 2014; Hailemariam et al., 2020).

Some factors make bacteria isolation and culture difficult, such as symbiosis (St. John et al., 2019), growth factors from other bacteria (D'Onofrio et al., 2010), slow growth (Pulschen et al., 2017), dormancy (Oliver, 2010), competition among strains (Lewis et al., 2020), and media eutrophication (Zhang et al., 2020). There are 1,000 of bacterial species in the rumen (Makkar and Mcsweeney, 2005), but less than 30 ureolytic bacterial species have been isolated (Gibbons and Doetsch, 1958; Slyter et al., 1968; John et al., 1974; Vanwyk and Steyn, 1975; Cook, 1976; Wozny et al., 1977; Lauková and Koniarová, 1995; Cook et al., 2007; Kim et al., 2014; Hailemariam et al., 2020). With the development of sequencing technology, progressively more bacteria have been discovered through culture-free technology and their functional genes have been studied, but the isolation of bacteria is still crucial. Without successful isolation and cultivation of bacteria, the metabolic pathways, physiological characteristics, and ecological functions based on omics data cannot be studied and verified (Gutleben et al., 2018). Therefore, it is crucial to isolate more novel ureolytic bacteria from the rumen to better understand urea metabolism.

In this study, we isolated a new strain of *E. hormaechei* from medium plus urea and named it Z129. We compared Z129 with other strains in the same species and found its unique genes related to nitrogen and carbohydrate metabolisms. We focused on the genes involved in urea metabolism and carbohydrates to explore its ability for urea and carbon metabolisms.

2. Materials and methods

2.1. Culture media

The liquid media contained 20 ml of clarified rumen fluid, 0.05 g of glucose, 0.05 g yeast extract, 2 g of urea, 0.05 g of cellobiose, 15 mL of solution 4 (0.3% K_2HPO_4), 15 mL of solution 5 (0.3% KH_2PO_4, 0.6% NaC1, 0.06% $MgSO_2{\cdot}7H_2O$, and 0.06% $CaCl_2$), 0.1 mL of Pfennig trace elements (0.03% H_3BO_3, 0.01% $ZnSO_4{\cdot}7H_2O$, 0.003% $MnCl_2{\cdot}4H_2O$, 0.002% $CoCl_2{\cdot}6H_2O$, 0.003% $Na_2MoO_4{\cdot}2H_2O$, 0.001% Na_2SeO_3, 0.002% $NiCl_2$, 0.001% $CuCl_2{\cdot}2H_2O$, and 0.015% $FeCl_2{\cdot}4H_2O$), 5 mL of hemin (0.05%), 0.31 mL of VFA mix (17% acetic, 6% propionic, 4% n-butyric, 1% n-valeric, 1% isovaleric, 1% isobutyric, and 1% 2-methyl butyric acids), 0.6 g of $NaHCO_3$, 0.1 mL of resazurin (0.1%), 0.05 g of L-cysteine HCl, and 0.0012 g of phenol red per 100 mL. The solid media were the corresponding basal media plus 2 g of agar. After boiling, high-purity nitrogen was blown into the solution for almost 2 h to exhaust the oxygen. After adjusting to pH 6.8, the solution was transferred into an anaerobic workstation (DWS, West Yorkshire, United Kingdom). For liquid media, 10 mL of solution was dispensed into Hungate tubes, and autoclaved at 100 kPa and 121°C lasting for 15 min. After cooling, all media were stored at 4°C. For solid media, agar was added to liquid media and autoclaved at 100 kPa and 121°C lasting for 15 min. After cooling to 40°C–50°C, the solution was poured into 90-mm culture dishes in the anaerobic workstation. Noticeably, after autoclaving, urea and phenol red were added to the media through a 2 μm sterile filter membrane.

2.2. Isolation of ureolytic strain

The rumen liquid samples were collected from Holstein dairy cattle (No. IAS2019-14). The inoculum was prepared and stored as described by Hailemariam et al. (2020). The diluent solution contained 15 mL of solution 4, 15 mL of solution 5, 0.6 g of $NaHCO_3$, 0.1 mL of resazurin (0.1%), and 0.05 g of L-cysteine HCl per 100 mL, and was prepared under anaerobic conditions in the anaerobic workstation. The inoculum was diluted 10 to 10^5 times with the anaerobic diluent. Then 200 μL of diluted inoculum in each dilution was spread on the solid media. After incubating for 72 h at 39°C, colonies with pink color were selected and streaked in new solid media. The single pink colonies were inoculated into liquid media. The strain was mixed with equal anaerobic diluent solution containing 30% glycerin, and stored at −80°C.

2.3. 16S rRNA gene sequencing

The DNA of isolated strains was extracted by cetyltrimethylammonium bromide (CTAB) plus bead beating method as described by Minas et al. (2011). The primers were 27F (5′-AGAGTTTGATCMTGGCTCA-3′) and 1492R (5′-TACGGYTTACCTTGTTACGACTT-3′). The PCR mixture included 25 μL of PCR Master Mix (Takara, Japan), 0.1 μL of 27F, 0.1 μL of 1492R, 5 μL of microbial DNA, and 19.8 μL of ddH_2O. The process of amplification was same as the described by Hailemariam et al. (2020). The PCR products were sequenced by Sanger sequencing (Applied Biosystems 3730XL, Foster City, CA, United States). The chromas were used to determine whether the strain was pure or contaminated according to single or double peak on the position of each base. Only pure strains were selected for genome sequencing and biochemical analysis. The 16S rRNA sequences were blasted with the rRNA/ITS database of NCBI for taxonomic annotation.

2.4. Physiological characterization

The test for Gram-staining was performed using a kit, following the manufacturer's instructions (RealTime Biotechnology, Beijing, China). The Gram-staining result was observed using a bright-field microscope (Axiocam ERc 5 s, ZEISS, Oberkochen, Germany).

The enzyme activity and the ability for sugar fermentation were tested by API ZYM (Merieux, Lyon, France) and API 20 STREP (Merieux, Lyon, France). The inoculum samples were taken at 0 and 48 h of incubation to measure the content of urea nitrogen using a BUN test kit with the diacetyloxime colorimetric method (Jiancheng, Nanjing, China).

2.5. Whole genome sequencing and analysis

The DNA of Z129 was extracted as the above and quantified using Qubit 2.0 (Invitrogen, Carlsbad, CA, United States). The sequencing library was constructed using a MGIEasy Universal DNA library kit (BGI, Shenzhen, China). The libraries were paired-end (2 × 150 bp) sequenced by DNBseq-T7 (BGI). The low quality reads including reads with length lower than 50 bp and reads with average mass lower than 20 were removed by TrimGalore (Martin, 2011). The qualified data were assembled to produce genomes using Megahit (Li et al., 2015), then the quality and contamination were checked using checkM (Parks et al., 2015). The taxonomy was annotated by GTDB-tk (Chaumeil et al., 2020) based on the GTDB RS202 database. The genes were predicted using Prokka (Seemann, 2014). The proteins were aligned to the eggNOG 5.0 database using diamond (Buchfink et al., 2014). The genes related to urea nitrogen and carbohydrate metabolisms were identified. CAZymes annotation was performed on the dbCAN meta server (Yin et al., 2012).

2.6. Pan-genome analysis

Based on the taxonomic result of whole genome sequencing, the genome sequences of 12 strains (*E. hormaechei* YT3, *E. hormaechei* YT2, *E. hormaechei* subsp. *hoffmannii* UCICRE 9, *E. hormaechei* subsp. *hoffmannii* UCICRE 3, *E. hormaechei* subsp. *hoffmannii* UCI 50, *E. hormaechei* subsp. *hoffmannii* MGH 13, *E. hormaechei* subsp. *hoffmannii* ECNIH3, *E. hormaechei* subsp. *hoffmannii* ECR091, *E. hormaechei* subsp. *steigerwaltii*, *E. hormaechei* subsp. *xiangfangensis*, *E. hormaechei* ATCC 49162, *E. hormaechei* subsp. *oharae*) on NCBII database were downloaded. Pan-genome analysis was performed using IPGA (Liu et al., 2022). The AA sequences of UreC of these strains were also downloaded from the NCBI database and aligned using the CLUSTAL W package in BioEdit (Alzohairy, 2011).

3. Results

3.1. Isolation and genome of ureolytic strain

According to the color indicator and 16S rRNA gene sequence chromas, one isolate Z129 with ureolytic activity was finally obtained, and was identified as a gram-positive bacterium (Figure 1). The genome of the isolate had a size of 4.52 Mbp, with completeness of 99.97% and contamination of 0.33% assessed by checkM (Figure 2A). There were 177 contigs with maximum length of 426 kb, minimum of 201 bp, and average of 25.5 kb. The number of predicted genes was 4,223, of which 4,074 were annotated using eggNOG database (Figure 2A). The GTDB-tk taxonomic analysis showed that Z129 belonged to *E. hormaechei*. The 16S rRNA gene showed that Z129 was most closely related to *E. hormaechei* subsp. *xiangfangensis* with identity of 99.36%.

3.2. Genes involved in urea and carbohydrate metabolism

A total of 30 GH families involved in carbohydrate metabolism were identified in the genome of Z129. The predominant members were GH13, GH19, GH28, and GH154 and account for 40% of the total (Figure 2B). The genes involved in urea hydrolysis including *ure*A (302 bp), *ure*B (314 bp), *ure*C (1,703 bp), *ure*D (827 bp), *ure*E (464 bp), *ure*F (674 bp), and *ure*G (617 bp) and the gene *ure*J (539 bp) involved in nickel ion transport were clustered together (Figure 2C). Additionally, genes involved in urea transport were also clustered together: *urt*A-*urt*E (Figure 2D). Urea metabolism related genes in the Z129 genome were grouped as genes involved in urea transport (*urt*A-*ure*E), nickel ions transport (*ure*J, *ton*B, *nix*A, *exb*B, *exb*D, and *rcn*A), urea hydrolysis (*ure*A, −*ure*G, and *ure*J), ammonia assimilation (*gdh*A, *gln*A, *gln*B, *gln*E, *gln*L, *gls*A, *glt*B, and *glt*D; Figure 2E). Carbohydrate metabolism related genes were grouped as genes related to starch hydrolysis (*amy*E), cellulose hydrolysis (*cel*B and *bgl*X), glucose transport (*crr*, *prs*G, and *mal*X), xylose transport (*xyl*F-*xyl*H), glycolysis (*pgi*, *pgk*, *fba*A, *eno*, *pfk*A, *gap*, *pyk*, and *gpm*L), and short-chain fatty acid biosynthesis (*ace*E, *ace*F, *frd*A, *sfc*A, *suc*C, *scp*B, *acka*, and *pta*; Figure 2E). As for the biosynthesis of amino acids such as asparagine, cysteine, methionine, lysine, alanine, and threonine, genes including *asn*B, *san*A, *cysk1*, *yxj*G, *dapf*, *isc*S, and *thr*C were also identified in the Z129 genome.

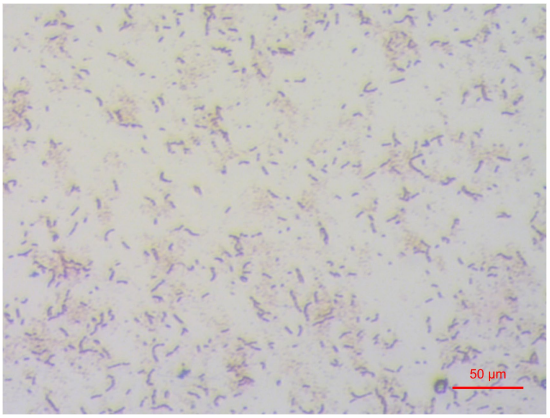

FIGURE 1
Gram-staining of strain Z129 observed by a bright-field microscope.

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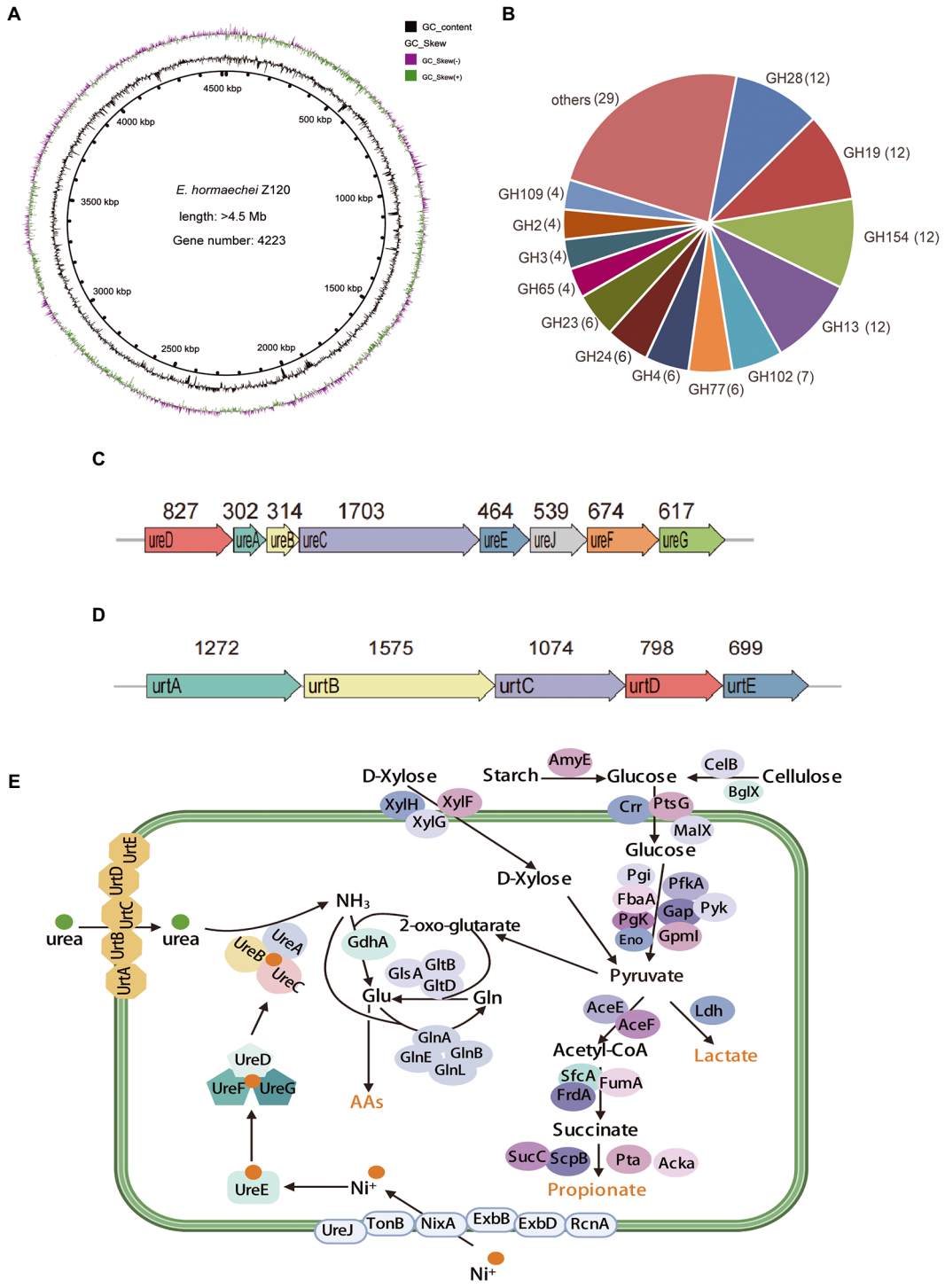

FIGURE 2
Functional genes related to urea and carbohydrate metabolism of strain Z129. **(A)** Circle diagram showing the genome. From the outside to the inside are GC-skew and GC-content. **(B)** The number of annotated genes in different GH families. The GH families with less than four genes were combined into the others. **(C)** Gene clusters for urea hydrolysis. The number indicates the length of the gene, the unit is bp. **(D)** Gene clusters for urea transportation. The number indicates the length of the gene, the unit is bp. **(E)** Genes involved in urea transport, nickel ions transport, urea hydrolysis, ammonia assimilation, starch metabolism, cellulose metabolism, xylose metabolism, and pyruvate metabolism.

3.3. Pan-genome analysis of *Enterobacter hormaechei*

Twelve genomes of known *E. hormaechei* strains and Z129 were used for pan-genome analysis. The number of pan gene clusters increased to 8,814 and core gene clusters decreased to 3,388, but the curve gradually flattened out (Figure 3A). The genomic phylogenetic tree analysis of *E. hormaechei* strains also showed that strain Z129 was most closely related to *E. hormaechei* ATCC 49162 (Figure 3B). The average nucleotide identity (ANI)values between Z129 and other

strains are as following: *E. hormaechei* YT3 (95.00%), *E. hormaechei* YT2 (94.83%), *E. hormaechei* subsp. *hoffmannii* UCICRE 9 (94.34%), *E. hormaechei* subsp. *hoffmannii* UCICRE 3 (94.24%), *E. hormaechei* subsp. *hoffmannii* UCI 50 (94.27%), *E. hormaechei* subsp. *hoffmannii* MGH 13 (94.25%), *E. hormaechei* subsp. *hoffmannii* ECNIH3 (94.28%), *E. hormaechei* subsp. *hoffmannii* ECR091 (94.25%), *E. hormaechei* subsp. *steigerwaltii* (94.80%), *E. hormaechei* subsp. *xiangfangensis* (94.75%), *E. hormaechei* ATCC 49162 (98.69%), *E. hormaechei* subsp. *oharae* (95.06%; Figure 3C). The ANI value between Z129 and *E. hormaechei* ATCC 49162 was the highest. The pan-genome analysis among 13 genomes showed that the amount of unique genes in each genome was in the range of 8–440 (Figure 4A). Strain Z129 provided 238 new genes representing 2.7% of genes of the species, and 5.6% of genes of its genome (Figure 4A). Compared with the other 12 strains, Z129 shared 110 genes with the most closely related strain *E. hormaechei* ATCC 49162 and shared no genes with *E. hormaechei* YT3, *E. hormaechei* YT2, *E. hormaechei* subsp. *hoffmannii* ECR091, UCICRE 3, and ECNIH3 (Figure 4A). The COG annotation showed that the core gene clusters included 1,438 genes for metabolism, 767 for cellular processes and signaling, 573 for information storage and processing and 610 for not annotated and poorly characterized (Figure 4B). The shared genes between Z129 and *E. hormaechei* ATCC 49162 included 19 genes for metabolism, 6 for cellular processes and signaling, 31 for information storage and

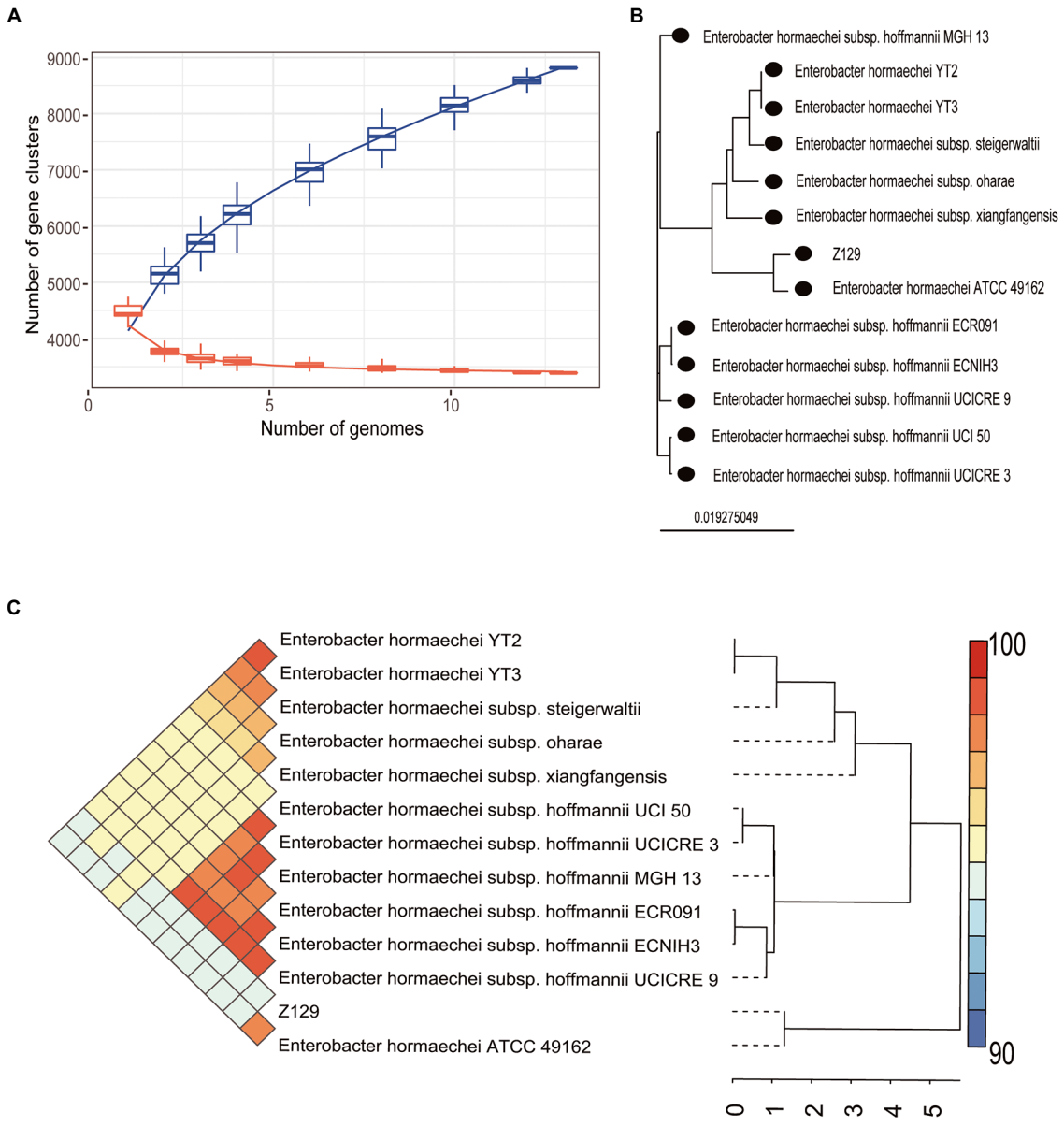

FIGURE 3
Pan-genome analysis of Z129 and 12 other *Enterobacter hormaechei* strains. **(A)** The number of pan gene clusters and core gene clusters along with the addition of new strains of *E. hormaechei*. **(B)** Phylogenetic tree highlighting the relationship of Z129 with other *E. hormaechei* strains based on the genome sequence. **(C)** Pairwise comparisons of average nucleotide identity (ANI). The color indicates the value of ANI, the value range is 90–100 with color turning from blue to red.

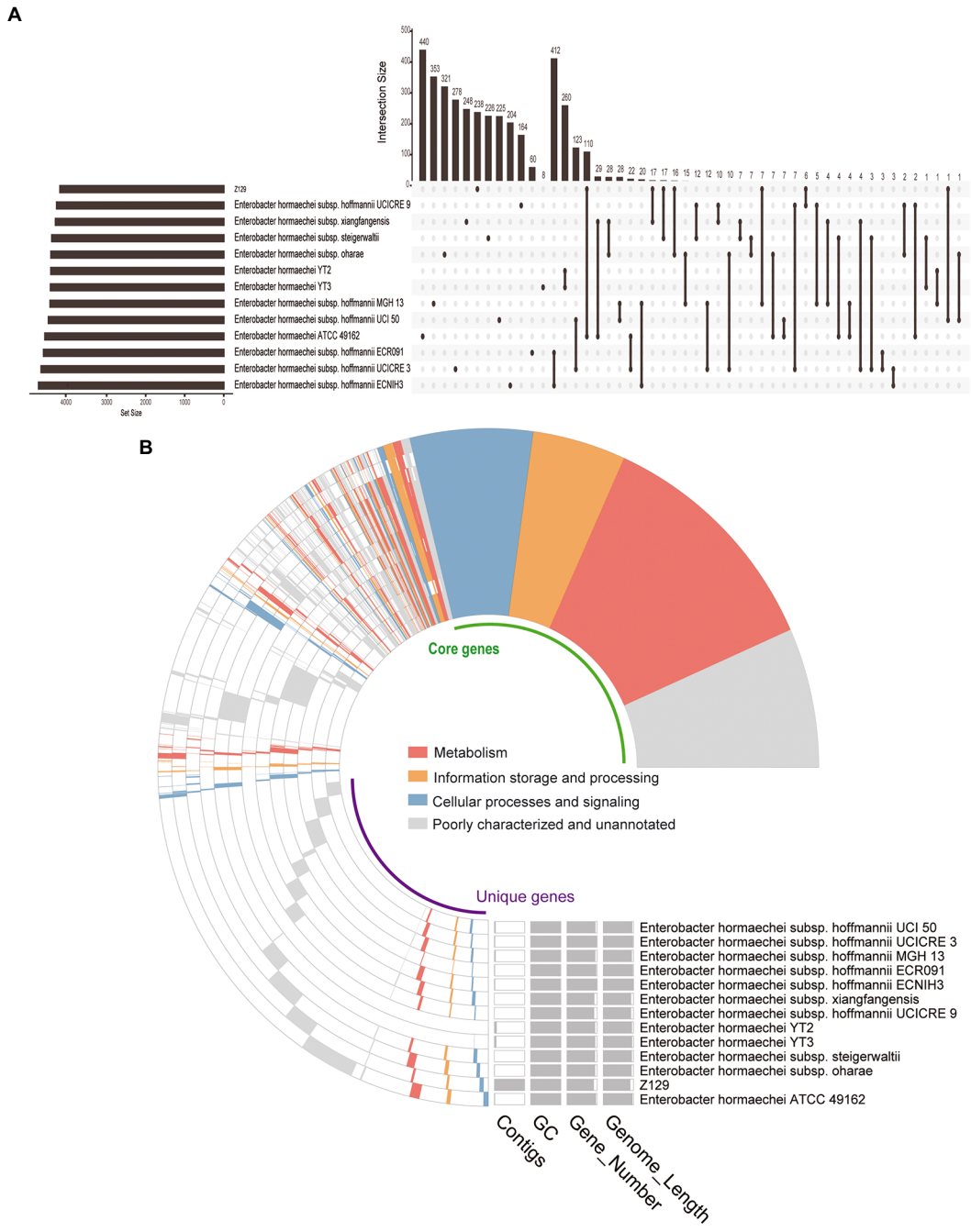

FIGURE 4
Distribution of genes among *Enterobacter hormaechei* strains. **(A)** Upset figure showing the unique genes of each strain and genes shared between any two strains. **(B)** COG annotation showing the core genes, unique genes, number of contigs, GC content, and genome length.

processing and 54 for not annotated and poorly characterized (Figure 4B). Some of unique genes, such as *appA*, *nrfa*, and *wecE*, of Z129 were related to phosphorus and nitrogen metabolisms (Supplementary Table 1). Additionally, urease protein UreC existed in all *E. hormaechei* strains and the phylogenetic tree of AA sequences showed that Z129 was most closely related to *E. hormaechei* ATCC 49162 followed by *E. hormaechei* subsp. *xiangfangensis* (Figure 5A). Two sites in the UreC AA sequence differed between Z129 and *E. hormaechei* (Figure 5B).

3.4. Functional activity of Z129

The enzyme activity test using API ZYM and API 20 STREP showed that Z129 was positive for alkaline phosphatase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, β-glucosidase, and pyrrolidone arylaminase, but negative for esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase,

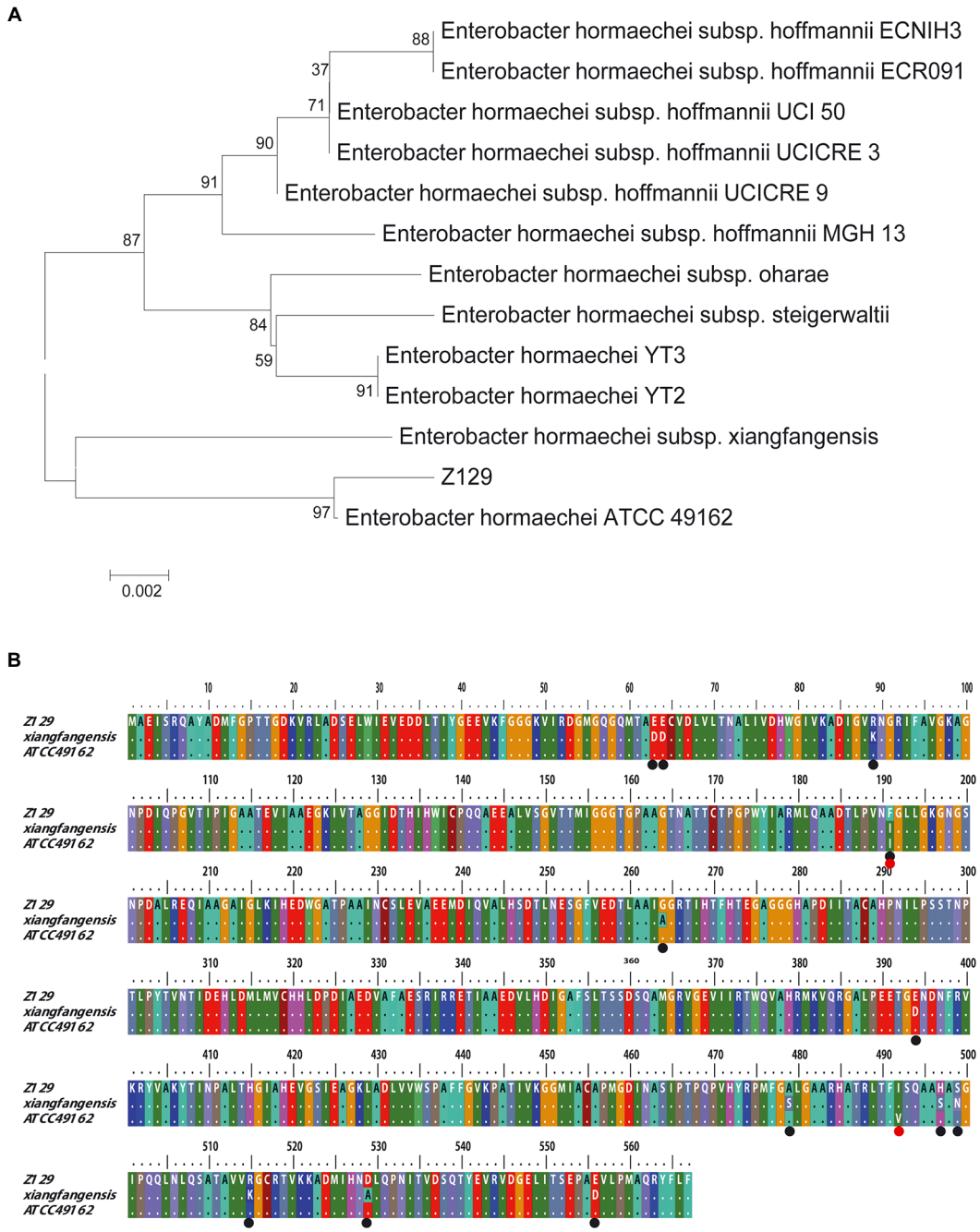

FIGURE 5
Comparative analysis of Z129 and 12 other *Enterobacter hormaechei* strains based on UreC AA sequence. **(A)** Phylogenetic tree based on UreC AA sequence. **(B)** Alignment of UreC AA sequences among Z129, *E. hormaechei* subsp. *xiangfangensis* and *E. hormaechei* ATCC 49162. The black circles indicate the different sites between Z129 and *E. hormaechei* subsp. *xiangfangensis*, and the red circles indicate the different sites between Z129 and *E. hormaechei* ATCC 49162.

β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase (Figures 6A, B). The sugar utilization assay of API 20 STREP revealed that Z129 took advantage of D-ribose, L-arabinose, D-lactose, and starch, but not D-mannitol, D-sorbitol, D-trehalose, inulin, D-raffinose, and glycogen. The urea nitrogen during Z129 incubation was decreased by 55.37% at 48 h indicating high ureolytic activity (Figure 6C).

4. Discussion

We isolated a new strain Z129 belonging to *E. hormaechei* that showed an ANI of 98.69% with *E. hormaechei* ATCC 49162. Compared with the strains of this species, Z129 possessed unique genes including *app*A, *nrf*A, and *wec*E. The expression of *app*A significantly improves phytase activity (Chiera et al., 2004), which can increase the efficiency of phytate phosphorus (Beaulieu et al., 2007).

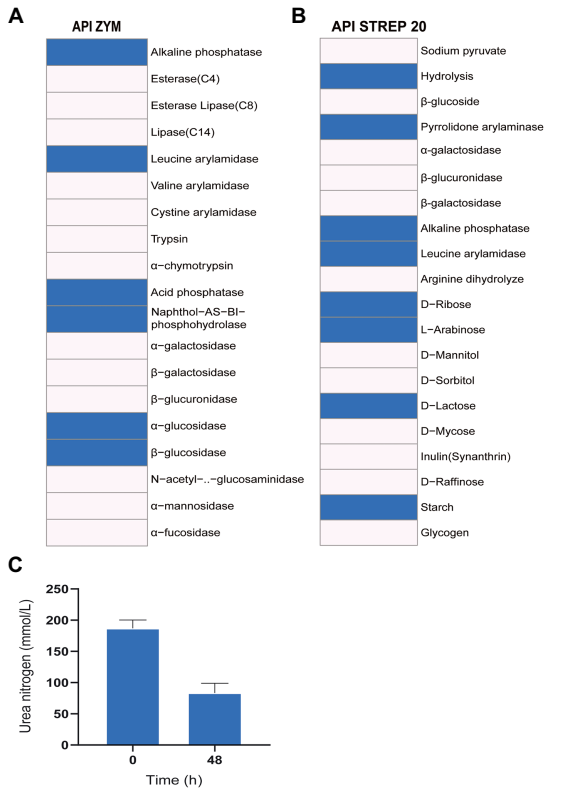

FIGURE 6
The enzyme activity and utilization of carbohydrates and urea. **(A)** Results of API ZYM, indicating 19 kinds of enzyme activities. The blue presents positive, the blank presents negative. **(B)** The results of API STREP 20. The result indicates enzyme activities and utilization of carbohydrates. The blue presents positive, the blank presents negative. **(C)** Change of urea nitrogen with the increased culturing time.

The addition of phytases improves the final body weight and feed conversion ratio of weaned piglets fed with a diet lacking in calcium and phosphorus (Wiśniewska et al., 2020). Gene *nrf*A can be involved in catalysis of the second step of dissimilatory nitrate reduction to ammonium to reduce nitrogen loss and protects the environment (Tiedje, 1988). Expression of *nrf*A is induced in a low-nitrate environment (Wang and Gunsalus, 2000). Gene *wec*E has been identified in *Escherichia coli* and encodes a sugar aminotransferase regulating synthesis of TDP-4-amino-4,6-dideoxy-D-galactose (Hwang et al., 2004). Therefore, the isolation of Z129 enriches taxonomic information, and the discovery of these unique genes expands the known genetic diversity within *E. hormaechei*.

Strains of *E. hormaechei* can produce urease (Roslan et al., 2020) and all *E. hormaechei* strains possesses genes encoding urease. Our results also showed that Z129 could utilize urea nitrogen. However, there is limited research concerning the genes involved in the whole urea metabolism within *E. hormaechei*. In this study, we successfully explored these genes. Urease is crucial in urea metabolism. Although urease gene clusters differ for different bacteria, they usually include structural genes (e.g., *ure*A-*ure*C) and accessory genes (e.g., *ure*D-*ure*I). Urease is a metalloenzyme with two nickel ions in its active center (Alfano and Cavazza, 2020). In fact, urease activation is a metal assembly process between nickel ions and urease structural proteins. Firstly, UreE transfers nickel ions to UreG (Yang et al., 2015), then UreG passes nickel ions to UreF (Zambelli et al., 2014), and nickel ions finally enter urease through UreD channels to complete the assembly of metal centers (Carter and Hausinger, 2010). Therefore, these helper genes and nickel ions are essential for urea metabolism. The entry of nickel ions is aided by nickel ion transporters encoded by *nik*A-*nik*E, *fec*ADE, *frpB4*, *ton*B, *Exb*BD, *nix*A, *ceu*E, *hpn*, and *ure*J (Dmitry et al., 2006; Hilde et al., 2013; Haley and Gaddy, 2015; Vinella et al., 2015). In this study, genes related to urease (*ure*A-*ure*G) and related to nickel ion transport (*ure*J, *ton*B, *nix*A, *exb*B, *exb*D, and r*cn*A) were all detected. The urease of rumen bacteria is located in the cytoplasm (McLean et al., 1985), meaning that urea must enter the bacteria before it is hydrolyzed by urease. Rumen bacteria usually possess three types of urea transporters: one is pH-independent, such as Yut (Sebbane et al., 2002), the other is ATP-activated encoded by *urt*A-*urt*E (Hailemariam et al., 2021). In strain Z129, we identified genes including *urt*A-*urt*E related to urea transport. The urea is hydrolyzed to ammonia which is not used directly by ruminants. The ammonia should be assimilated to produce other forms of nitrogen for the growth and production of ruminants. The nitrogen assimilation in bacteria is controlled by *gdh*A, *gln*A, *gln*N, *gln*B, *gln*E, *gln*G, *gln*L, *gln*K, *gln*R, and *gls*A (Herrero et al., 2019; Liu et al., 2020), which are also found in Z129 genome. All these results revealed that strain Z129 contained all the genes involved in urea metabolism including urease activation, urea transport, and ammonia assimilation, which meant that Z129 possessed the ability for urea metabolism. The rumen fermentation *in vitro* also showed that Z129 had ureolytic activity.

In addition to the urea metabolism, we also focused on the carbohydrate utilization of Z129. The *E. hormaechei* species can ferment various carbohydrates including D-glucose, L-arabinose, cellobiose, dulcitol, D-galactose, maltose, D-mannitol, D-mannose, L-rhamnose, sucrose, trehalose, and D-xylose (O'Hara et al., 1989). The sugar utilization test also showed that Z129 fermented D-ribose, L-arabinose, and D-lactose but not D-mannitol, D-sorbitol. D-trehalose, inulin, D-raffinose, and glycogen. Although there were some differences from the result of O'Hara et al. (1989), Z129 showed the ability to ferment various carbohydrates. The CAZymes annotation also showed that there were various GH families including GH13, GH19, GH154, GH28, GH102, GH77, GH4, GH24, and GH23 in the Z129 genome. The GH28 family plays important roles in pectin degradation (Zhao et al., 2013) and contains all pectin-degrading hydrolases (Anuradha and Bhawaniprasad, 2019). The GH19 family contains endo-chitinases which hydrolyzes the chitinoside bond to produce N-acetyl-D-glucosamine (Nakagawa et al., 2013). The most typical and studied enzyme among GH13 is α-amylase, which specifically catalyzes hydrolysis of α-1,4-glycosidic linkages of starch to produce small molecular products including glucose, maltose, and maltotriose (Gangadharan et al., 2008). In addition, genes related to starch, cellulose and hemicellulose metabolism including *amy*E, *cel*B, *bgl*X, *xyl*F-*xyl*H, *fba*A, *ace*A, and *suc*C were also identified in the Z129 genome. The nitrogen from urea metabolism and the carbon from carbohydrate fermentation are used together to produce various AAs.

In conclusion, Z129 is a new gram-positive strain of *E. hormaechei*, carrying unique genes including *app*A, *nrf*A, and *wec*E related to feed

conversion ratio, nitrogen dissimilatory reduction and lactose synthesis. Strain Z129 possesses the genes of each step of urea metabolism and various genes involved in cellulose, hemicellulose, and starch fermentation.

Data availability statement

The names of the repository/repositories and accession number(s) can be found at: https://nmdc.cn/en, SUB1675681571147 and https://www.ncbi.nlm.nih.gov/, GCF_001875655.1, GCF_000750225.1, GCF_000750275.1, GCF_000534575.1, GCF_000534035.1, GCF_000492615.1, GCF_000492495.1, GCF_020097195.1, GCF_001729725.1, GCF_001729785.1, GCF_000328905.1, and GCF_000328885.1.

Ethics statement

The animal study was reviewed and approved by the Ethics Committee of Institute of Animal Sciences of CAAS. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

SZ designed the study and reviewed the paper. HZ performed the experiments and wrote the paper. SZ and HZ analyzed the genome equally. JW and NZ contributed to project administration and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1169973/full#supplementary-material

References

Alfano, M., and Cavazza, C. (2020). Structure, function, and biosynthesis of nickel-dependent enzymes. *Protein Sci.* 29, 1071–1089. doi: 10.1002/pro.3836

Alzohairy, A. (2011). Bioedit: an important software for molecular biology. *GERF Bull. Biosci.* 2, 60–61.

Anuradha, K., and Bhawaniprasad, B. (2019). Structural insights into the molecular mechanisms of pectinolytic enzymes. *J. Proteins Proteom.* 10, 325–344. doi: 10.1007/s42485-019-00027-5

Beaulieu, A., Bedford, M., and Patience, J. (2007). Supplementing corn or corn-barley diets with an e. coli derived phytase decreases total and soluble p output by weanling and growing pigs. *Can. J. Anim. Sci.* 87, 353–364. doi: 10.4141/CJAS06032

Buchfink, B., Xie, C., and Huson, D. H. (2014). Fast and sensitive protein alignment using diamond. *Nat. Methods* 12, 59–60. doi: 10.1038/nmeth.3176

Calsamiglia, S., Ferret, A., Reynolds, C. K., Kristensen, N. B., and van Vuuren, A. M. (2010). Strategies for optimizing nitrogen use by ruminants. *Animal* 4, 1184–1196. doi: 10.1017/S1751731110000911

Carter, E. L., and Hausinger, R. P. (2010). Characterization of the klebsiella aerogenes urease accessory protein ured in fusion with the maltose binding protein. *J. Bacteriol.* 192, 2294–2304. doi: 10.1128/jb.01426-09

Chaumeil, P., Mussig, A. J., Hugenholtz, P., and Parks, D. H. (2020). Gtdb-tk: a toolkit to classify genomes with the genome taxonomy database. *Bioinformatics* 36, 1925–1927. doi: 10.1093/bioinformatics/btz848

Cherdthong, A., and Wanapat, M. (2010). Development of urea products as rumen slow-release feed for ruminant production: a review. *Aust. J. Basic Appl. Sci.* 4, 2232–2241.

Chiera, J. M., Finer, J. J., and Grabau, E. A. (2004). Ectopic expression of a soybean phytase in developing seeds of glycine max to improve phosphorus availability. *Plant Mol. Biol.* 56, 895–904. doi: 10.1007/s11103-004-5293-6

Cook, A. R. (1976). Urease activity in the rumen of sheep and the isolation of ureolytic bacteria. *J. Gen. Microbiol.* 92, 32–48. doi: 10.1099/00221287-92-1-32

Cook, A. R., Riley, P. W., Murdoch, H., Evans, P. N., and McDonald, I. R. (2007). Howardella ureilytica gen. Nov., Sp. Nov., a gram-positive, coccoid-shaped bacterium from a sheep rumen. *Int. J. Syst. Evol. Microbiol.* 57, 2940–2945. doi: 10.1099/ijs.0.64819-0

Dmitry, A. R., Peter, H., Mikhail, S. G., and Thomas, E. (2006). Comparative and functional genomic analysis of prokaryotic nickel and cobalt uptake transporters: evidence for a novel group of atp-binding cassette transporters. *J. Bacteriol.* 188, 317–327. doi: 10.1128/JB.188.1.317

D'Onofrio, A., Crawford, J. M., Stewart, E. J., Witt, K., Gavrish, E., Epstein, S., et al. (2010). Siderophores from neighboring organisms promote the growth of uncultured bacteria. *Chem. Biol.* 17, 254–264. doi: 10.1016/j.chembiol.2010.02.010

Gangadharan, D., Sivaramakrishnan, S., Nampoothiri, K. M., Sukumaran, R. K., and Pandey, A. (2008). Response surface methodology for the optimization of alpha amylase production by bacillus amyloliquefaciens. *Bioresour. Technol.* 99, 4597–4602. doi: 10.1016/j.biortech.2007.07.028

Gibbons, R. J., and Doetsch, R. N. (1958). Physiological study of an obligately anaerobic ureolytic bacterium. *J. Bacteriol.* 77, 417–428. doi: 10.1128/jb.77.4.417-428.1959

Gutleben, J., Chaib De Mares, M., van Elsas, J. D., Smidt, H., Overmann, J., and Sipkema, D. (2018). The multi-omics promise in context: from sequence to microbial isolate. *Crit. Rev. Microbiol.* 44, 212–229. doi: 10.1080/1040841x.2017.1332003

Hailemariam, S., Zhao, S., He, Y., and Wang, J. (2021). Urea transport and hydrolysis in the rumen: a review. *Anim. Nutr.* 7, 989–996. doi: 10.1016/j.aninu.2021.07.002

Hailemariam, S., Zhao, S., and Wang, J. (2020). Complete genome sequencing and transcriptome analysis of nitrogen metabolism of succinivibrio dextrinosolvens strain z6 isolated from dairy cow rumen. *Front. Microbiol.* 11:1826. doi: 10.3389/fmicb.2020.01826

Haley, K. P., and Gaddy, J. A. (2015). Metalloregulation of helicobacter pylori physiology and pathogenesis. *Front. Microbiol.* 6:911. doi: 10.3389/fmicb.2015.00911

Herrero, A., Flores, E., and Imperial, J. (2019). "Nitrogen assimilation in bacteria" in *Encyclopedia of microbiology*. ed. T. M. Schmidt. *4th* ed (United Kingdom: Academic Press, Oxford), 280–300.

Hilde, D. R., Daniel, V., and Christine, C. (2013). Common themes and unique proteins for the uptake and trafficking of nickel, a metal essential for the virulence of helicobacter pylori. *Front. Cell. Infect. Microbiol.* 3:94. doi: 10.3389/fcimb.2013.00094

Hwang, B., Lee, H., and Yang, Y. (2004). Characterization and investigation of substrate specificity of the sugar aminotransferase wece from e. coli k12. *Chem. Biol.* 11, 915–925. doi: 10.1016/j.chembiol

Jin, D., Zhao, S., Wang, P., Zheng, N., Bu, D., Beckers, Y., et al. (2016). Insights into abundant rumen ureolytic bacterial community using rumen simulation system. *Front. Microbiol.* 7:1006. doi: 10.3389/fmicb.2016.01006

John, A., Isaacson, H. R., and Bryant, M. P. (1974). Isolation and characteristics of a ureolytic strain of selenomonas ruminantium. *J. Dairy Sci.* 57, 1003–1014. doi: 10.3168/jds.s0022-0302(74)85001-0

Kim, J., Henriksen, E., Cann, I., and Mackie, R. (2014). Nitrogen utilization and metabolism in ruminococcus albus 8. *Appl. Environ. Microbiol.* 80, 3095–3102. doi: 10.1128/AEM.00029-14

Lauková, A., and Koniarová, I. (1995). Survey of urease activity in ruminal bacteria isolated from domestic and wild ruminants. *Microbios* 84, 7–11.

Lewis, W., Tahon, G., Geesink, P., Sousa, D., and Ettema, T. (2020). Innovations to culturing the uncultured microbial majority. *Nat. Rev. Microbiol.* 19, 225–240. doi: 10.1038/s41579-020-00458-8

Li, D., Liu, C., Luo, R., Sadakane, K., and Lam, T. (2015). Megahit: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de bruijn graph. *Bioinformatics* 31, 1674–1676. doi: 10.1093/bioinformatics/btv033

Liu, G., Vijayaraman, S. B., Dong, Y., Li, X., Andongmaa, B. T., Zhao, L., et al. (2020). Bacillus velezensis lg37: transcriptome profiling and functional verification of glnk and mnra in ammonia assimilation. *BMC Genomics* 21:215. doi: 10.1186/s12864-020-6621-1

Liu, D., Zhang, Y., Fan, G., Sun, D., Zhang, X., Yu, Z., et al. (2022). Ipga: a handy integrated prokaryotes genome and pan-genome analysis web service. *iMeta.* 1:e55. doi: 10.1002/imt2.55

Makkar, H. P. S., and Mcsweeney, C. S. (2005). *Methods in gut microbial ecology for ruminants*. Netherlands: Springer.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet j.* 17, 10–12. doi: 10.14806/ej.17.1.200

McLean, R. J. C., Cheng, K. J., Gould, W. D., and Costerton, J. W. (1985). Cytochemical localization of urease in a rumen staphylococcus sp. by electron microscopy. *Appl. Environ. Microbiol.* 49, 253–255. doi: 10.1128/AEM.49.1.253-255.1985

Minas, K., McEwan, N. R., Newbold, C. J., and Scott, K. P. (2011). Optimization of a high-throughput ctab-based protocol for the extraction of qpcr-grade dna from rumen fluid, plant and bacterial pure cultures. *FEMS Microbiol. Lett.* 325, 162–169. doi: 10.1111/j.1574-6968.2011.02424.x

Nakagawa, Y., Eijsink, V., Totani, K., and Vaaje-Kolstad, G. (2013). Conversion of α-chitin substrates with varying particle size and crystallinity reveals substrate preferences of the chitinases and lytic polysaccharide monooxygenase of serratia marcescens. *J. Agric. Food Chem.* 61, 11061–11066. doi: 10.1021/jf402743e

O'Hara, C. M., Steigerwalt, A. G., Hill, B. C., Farmer, J. J. I., Fanning, G. R., and Brenner, D. J. (1989). Enterobacter hormaechei, a new species of the family enterobacteriaceae formerly known as enteric group 75. *J. Clin. Microbiol.* 27, 2046–2049. doi: 10.1128/JCM.27.9.2046-2049.1989

Oliver, J. D. (2010). Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiol. Rev.* 34, 415–425. doi: 10.1111/j.1574-6976.2009.00200.x

Owens, F., Qi, S., and Sapienza, D. A. (2014). Invited review: applied protein nutrition of ruminants—current status and future directions1. *Profess Anim Sci.* 30, 150–179. doi: 10.15232/S1080-7446(15)30102-9

Parks, D., Imelfort, M., Skennerton, C., Philip, H., and Tyson, G. (2015). Checkm: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res.* 25, 1043–1055. doi: 10.1101/gr.186072.114

Pulschen, A., Bendia, A., Fricker, A., Pellizari, V., Galante, D., and Rodrigues, F. (2017). Isolation of uncultured bacteria from Antarctica using long incubation periods and low nutritional media. *Front. Microbiol.* 8:1346. doi: 10.3389/fmicb.2017.01346

Reynolds, C., and Kristensen, N. (2008). Nitrogen recycling through the gut and the nitrogen economy of ruminants: an asynchronous symbiosis. *J. Anim. Sci.* 86, E293–E305. doi: 10.2527/jas.2007-0475

Roslan, M. A. M., Zulkifli, N. N., Sobri, Z. M., Zuan, A. T. K., Cheak, S. C., and Abdul Rahman, N. A. (2020). Seed biopriming with p- and k-solubilizing enterobacter hormaechei sp. improves the early vegetative growth and the p and k uptake of okra (abelmoschus esculentus) seedling. *PLoS One* 15:e232860. doi: 10.1371/journal.pone.0232860

Schwab, C., and Broderick, G. (2017). A 100-year review: protein and amino acid nutrition in dairy cows. *J. Dairy Sci.* 100, 10094–10112. doi: 10.3168/jds.2017-13320

Sebbane, F., Bury-Moné, S., Cailliau, K., Browaeys-Poly, E., Reuse, H., and Simonet, M. (2002). The yersinia pseudotuberculosis yut protein, a new type of urea transporter homologous to eukaryotic channels and functionally interchangeable in vitro with the helicobacter pylori urei protein. *Mol. Microbiol.* 45, 1165–1174. doi: 10.1046/j.1365-2958.2002.03096.x

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30, 2068–2069. doi: 10.1093/bioinformatics/btu153

Slyter, L. L., Oltjen, R. R., Kern, D. L., and Weaver, J. M. (1968). Microbial species including ureolytic bacteria from the rumen of cattle fed purified diets. *J. Nutr.* 94, 185–192. doi: 10.1093/jn/94.2.185

St. John, E., Liu, Y., Podar, M., Stott, M. B., Meneghin, J., Chen, Z., et al. (2019). A new symbiotic nanoarchaeote (candidatus nanoclepta minutus) and its host (zestosphaera tikiterensis gen. Nov., Sp. Nov.) from a New Zealand hot spring. *Syst. Appl. Microbiol.* 42, 94–106. doi: 10.1016/j.syapm.2018.08.005

Tiedje, J. (1988). "Ecology of denitrification and dissimilatory nitrate reduction to ammonium" in *Biology of anaerobic microorganisms*. ed. A. J. B. Zehnder (New York: Wiley), 179–244.

Vanwyk, L., and Steyn, P. L. (1975). Ureolytic bacteria in sheep rumen. *J. Gen. Microbiol.* 91, 225–232. doi: 10.1099/00221287-91-2-225

Vinella, D., Fischer, F., Vorontsov, E., Gallaud, J., Malosse, C., Michel, V., et al. (2015). Evolution of helicobacter: acquisition by gastric species of two histidine-rich proteins essential for colonization. *PLoS Pathog.* 11:e1005312. doi: 10.1371/journal.ppat.1005312

Wang, H., and Gunsalus, R. P. (2000). The nrfa and nirb nitrite reductase operons in escherichia coli are expressed differently in response to nitrate than to nitrite. *J. Bacteriol.* 182, 5813–5822. doi: 10.1128/JB.182.20.5813-5822.2000

Wiśniewska, Z., Nollet, L., Lanckriet, A., Vanderbeke, E., Petkov, S., Outchkourov, N., et al. (2020). Effect of phytase derived from the e. coli appa gene on weaned piglet performance, apparent total tract digestibility and bone mineralization. *Animals* 10:121. doi: 10.3390/ani10010121

Wozny, M. A., Bryant, M. P., Holdeman, L. V., and Moore, W. E. C. (1977). Urease assay and urease-producing species of anaerobes in the bovine rumen and human feces. *Appl. Environ. Microbiol.* 33, 1097–1104. doi: 10.1128/aem.33.5.1097-1104.1977

Yang, X., Li, H., Lai, T., and Sun, H. (2015). Uree-ureg complex facilitates nickel transfer and preactivates gtpase of ureg in helicobacter pylori. *J. Biol. Chem.* 290, 12474–12485. doi: 10.1074/jbc.M114.632364

Yin, Y., Mao, X., Yang, J., Chen, X., Mao, F., and Xu, Y. (2012). Dbcan: a web resource for automated carbohydrate-active enzyme annotation. *Nucleic Acids Res.* 40, W445–W451. doi: 10.1093/nar/gks479

Zambelli, B., Berardi, A., Martin-Diaconescu, V., Mazzei, L., Musiani, F., Maroney, M. J., et al. (2014). Nickel binding properties of helicobacter pylori uref, an accessory protein in the nickel-based activation of urease. *J. Biol. Inorg. Chem.* 19, 319–334. doi: 10.1007/s00775-013-1068-3

Zhang, X., Ahmad, W., Zhu, X., Chen, J., and Austin, B. (2020). Viable but nonculturable bacteria and their resuscitation: implications for cultivating uncultured marine microorganisms. *Mar. Life Sci. Tech.* 3, 189–203. doi: 10.1007/s42995-020-00041-3

Zhao, Z., Liu, H., Wang, C., and Xu, J. (2013). Comparative analysis of fungal genomes reveals different plant cell wall degrading capacity in fungi. *BMC Genomics* 14:274. doi: 10.1186/1471-2164-14-274

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Evaluation of the representative of using rumen fluid samples from lambs fed pelleted TMR for analysis of prokaryotic communities

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The pelleted TMR pulverized the grass during processing, which may result in more solid attached microorganisms in the filtered rumen fluid. The objective of this study was evaluating the necessity of distinguishing physical phases of rumen contents for analysis of prokaryotes communities in rumen of lambs fed pelleted TMR, considering the dissimilarity of diversity and community of bacteria and archaea between fluid and mixed rumen contents. The yield of microbial DNA, bacterial diversity, abundances of fibrolytic bacteria of phylum Fibrobacterota and Spirochaetota, as well as genus *Ruminococcus*, *Lachnospiraceae*_NK3A20, *Fibrobacter*, and F082, and abundance of archaeal *Methanimicrococcus* in rumen fluid were lower than those in mixed phase of rumen contents ($p \leq 0.05$). In conclusion, it is necessary to consider rumen content physical phases when studying the prokaryotic community in rumen of lambs fed pelleted TMR.

KEYWORDS

rumen fluid, rumen contents, pelleted TMR, bacteria, archaea

Introduction

It has been well-recognized that the microbial communities inhabiting the fluid and solid phases of rumen contents are distinguishing (Henderson et al., 2013). Bacterial communities associated with solid phase was fibrolytic phyla *Fibrobacteres* and *Firmicutes*, particularly genus *Butyrivibrio*, *Succiniclasticum* and *Lachnospiraceae*, while the predominant bacterial community member in fluid phase was *Prevotella* (Henderson et al., 2013). A hydrotrophic archaea *Methanobrevibacter* is prevalent in the rumen, accounting for 60%–95% of the total archaea (Hook et al., 2010; Ma et al., 2019). Interestingly, the structure of the archaeal community is also different between the solid and liquid phases of rumen contents, and minority archaea such as *Methanosphaera* are less abundant in the solid phase (Henderson et al., 2013; Vaidya et al., 2018).

The solid and fluid phases of rumen contents are generally separated by filtration through cheesecloth. In regular TMR for sheep, the length of the forage is generally 2–10 cm (Nielsen et al., 2017). The usual mesh size of cheesecloth used to separate rumen fluid is 250–350 μm (Ma et al., 2019), through which forage in regular TMR can be easily intercepted. However, in the pelleted TMR commonly used in lambs, the forage is pre-crushed to less than 8 mm to meet the homogenization requirements before pelleting (Malik et al., 2021). To some extent, this adds the probability of forage particles escaping into the liquid phase during squeezing filtration of cheesecloth. It is possible that the rumen fluid obtained for determination of the microbial composition could represent the rumen contents when lambs are fed pelleted TMR. Since rumen sample fractions differed substantially in terms of their physical natures and associated

microorganisms, particular attention should be paid to studies involving the composition of rumen prokaryotes based on sequencing technology. Rumen solid phase is a better choice when studying rumen fibrolitic population (Gharechahi et al., 2020), while rumen fluid is undoubtedly more advantageous in animal welfare and application because of its convenience and safety (Henderson et al., 2015). The rumen contents of lambs that are fed finely ground feed are homogenized and more solid fragments escape into the liquid phase even after being filtered by gauze. Therefore, it is necessary to determine whether it is essential to distinguish between the solid and liquid phases when examining the rumen contents of lambs fed this finely ground diet.

Our hypothesis was that the characteristics of the physical form in pelleted TMR causes the solid phase of rumen contents to more easily escape cheesecloth in the process of separation. In this study, the need to distinguish physical phase of rumen contents was evaluated from the perspective of differences in prokaryotic community composition between rumen contents and rumen fluid for lambs fed pelleted TMR.

Methods and materials

Experimental design and animals

The animal study and sample collection were approved by the Ethics Committee of Gansu Agriculture University, Gansu, China (Approval number: GSAU-LIU-2018-02).

Under a paired design, fluid and mixed phases of rumen contents were collected from 10 *Hu* lambs (about 70-day-old, average body weight = 15.8 ± 0.76 kg) fed a same pelleted TMR. The pelleted TMR contained 5% alfalfa hay, 55.90% corn, 11% soybean meal, 1.5% whey powder, 7% expended soybean, 17% dried malt root, 1.20% limestone, 1% premix, 0.3% NaCl, and 0.1% feed attractant. All lambs had free access to water and feed.

Animals were slaughtered after a 12-h fasting period. After slaughter, about 200 g of the contents from the middle of rumen and used as the mixed phase. The liquid phase of rumen contents was achieved after filtering through four layers of cheesecloth. All samples were immersed in liquid nitrogen immediately, and then stored at −80°C until DNA extraction.

DNA extraction

Microbial DNA were extracted from fluid and mixed phases of rumen contents using YM + SB method (Ma et al., 2020). The integrity of the DNA was validated using 1% (w/v) agarose gel electrophoresis. The DNA yields and purities were evaluated by measuring the $OD_{260/280}$ and $OD2_{60/230}$ ratios using a spectrophotometer (NanoDrop, Thermo Fisher Scientific, Waltham, MA, United States).

16S rRNA amplicon sequencing

Amplicon sequencing with single-end reads was conducted on an Ion S5 XL platform (Thermo Fisher Scientific, Waltham, MA, United States) by Novogene Co., Ltd. (Tianjing, China) according to the standard procedure of the company. In brief, microbial DNA samples were diluted to 1 ng/μL before amplifying the designated regions of 16S rRNA genes of prokaryotes. The V3-V4 bacterial 16S rRNA genes were amplified using primers of 341F (5′-CCTAYGGGRB GCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) (Zakrzewski et al., 2012). The V8 regions of archaeal 16S rRNA genes were amplified using 1106F (5′-TTWAGTCAGGCAACGAGC-3′) and 1378R (5′-TGTGCAAGGAGCAGGGAC-3′) (Feng et al., 2013).

Bioinformatic analysis

Quality control on the raw reads was performed by *vsearch* by setting -fastq_maxee *1* and -fastq_maxee_rate *0.01* (Rognes et al., 2016). Chimera sequences were detected and removed by *usearch -uchime_denovo* (Edgar, 2010). The zero-radius OTUs (ZOTUs) were identified by setting *usearch -unoise3* (Edgar, 2010). Representative ZOTU sequences were annotated with *Mothur* (Schloss et al., 2009) referring *Silva.nr. 138* for bacteria (Elmar et al., 2007) and *RIM14.6* for archaea (Seedorf et al., 2014).

The alpha diversity of ZOTUs was estimated by using observed ZOTUs and faith's phylogenetic diversity (PD) index (Armstrong et al., 2021). The principal coordinate analysis (PCoA) was conducted based on Bray–Curtis dissimilarity matrix (Bray and Curtis, 1957) to obtain and visual principal coordinates from complex multidimensional data. The diversity calculations were performed in *R* (R Core Team, 2020), and the relevant codes is accessible at Ma (2021).

Statistical analysis

Paired t-test were conducted using *R* with the parameter of *paired* = *TRUE* (R Core Team, 2020). To test for differences in overall bacterial or archaeal community between fluid and mixed phases of rumen contents, the Bray–Curtis dissimilarity matrix among sources of variation was parted, and a permutational ANOVA (PMANOVA) was performed by *vegan* with 999 permutation (Oksanen et al., 2007). All *p*-values of relative abundances of amplicon data were adjusted according to the method of Benjamini and Hochberg (1995) by *p.adjust()* function of *R* (R Core Team, 2020). A probability of $p < 0.05$ was considered to indicate a significant difference.

Results and discussion

In our experience, rumen contents of lambs fed pelleted TMR are more viscous and more homogeneous than those of adult ruminants. This is in line with reports that pelleted TMR increases organic acid concentration (Trabia et al., 2020; Li et al., 2021) and rumen absorption capacity (Malik et al., 2021). So, we compared rumen fluid with rumen mixed contents to determine its representative.

In this study, high yield DNA was extracted from rumen fluid and rumen mixed contents. The yield of DNA extracted from adult goat rumen fluid was only one third of that in this study using the same DNA extraction method (Ma et al., 2020). This suggests that more solid contents in lambs fed pelleted TMR escaped through the cheesecloth. The microbial DNA yield in the rumen mixed content was higher than that in the rumen liquid ($p = 0.003$, Table 1). It was expected because the microbes in the solid phase are much denser than those in rumen fluid (Vaidya et al., 2018). However, neither extracted DNA from rumen fluid nor mixed contents was achieve

the ideal value of 2, as indicated by $OD_{260/230}$, the key indicators related to DNA quality, were 1.66 and 1.56, respectively. This level is close to that of DNA extracted from adult goats using the same method (Ma et al., 2020), and higher than seven out of fifteen commercially available DNA extractions methods (Henderson et al., 2013).

A total of 1,594,679 bacterial reads and 160, 0916 archaeal reads were obtained by sequencing, with an average of 79, 733 and 80,045 reads per sample, respectively. After the denoise algorithm, the reads collapsed into 1, 911 bacterial ZOTUs and 225 archaeal ZOTUs. The bacterial alpha-diversity was higher in mixed rumen contents than in rumen fluid, as indicated by higher observed ZOTUs and PD index (Table 1, $p = 0.02$). But archaeal alpha-diversity was not influenced by physical phase of rumen contents, as indicated by similar observed ZOTUs and PD index ($p \geq 0.25$). This result was similar to that of adult ruminants fed regular TMR (Vaidya et al., 2018), but did not align with our hypothesis. This suggests that if research needs to cover more bacteria, mixed content is a better choice.

TABLE 1 Microbial DNA quality and alpha-diversity of fluid and mixed phase of content in rumen of lambs fed pelleted TMR starter.

Items[1]	Rumen content phase		SEM	*P*-value	
	Fluid	Mixed		Rumen content phase	Animal
DNA yield, μg/g	175	335	26.1	0.003	0.005
$OD_{260/280}$	2.05	2.10	0.042	0.45	0.39
$OD_{260/230}$	1.66	1.56	0.056	0.32	0.11
Bacteria					
Observed ZOTUs	1,755	1,825	18.8	0.02	0.07
PD	33.8	34.4	0.18	0.02	0.01
Archaea					
Observed ZOTUs	150	158	4.4	0.25	0.30
PD	12.3	13.5	1.23	0.48	0.57

[1]Faith's phylogenetic diversity.

Principal coordinate analysis at ZOTU level shown both overall communities of bacteria and archaea in rumen fluid and mixed rumen contents were different ($p < 0.001$, Figure 1). Further taxonomic abundance analysis showed that the rumen fluid had lower abundances of phylum *Fibrobacterota* and *Spirochaetota*, as well as genus *Ruminococcus*, *Lachnospiraceae*_NK3A20, *Fibrobacter,* and F082, which are related to fiber degradation, than the mixed rumen contents ($p \leq 0.03$, Figure 2; Supplementary Tables S1, S2). Populations attached to feed particles can infiltrate surface of feed plants and have more activity in degrading carbohydrate than the planktonic population (McAllister et al., 1994). It is well recognized that the population of fibrolytic bacteria on rumen fluid is much lower than on forage grass (De Mulder et al., 2017; Vaidya et al., 2018). Unfortunately, the relative abundances of fibrolytic bacteria in rumen fluid was not representative of the rumen contents, although pelleted TMR greatly reduced the size of the forage.

We detected that 99.9% of the archaea were *Euryarchaeota*, so they were not analyzed at the phyla level. Archaea in the rumen are hydrogen-trophic methanogens that usually coexist with hydrogen-producing microorganisms such as fibrolytic bacteria (Czerkawski et al., 1972). The second largest archaeal genus *Methanosphaera* in the rumen has been reported to be more abundant in rumen fluid than in solid rumen contents (Henderson et al., 2013; Vaidya et al., 2018). However, we only observed a numerical change ($p = 0.87$; Figure 2 and Supplementary Table S3). Our comparison of rumen fluid with mixed rumen contents rather than solid phase may have reduced this

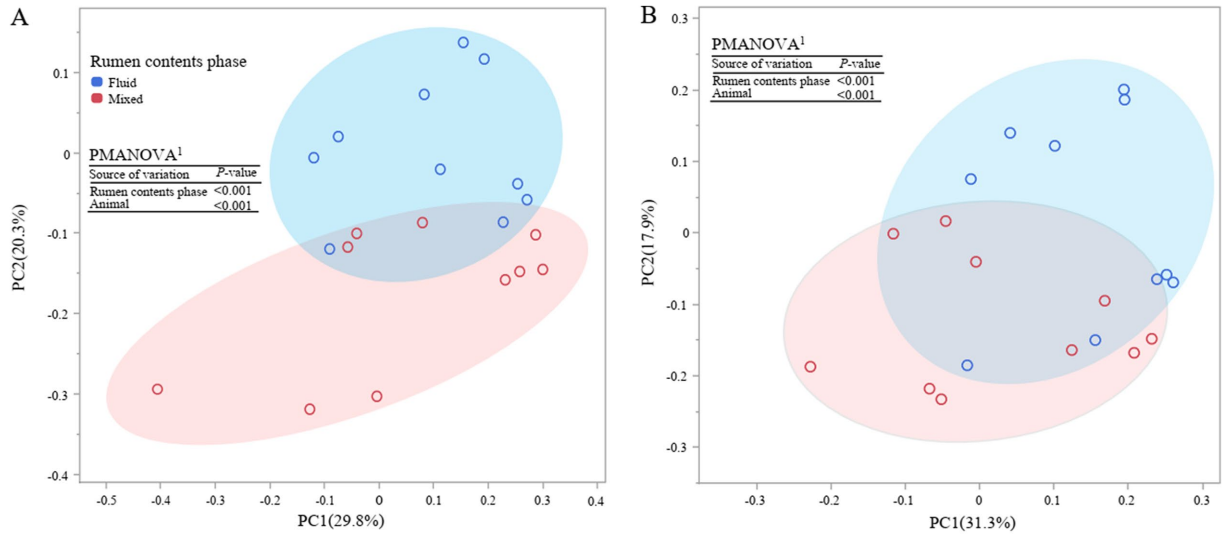

FIGURE 1
Principal coordinate analysis (PCoA) of bacterial **(A)** and archaeal **(B)** community at ZOTU level. PMANOVA, permutational multivariate analysis of variance with 999 permutations. The ellipses show contrasting prokaryotic community in mixed and liquid phases of rumen content.

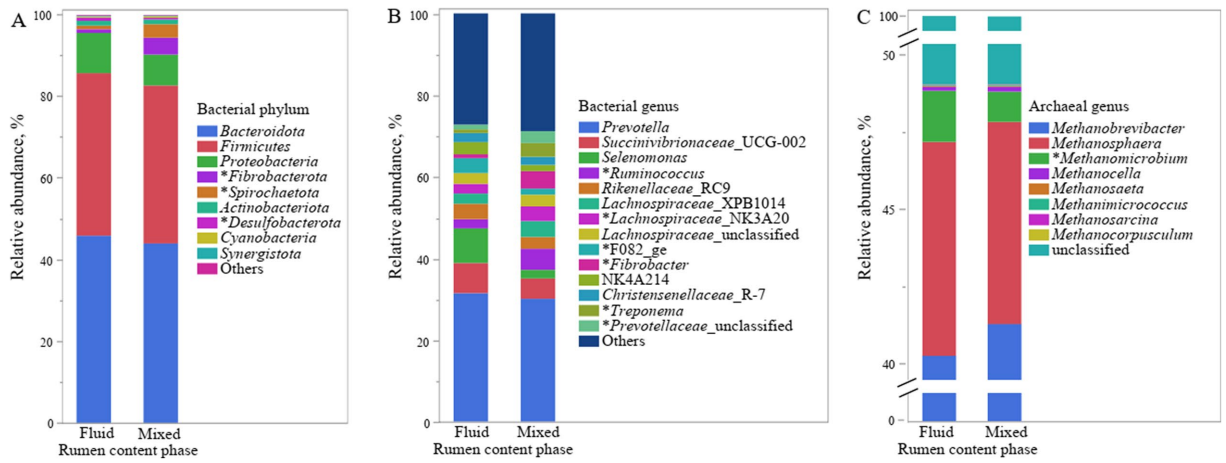

FIGURE 2
Relative abundance of bacterial phyla **(A)** and genera **(B)** and archaeal genera **(C)** in fluid and mixed phase of content in rumen of lambs fed pelleted TMR starter. The asterisk (*) before the taxonomic name indicates that its relative abundances in the mixed and liquid phases are different ($p \leq 0.05$).

discrepancy to some extent. We observed that archaeal genus *Methanimicrococcus* was less abundant in rumen fluid than in mixed rumen contents ($p = 0.01$). Genus *Methanimicrococcus* has been reported to decrease in abundance with the increase of dietary forage (Huo et al., 2020), suggesting that it may be symbiotic with planktonic hydrogen-producer. Such speculation was contradicted with the lower relative abundance of genus *Methanimicrococcus* in rumen fluid than in mixed rumen contents. More research is needed to understand this phenomenon.

In conclusion, the yield of microbial DNA, bacterial diversity, abundance of fibrolytic bacteria, and abundance of archaeal *Methanimicrococcus* in rumen fluid were lower than those in mixed rumen contents. Therefore, this study emphasizes the need for careful consideration of sample collection methods in rumen microbial studies, especially in young ruminants like lambs.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Ethics Committee of Gansu Agriculture University.

Author contributions

ZM: conceptualization, investigation, software, data curation, writing, visualization, and funding acquisition. JZ: investigation, methodology, and data curation. TL: conceptualization, data curation, supervision, funding acquisition, and project administration. CZ: investigation, writing—review and editing, and supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1190253/full#supplementary-material

References

Armstrong, G., Cantrell, K., Huang, S., McDonald, D., Haiminen, N., Carrieri, A. P., et al. (2021). Efficient computation of faith's phylogenetic diversity with applications in characterizing microbiomes. *Genome Res.* 31, 2131–2137. doi: 10.1101/gr.275777.121

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc.* 57, 289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x

Bray, J. R., and Curtis, J. T. (1957). An ordination of the upland forest communities of southern Wisconsin. *Ecol. Monogr.* 27, 325–349. doi: 10.2307/1942268

Czerkawski, J. W., Harfoot, C. G., and Breckenridge, G. (1972). The relationship between methane production and concentrations of hydrogen in the aqueous and gaseous phases during rumen fermentation in vitro. *J. Appl. Biomater.* 35, 537–551. doi: 10.1111/j.1365-2672.1972.tb03735.x

De Mulder, T., Goossens, K., Peiren, N., Vandaele, L., Haegeman, A., De Tender, C., et al. (2017). Exploring the methanogen and bacterial communities of rumen environments: solid adherent, fluid and epimural. *FEMS Microbiol. Ecol.* 93:w251. doi: 10.1093/femsec/fiw251

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than blast. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Elmar, P., Christian, Q., Katrin, K., Fuchs, B. M., Wolfgang, L., Jrg, P., et al. (2007). SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res.* 35, 7188–7196. doi: 10.1093/nar/gkm864

Feng, Y., Lin, X., Yu, Y., Zhang, H., Chu, H., and Zhu, J. (2013). Elevated ground-level o3 negatively influences paddy methanogenic archaeal community. *Sci. Rep.* 3:3193. doi: 10.1038/srep03193

Gharechahi, J., Vahidi, M. F., Bahram, M., Han, J., Ding, X., and Salekdeh, G. H. (2020). Metagenomic analysis reveals a dynamic microbiome with diversified adaptive functions to utilize high lignocellulosic forages in the cattle rumen. *ISME J.* 15, 1108–1120. doi: 10.1038/s41396-020-00837-2

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., and Janssen, P. H. (2015). Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Sci. Rep.* 5:14567. doi: 10.1038/srep14567

Henderson, G., Cox, F., Kittelmann, S., Miri, V. H., Zethof, M., Noel, S. J., et al. (2013). Effect of DNA extraction methods and sampling techniques on the apparent structure of cow and sheep rumen microbial communities. *PLoS One* 8:e74787. doi: 10.1371/journal.pone.0074787

Hook, S. E., Wright, A. G., and McBride, B. W. (2010). Methanogens: methane producers of the rumen and mitigation strategies. *Archaea* 2010, 1–11. doi: 10.1155/2010/945785

Huo, J., Fang, S., Wu, P., Zhong, X., and Zhan, J. (2020). Effects of diets with different concentration-roughage ratios on the microbial community structure of Nubian goat rumen (in Chinese). *Pratacultural Science.* 37, 2558–2566. doi: 10.11829/j.issn.1001-0629.2020-0303

Li, B., Sun, X., Huo, Q., Zhang, G., Wu, T., You, P., et al. (2021). Pelleting of a total mixed ration affects growth performance of fattening lambs. *Front. Vet. Sci.* 8:629016. doi: 10.3389/fvets.2021.629016

Ma, Z. Y. (2021). Microbial diversity. Available at: https://github.com/sleepvet/MicrobialDiversity (Accessed December 10, 2021).

Ma, Z. Y., Zhang, X. M., Wang, M., Wang, R., Jiang, Z. Y., Tan, Z. L., et al. (2019). Molecular hydrogen produced by elemental magnesium inhibits rumen fermentation and enhances methanogenesis in dairy cows. *J. Dairy Sci.* 102, 5566–5576. doi: 10.3168/jds.2018-15647

Ma, Z. Y., Zhang, X. M., Wang, R., Wang, M., Liu, T., and Tan, Z. L. (2020). Effects of chemical and mechanical lysis on microbial DNA yield, integrity, and downstream amplicon sequencing of rumen bacteria and protozoa. *Front. Microbiol.* 11:581227. doi: 10.3389/fmicb.2020.581227

Malik, M. I., Rashid, M. A., Yousaf, M. S., Naveed, S., and Rehman, H. U. (2021). Rumen morphometry and sorting behavior of fattening male goat fedpelleted and unpelleted TMR with two levels of wheat straw. *Small Rumin. Res.* 196:106316. doi: 10.1016/j.smallrumres.2021.106316

McAllister, T. A., Bae, H. D., Jones, G. A., and Cheng, K. J. (1994). Microbial attachment and feed digestion in the rumen. *J. Anim. Sci.* 72, 3004–3018. doi: 10.2527/1994.72113004x

Nielsen, M. V., Nadeau, E., Markussen, B., Helander, C., Eknæs, M., and Nørgaard, P. (2017). The relation between energy intake and chewing index of diets fed to nursing ewes. *Small Rumin. Res.* 153, 180–188. doi: 10.1016/j.smallrumres.2017.06.017

Oksanen, J., Kindt, R., Legendre, P., O Hara, B., Stevens, M. H. H., Oksanen, M. J., et al. (2007). Vegan: community ecology package. R package version 2.5-6. Available at: https://CRAN.R-project.org/package=vegan (Accessed September 3, 2020).

R Core Team. (2020). R: a language and environment for statistical computing. Available at: https://www.R-project.org/ (Accessed September 3, 2020).

Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahe, F. (2016). Vsearch: a versatile open source tool for metagenomics. *Peer J.* 4:e2584. doi: 10.7717/peerj.2584

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/AEM.01541-09

Seedorf, H., Kittelmann, S., Henderson, G., and Janssen, P. H. (2014). RIM-DB: a taxonomic framework for community structure analysis of methanogenic archaea from the rumen and other intestinal environments. *Peer J.* 2:e494. doi: 10.7717/peerj.494

Trabia, E. B., Seddika, H., Xie, F., Wang, X., Liu, J., and Mao, S. (2020). Effect of pelleted high-grain total mixed ration on rumen morphology, epithelium-associated microbiota and gene expression of proinflammatory cytokines and tight junction proteins in Hu sheep. *Anim. Feed Sci. Technol.* 263:114453. doi: 10.1016/j.anifeedsci.2020.114453

Vaidya, J. D., van den Bogert, B., Edwards, J. E., Boekhorst, J., van Gastelen, S., Saccenti, E., et al. (2018). The effect of DNA extraction methods on observed microbial communities from fibrous and liquid rumen fractions of dairy cows. *Front. Microbiol.* 9:92. doi: 10.3389/fmicb.2018.00092

Zakrzewski, M., Goesmann, A., Jaenicke, S., Jünemann, S., Eikmeyer, F., Szczepanowski, R., et al. (2012). Profiling of the metabolically active community from a production-scale biogas plant by means of high-throughput metatranscriptome sequencing. *J. Biotechnol.* 158, 248–258. doi: 10.1016/j.jbiotec.2012.01.020

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Modulating gastrointestinal microbiota to alleviate diarrhea in calves

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The calf stage is a critical period for the development of heifers. Newborn calves have low gastrointestinal barrier function and immunity before weaning, making them highly susceptible to infection by various intestinal pathogens. Diarrhea in calves poses a significant threat to the health of young ruminants and may cause serious economic losses to livestock farms. Antibiotics are commonly used to treat diarrhea and promote calf growth, leading to bacterial resistance and increasing antibiotic residues in meat. Therefore, finding new technologies to improve the diarrhea of newborn calves is a challenge for livestock production and public health. The operation of the gut microbiota in the early stages after birth is crucial for optimizing immune function and body growth. Microbiota colonization of newborn animals is crucial for healthy development. Early intervention of the calf gastrointestinal microbiota, such as oral probiotics, fecal microbiota transplantation and rumen microbiota transplantation can effectively relieve calf diarrhea. This review focuses on the role and mechanisms of oral probiotics such as *Lactobacillus*, *Bifidobacterium* and *Faecalibacterium* in relieving calf diarrhea. The aim is to develop appropriate antibiotic alternatives to improve calf health in a sustainable and responsible manner, while addressing public health issues related to the use of antibiotics in livestock.

KEYWORDS

calf diarrhea, gastrointestinal microbiota, gut health, rumen, probiotics

1. Introduction

Neonatal calf diarrhea (NCD) is a common cause of growth disorder and death of newborn calves and leads to economic losses in the animal husbandry (Cho and Yoon, 2014). The main age affected by intestinal diseases is calves under 30 days old (Dall Agnol et al., 2021). According to the National Animal Health Monitoring Program for Dairy Products in the United States, diarrhea is responsible for 57% of weaned calf mortality, and 20% calf mortality can result in a 38% reduction in net income (Fentie et al., 2020). Neonatal diarrhea can also reduce the growth performance of animals, reduce reproductive performance and milk production in the advanced stage of lactation (Aghakeshmiri et al., 2017). In animal husbandry, antibiotics have been widely used to treat calf diarrhea and promote livestock growth. However, there is becoming increasingly clear that there are many side effects of antibiotic use, with the emergence of drug-resistant bacteria and the residue of antibiotics in meat being major concerns. More importantly, the misuse of antibiotics during the calf stage and repeated diarrhea before weaning can lead to immature rumen and intestinal flora, which can have a lasting negative impact on the digestion and absorption of the calf growing diet (Ji et al., 2018). Considering the aforementioned risks,

there is an urgent need to expeditiously develop and implement innovative strategies to prevent and treat infectious diarrhea in calves. This objective seeks to minimize the requirement for antibiotic intervention and mitigate the propagation of antibiotic-resistant bacteria to both animal and human populations. Therefore, it is extremely important to reduce the use of antibiotics in calves and identify alternatives to antibiotic treatment.

The early stages of animal growth have a significant impact on their health, with contact with beneficial microbiota being particularly important. The early intestinal microbiota plays a crucial role in the long-term health of the host, especially in young animals whose gut microbiota is more vulnerable to external influences. Research has shown that the early postnatal period is a critical window for manipulating the gut microbiota to optimize immunity in individual newborns (Torow and Hornef, 2017; Figure 1). The symbiotic relationship between the host and gut microbiota is vital for regulating mucosal immunity and preventing pathogen colonization. Early colonization of the gut microbiota is crucial for promoting the establishment of intestinal barrier function and the maturation of the host immune system, which are essential for maintaining overall host health (Gensollen et al., 2016). The underdeveloped immune system in newborn animals is often associated with a range of early-onset ailments, such as early diarrhea and weaning stress (Chen et al., 2018). Therefore, it is crucial to foster the maturation of the intestinal immune system during the early stages of life to enhance the growth, development, and disease resistance of newborn mammals.

Timely intervention on calves through the addition of probiotics and other methods is essential in promoting their growth and metabolism. Probiotics and prebiotics have emerged as potential alternatives to antibiotics for promoting intestinal health and reducing the incidence of calf diarrhea. A comprehensive understanding of the structure and function of gastrointestinal microbiota can help in identifying reasonable antibiotic substitutes, such as probiotics and prebiotics (Mayer et al., 2012). Evidence suggests that probiotic supplementation can decrease the incidence of diarrhea, improve average daily weight gain, and enhance feed efficiency (Timmerman et al., 2005). This review aimed to investigate early intervention of calf gastrointestinal microbiota to relieve calf diarrhea.

2. Current status of calf diarrhea

Calf diarrhea is a significant cause of growth disorders and mortality in newborn calves, resulting in substantial economic losses in livestock farms (Cho and Yoon, 2014). The pre-weaning phase is a critical time for calves as they are highly susceptible to infectious pathogens, which can have a detrimental impact on their intestinal health (Kim et al., 2011). Digestive disorders including diarrhea are the most common diseases of pre-weaning dairy heifers, affecting 38.5% (Urie et al., 2018). In 2018, the National Animal Health Monitoring System of the United States published research results indicating that diarrhea is the cause of 39% of calf deaths in the first 3 weeks after birth (Kim et al., 2021). The mortality rate has been recently determined to be 7.6% in Canada and 5.3% in Belgium, with 25.4% of the calves experiencing at least one disease between arrival and slaughter (Pardon et al., 2012; Winder et al., 2016). Although the mortality rate of dairy heifers in the United States decreased from 11% in 2007 to 5% in 2014, the overall morbidity rate 33.9% is still alarmingly high (Urie et al., 2018). In summary, calf diarrhea remains a practical problem that the cattle industry needs to solve urgently.

3. Causes of calf diarrhea

Causes of calf diarrhea are complex and multifactorial, with numerous factors contributing to its development (Cho and Yoon, 2014). Infectious agents, such as Rotavirus, Coronavirus, *Escherichia coli* and *Cryptosporidium*, are significant intestinal pathogen that cause NCD (Gulliksen et al., 2009). Calves with diarrhea are generally divided into infectious and non-infectious cases, with infectious causes being more serious and destructive to cattle husbandry. Several variables can lead to the emergence of calf diarrhea, including autoimmune disorders, malnutrition, environmental and management

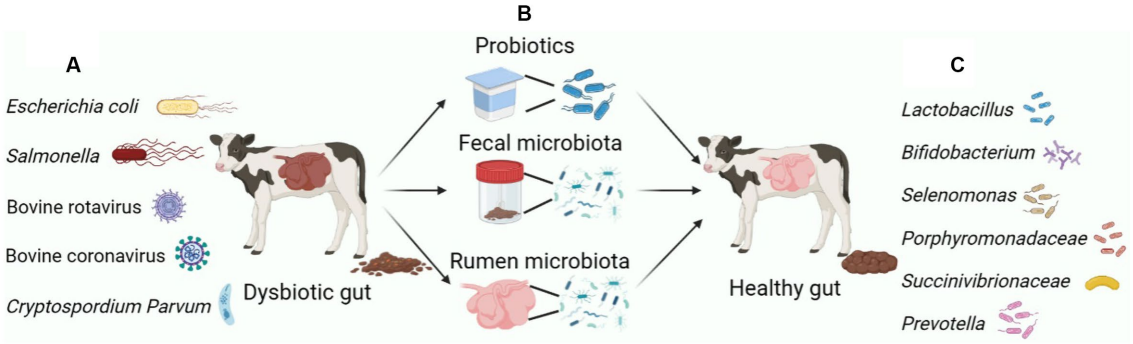

FIGURE 1
Major microbiota manipulation measures to remodel the dysbiosis of the gastrointestinal microbiota of calves. **(A)** The gastrointestinal tract of calves is susceptible to infection by pathogenic bacteria, leading to diarrhea. The main pathogenic bacteria causing gastrointestinal inflammation and diarrhea in calves include: *Escherichia coli*, *Salmonella*, BRV, BCV and *C. parvum*. **(B)** Measures to prevent and alleviate calf diarrhea through early intervention in the calf gut microbiota mainly include: Feed probiotics directly, FMT and RMT. **(C)** Probiotics mainly include *Lactobacillus*, *Bifidobacterium*, etc. The marker bacteria for normalizing the gastrointestinal microbiome of calves contain *Selenomonas*, *Porphyromonadaceae*, *Succinivibrionaceae* and *Prevotella*. Figure was created in biorender (http://biorender.io).

stress and pathogens (Cho and Yoon, 2014). During the fetal period, calves cannot obtain immuneoglobulins from the maternal circulatory system due to the placenta's special, leading to functionally immature autoimmune systems that are easily attacked by various pathogens (Lopez and Heinrichs, 2022). An imbalance of the gastrointestinal microbiota tends to induce calf diarrhea. Currently, primary infectious agents responsible for pre-weaning diarrhea in calves include Enterotoxigenic *Escherichia coli* (ETEC), *Cryptosporidium parvum*, Rotavirus, Circovirus, and Coronavirus (Foster and Smith, 2009; Table 1). Preventing both pathogenic and non-pathogenic diarrhea during calf rearing is crucial, as illness during this stage can delay growth, impacting productivity and even resulting in death.

3.1. Infectious factors

3.1.1. ETEC

ETEC is a bacterial disease that infects newborn calves and is the leading cause of diarrhea after 4 days of birth (Ji et al., 2018). Although it rarely affects older calves or cows, severe contamination in the environment can lead to ingestion of ETEC and subsequent production of two virulence factors K99 fimbriae and heat-stable toxin (STa; Foster and Smith, 2009). The K99 antigen is expressed only at pH levels below 6.5, which STa is a primary mediator of ETEC and is secreted by many ETEC strains. Toxin production is limited when the environmental pH is below 7.0 (Saeed et al., 1986). Preventing contamination in the environment and maintaining a healthy pH level can help reduce the risk of ETEC infection in calves (Table 1). ETEC infects intestinal epithelial cells following ingestion and multiplies in the intestinal villi. The low pH of the distal small intestine creates a favorable environment for ETEC colonization, leading to villous atrophy and intrinsic damage to the lamellae (Francis et al., 1989). Upon reaching the ileum, ETEC expresses both K99 antigen and STa due to the increase in pH. Newborn calves, with higher proximal gastrointestinal tract (GIT) pH, can express the antigen more rapidly than older calves (Fossler et al., 2005). ETEC adhesion enables the bacteria to colonize and multiply in the ileum, subsequently spreading throughout the small intestine. Once established in the intestine, ETEC produces heat-stable toxins that cause secretory diarrhea (Foster and Smith, 2009).

3.1.2. *Salmonella*

Salmonella is a bacterial disease that affects calves primarily from 10 days to 3 months of age and also causes diarrhea in adult cattle and heifers. The clinical manifestations of *Salmonella* are characterized by watery and mucous diarrhea with fibrin and blood (Fossler et al., 2005; Table 1). The mechanisms of *Salmonella* virulence involve the invasion of the intestinal mucosa, multiplication in lymphoid tissues, and the ability to evade the host defense system, leading to systemic disease. The pathogenesis of *Salmonella* involves the invasion of intestinal epithelial cells, the ability to survive within macrophages, and the induction of intestinal pathogenicity (Tsolis et al., 1999). *Salmonella* can colonize various regions such as M cells, intestinal cells and tonsil tissue. Once the bacteria infect lymphoid tissues such as tonsil tissue, it can quickly disseminate throughout the body by invading monocytes and phagocytes (Holt, 2000; Reis et al., 2003).

3.1.3. Bovine rotavirus

Bovine rotavirus (BRV) is a significant pathogen that causes neonatal calf diarrhea, which can be severe enough to result in mortality among newborn calves (Jang et al., 2019). Typically, BRV affects calves 1–2 week old, and the short incubation period of 12–24 h cause acute diarrhea in calves. Following infection, calves can excrete significant amounts of the virus into their feces within 5 to 7 days,

TABLE 1 The main pathogens that cause diarrhea in calves and their mechanism of action.

Pathogens	Period of infection	Clinical symptoms	Mechanism of action	Citations
ETEC	Calves aged 4 days after birth	Watery diarrhea, passing pale yellow, gruel-like, foul-smelling stools	ETEC proliferates in the enterocytes of the intestinal villi, causing secretory diarrhea	Foster and Smith (2009)
Salmonella	Calves aged 10 days to 3 months	Watery and mucoid diarrhea, passing stools that are grayish-yellow liquid mixed with mucus and blood	*Salmonella* can invade the intestinal mucosa and proliferate in the lymphoid tissue, resulting in systemic disease	Tsolis et al. (1999)
BRV	1 to 2 weeks old calves	Severe watery diarrhea with undigested curds mixed in stool	BRV replicates in the cytoplasm of intestinal villous epithelial cells, which destroys intestinal epithelial cells and secretes viral enterotoxin, resulting in diarrhea caused by indigestion and absorption	Jang et al. (2019)
BCV	1 to 2 weeks old calves	Discharge yellow watery stool with respiratory symptoms	BCV attaches to intestinal epithelial cells through prickles and hemagglutinin glycoprotein, fusing the envelope of the virus with the cell membrane or endocytosis vesicle, leading to cell death	Hodnik et al. (2020)
C. parvum	Calves aged 4 days and 6 weeks	Severe diarrhea, gray white or yellow feces, containing a large amount of cellulose, blood and mucus	The direct cytotoxic effect of *C. parvum* on intestinal epithelial cells and apoptosis	Buret et al. (2003)

potentially contaminating the environment and facilitating the spread of the virus to other calves (Ammar et al., 2014). BRV infection mechanism involves replication within the cytoplasm of epithelial cells of the small intestinal villi. This result in the destruction of mature intestinal epithelial cells in the villi, activation of the enteric nervous system by the vasoactive component of the damaged cells, and the secretion of viral enterotoxins, which are the main factors contributing to rotavirus-induced dyspepsia or malabsorption leading to diarrhea (Table 1). Jang's study demonstrated that rotavirus infection has an impact on the diversity, homogeneity, and abundance of the intestinal microbiota, as well as on the membership and structure of the microbiota (Jang et al., 2019). Moreover, a study has shown that treatment with milk replacer-based probiotics is effective in preventing clinical signs of severe diarrhea and BRV infection in calves (Kayasaki et al., 2021).

3.1.4. Bovine coronavirus

Bovine coronavirus (BCV) causes calf enteritis in dairy and beef cattle. The age of the affected animals ranged from 1 day to about 3 months, with diarrhea usually occurring between 1 and 2 weeks of age and peaking between days 7 and 10 (Clark, 1993). Calves can become infected with BCV through the mouth and respiratory tract. Clinical signs usually appear 2 days after infection and last for 3 to 6 days (Hodnik et al., 2020; Table 1). Calves or calves lacking colostrum are particularly vulnerable to severe diarrhea caused by secondary coronaviruses. Coronavirus infection usually begins proximal to the small intestine and subsequently spreads throughout the small intestine (Vlasova and Saif, 2021). The virus attaches to intestinal epithelial cells via spikes and hemagglutinin glycoproteins, causing the viral envelope to fuse with cell membranes or intracellular vesicles (Schultze et al., 1991). The virus then replicates and induces cell death through release through normal secretory mechanisms, eventually leading to diarrhea (Clark, 1993).

3.1.5. *Cryptosporidium parvum*

C. parvum is a common gastrointestinal pathogen affecting dairy cows and immunocompromised individuals, has become a major cause of calf diarrhea worldwide (Mosier and Oberst, 2000). Calves between 4 days and 6 weeks old often experience digestive issues due to *C. parvum* (de Graaf et al., 1999). The parasite is typically transmitted via the fecal-oral route, causing acute diarrhea (Tzipori and Ward, 2002; Table 1). The life cycle of *C. parvum* involves five stages: trophozoite, schizonts, gametophyte, zygote and oocyst, of which the oocyst is the infection stage. *C. parvum* oocysts are highly infectious and can survive in the environment for extended periods of time. Thin-walled and thick-walled oocysts are the two types of oocysts. Sporozoites escape directly into the intestinal epithelium and form self-infection in the thin-walled oocysts, whereas thick-walled oocysts (approximately 80%) sporulate in the intestinal epithelium or intestinal lumen, and the formed sporozoites are excreted with the host feces (de Graaf et al., 1999). *C. parvum* infection can cause severe villi atrophy and crypt hyperplasia in calves and other animals. This is due to the loss of intestinal epithelial cells in the villi, resulting in secondary retraction of the villi to maintain the integrity of the epithelial barrier (Argenzio et al., 1990). The increase in epithelial cell loss during *C. parvum* infection is thought to result from the direct toxic effect of organisms on intestinal epithelial cells or cell apoptosis (Buret et al., 2003).

3.2. Non-infectious factors

Some management factors, such as colostrum feeding, housing, calving assistance, production system, herd size, season and micro-environmental hygiene, have a significant effect on calf morbidity and mortality (Fentie et al., 2020). It is well known that an adequate supply of colostrum and passive transfer of immuneoglobulins is very important for calf health. The quantity and quality of colostrum and the timing of the first colostrum feeding play an important role (Weaver et al., 2000). Calves have low immunity and are susceptible to various infections, especially when they do not consume enough colostrum (Gitau et al., 2010). Insufficient nutrition of calves will also affect the immunity of calves, thus affecting the incidence rate and mortality (Klein-Jöbstl et al., 2014). The study found that the incidence rate of diarrhea in cattle with large-scale breeding increased because greater housing density may lead to greater disease outbreaks. It is usually recommended to place cattle houses separately because it can reduce the load of pathogens (Frank and Kaneene, 1993). The length of time that cows and calves spend in the calving area is another factor that may affect the risk of diarrhea. Studies have shown that promptly clearing the area after each calving can significantly reduce the incidence of diarrhea in farm calves (Klein-Jöbstl et al., 2014).

4. Calf gastrointestinal microbiota

4.1. Calf gut microbiota

4.1.1. The importance of the gut microbiota in calves

The gut microbiota is made up of millions of genes that are essential for microbiota to survive in the gastrointestinal environment, with about 99% of them coming from bacteria (Qin et al., 2010). This fact demonstrates the importance of the gut microbiota and its indispensable role in maintaining the health and normal function of mammalian hosts (Gareau et al., 2010). The importance of gut microbiota in maintaining gastrointestinal development and function has been widely recognized, and differentiation and development of intestinal epithelial cells, mucosal layers, lymphoid structures, and immune cells are necessary for the presence of the gut microbiota (Malmuthuge and Guan, 2017). Furthermore, the establishment of the intestinal microbiota of neonatal calves is a complex process influenced by internal and external factors such as microbiota succession (Gomez et al., 2017). The symbiotic relationship between the gastrointestinal microbiota and the host is essential for maintaining mucosal immunity and defending against colonization of pathogens (Zeineldin et al., 2018). Studies have shown that the gut microbiota has a significant impact on the host immune system, and its presence early in development can have lasting effects on gut health in adults (Kerr et al., 2015). When the gut microbiota is disrupted, it leads to "ecological imbalances" that can lead to increased intestinal inflammation, impaired regulation of immune responses, reduced pathogens' ability to compete for nutrients, and in some cases, the restoration of normal microbiota contributes to recovery from such diseases (Rolhion and Chassaing, 2016; Gomez et al., 2017). Some studies have shown that restoring a healthy microbiota community is an effective way to prevent or treat gastrointestinal diseases. Microbial culture has been used in ruminant feeding as an alternative or

reduction of antibiotics for newborn calves, thereby improving cow growth performance, feed efficiency, daily gain and milk production (Krehbiel et al., 2003).

4.1.2. Microbiota colonization of calves in the gut

Microbiota colonization is influenced by the bidirectional interaction between the host and microbiota, as well as external factors such as the maternal microbiota, the delivery process, diet, and antibiotic usage. Regular exposure to host-specific microbiota is essential not only for the development and maturation of the mucosal immune system but also for the absorption of nutrients and the maintenance of the animal general health (Malmuthuge et al., 2015). Oxygen in the intestine is utilized to create a strictly anaerobic environment, allowing beneficial bacteria, such as *Bifidobacterium* and *Bacteroides*, the two primary types of bacteria found in the infant gut, to thrive and have a positive effect on the mucosal immune system (Jost et al., 2012). Initial microbiota colonization of the gut of calves is typically done by *Citrobacter*, *Lactococcus*, *Leuconostoc* and *Lactobacillus* shortly after birth, and the fecal microbiota of calves at 6 and 12 h after birth is generally quite similar. However, due to increased microbiota diversity, the composition of the intestinal microbiota changes significantly from 6 to 12 h after birth (Mayer et al., 2012).

Studies have shown that the similarity in fecal microbiota composition between individuals drops sharply after 24 h, suggesting that calves quickly establish a complex microbiota soon after birth. However, the individual differences observed in calves lessen with age, indicating that a more similar microbiota community is established in the calf gut (Mayer et al., 2012). Research has demonstrated that the relative abundance of *Bifidobacterium* and *Ruminococcus* tends to increase with advancing calf age, while the relative abundance of *Bacteroides* and *Lactobacillus* decreases as calf age increases (Malmuthuge et al., 2019; Figure 2). The lower bacterial diversity in diarrheic calves compared to healthy calves implies that the gut microbiota is related to the host health. Establishing a normal gut microbiota before weaning, which generally occurs within 7 weeks of birth, is essential for calf health and growth (Oikonomou et al., 2013). Therefore, it is necessary to cultivate a healthy gut microbiota in newborn calves in order to improve gut health and overall host health.

4.2. Rumen microbiota in calves

4.2.1. Rumen development

In ruminants, the rumen is an enlarged vestal lumen responsible for breaking down most food and is the source of major bacterial and fungal communities that ferment plant-derived lignocellulosic material and other substrates into short-chain organic acids, which are then absorbed by the host and contribute to the growth and maintenance of the animal (Flint et al., 2008). The structure and physiological traits of the rumen evolve with age, which is closely associated with the maturation of the rumen microbiota, as fermentation products participate in the formation of the villi lining the rumen wall (Beharka et al., 1998). Newborn calves have a much smaller proportion of the rumen compared to adult cows and the villi of the rumen wall, which it uses to absorb nutrients, have yet to form. During the animal lactation stage after birth, the rumen has no function and milk does not pass through it due to the reflexes of the esophageal groove. Therefore, the development of the rumen in newborn calves is necessary for optimal nutrient absorption and growth (Jami et al., 2013). As rumen development and microbiota colonization occurs, calves are physiologically transformed from pseudomonogastric animals to functional ruminants. It is notable that the development of the rumen in calves substantially affects feed intake, nutrient digestibility and overall growth. Even slight adjustments to early feeding programs and nutrition can have a major influence on the development of the rumen, leading to long-term impacts on the growth, health, and milk production of adult cattle. Therefore, the development of the rumen in newborn calves is one of the most critical aspects of calf nutrition (Diao et al., 2019).

4.2.2. Colonization of the rumen microbiota

The gastrointestinal tract of young ruminants is sterile at birth, and shortly after birth, a significant number of bacteria rapidly start to colonize the forestomach. Microbiota inoculation of the rumen of newborns can occur through the vaginal canal, feces, colostrum, skin and saliva. Members of typical functional rumen groups, such as methanogens, Fibrolytic bacteria or *Proteobacteria* were detected in calf rumen less than 20 min after birth, signifying that inoculation happened even before birth (Guzman et al., 2015). *Streptococcus* and *Enterococcus* had a role at the beginning of colonizing the rumen, assisting in the formation of a completely anaerobic environment and stimulating the fast development of strict anaerobic bacteria (Jami et al., 2013). It was found that cellulolytic bacteria and other bacterial species important for rumen function can be detected as early as 1 day after birth, demonstrating the establishment of these rumen bacteria prior to the introduction of concentrated feed to young ruminants (Jami et al., 2013). Some anaerobic genera, such as *Prevotella*, that were notably more abundant were identified as permanent members of the bacterial community in the mature rumen based on samples collected from 3-day-old calves (Stevenson and Weimer, 2007; Purushe et al., 2010). Thus, calf GIT colonization is initiated early in life and may even begin during parturition as in other mammals, however, this dynamic process changes greatly during the early life history of the animal (Dominguez-Bello et al., 2010; Figure 2). The findings revealed that the rumen of three-week-old calves had a substantial level of both *Bacteroides* and *Prevotella*, indicating that fermented feed might promote the maturation of the rumen microbiota community (Malmuthuge et al., 2014).

Members of the dominant populations in the rumen, the phylum *Bacteroides* and Firmicutes were also detected as the dominant populations in the fecal community compared to the rumen bacterial community (Whitford et al., 1998). In addition, the major Rumenic fibrogenic bacteria, such as *Fibrobacterium* and *Flavibacter*, increased with calf age, which may be related to the development of the digestive tract and increased fiber digestibility after birth (Uyeno et al., 2010). At two weeks of age, the rumen microbiota in calves becomes more diverse and consists of a greater abundance of long-lived bacterial species (Li et al., 2012). Another study revealed that the diversity of rumen microbiota, as well as the within-group similarity, increased with age. This suggests a transition of the rumen microbiota from less pronounced and heterogeneous communities to more diverse and homogeneous mature bacterial communities (Jami et al., 2013). Furthermore, a recent study further supports this point, suggesting that as animals grow older, the diversity of their intestinal flora

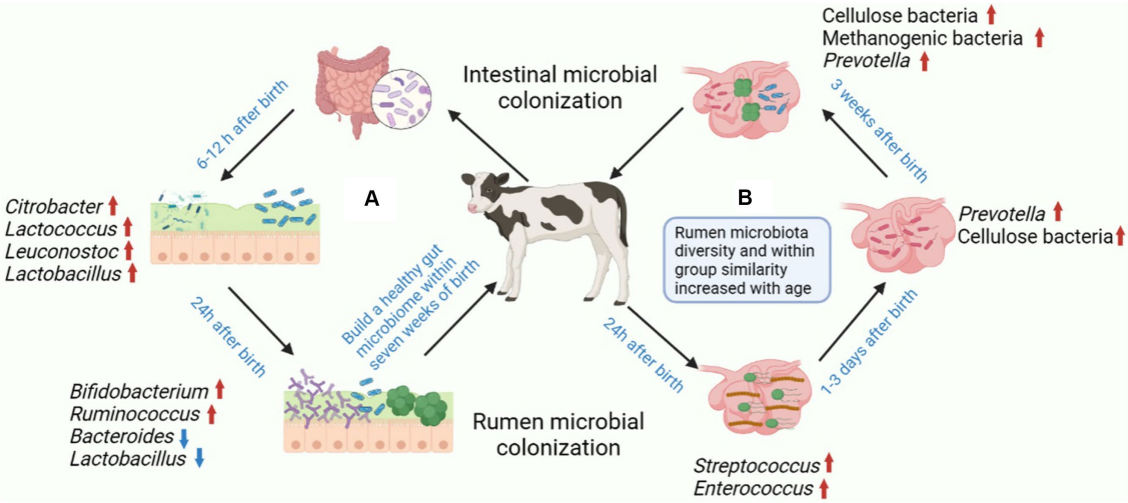

FIGURE 2
Postnatal colonization of gastrointestinal microbiota and establishment of healthy gastrointestinal microbiota in calves. **(A)** The colonization of gastrointestinal microbiota is a complex process, and the stable establishment of microorganisms is important for the host. The main colonizing bacteria in the intestines of calves 6–12 h after birth include *Citrobacter, Lactococcus, Leuconostoc* and *Lactobacillus*. The relative abundance of *Bifidobacterium* and *Ruminococcus* increased after 24 h after birth in calves, while the relative abundance of *Bacteroides* and *Lactobacillus* decreased with age. **(B)** The earliest colonizers of calf rumen were mainly *Streptococcus* and *Enterococcus*, which contributed to the transformation of the rumen into a completely anaerobic environment. The main form of rumen that can be detected in calves within 3 days of birth is *Prevotella*. Cellulose, *Methanogenic* bacteria and *Prevotella* can be detected in the rumen of calves aged 1–3 weeks. Figure was created in BioRender (http://biorender.io).

increases in terms of α diversity, but decreases in terms of β diversity (Dill-McFarland et al., 2017).

5. Early microbiota intervention to relieve calf diarrhea

5.1. Early supplement of probiotics to alleviate calf diarrhea

Probiotics are defined as living microorganisms that, if given in sufficient quantities, can provide health benefits to the host (Hill et al., 2014). Probiotics include bacteria, yeasts and fungi that have beneficial health effects on humans and animals. They have the ability to regulate the balance and activity of gastrointestinal microbiota, so they are considered beneficial to host animals and have been used as functional food (Uyeno et al., 2015). Currently, around 41% of pre-weaned heifers are using probiotics (Kim et al., 2011). Adding probiotics to the diet of young cattle can prevent imbalances in the intestinal microbiota, improve growth and prevent diarrhea, especially in stressful conditions (Frizzo et al., 2010).At present, the application of probiotics and symbiotic bacteria in young ruminants usually aims at the gastrointestinal system and minimizes the incidence of gastrointestinal diseases by stabilizing and enhancing the intestinal microbiota (Uyeno et al., 2015). Results from experiments indicate that supplementation with probiotic products can reduce the incidence of diarrhea and improve average daily gain and feed efficiency (Signorini et al., 2012).

Kim's research has demonstrated that direct feeding of microorganisms (including three strains of *Lactobacillus* and three *Bacillus* strains, as well as one strain of *Saccharomyces boulardii* and one strain of non-pathogenic *Escherichia coli*) as probiotics in calves after birth can significantly reduce the incidence of calf diarrhea (Kim et al., 2011; Table 2). Renaud et al. found that administering a multispecies probiotics and yeast bolus to calves at the onset of diarrhea reduced the duration of diarrhea (Renaud et al., 2019). Supplementation with a multispecies probiotic has been shown to reduce the incidence of diarrhea in neonatal calves from day 7 to 21 after birth (Wu et al., 2021). In addition, the difference of microbiota composition among young ruminants is higher than that of adult ruminants (Jami et al., 2013), and the intestinal microbiota of young ruminants may change more easily than that of adult ruminants in early life, making them more susceptible to probiotics (Abe et al., 1995). Compared with antibiotics in feed, probiotics in dairy substitutes can reduce diarrhea and serve as alternatives to antibiotics (Kim et al., 2011). Supplementing probiotics in dairy substitutes can help mitigate the adverse effects of the milk fed veal industry, especially when animals experience diarrhea (Villot et al., 2019). Thus, supplementing with probiotics offers an opportunity to improve early gut health and minimize calves' susceptibility to intestinal infections before weaning.

5.2. Fecal microbiota transplantation alleviates calf diarrhea

Fecal microbiota transplantation (FMT) is a promising treatment for dysbiosis related diseases. It involves transplanting the fecal contents of a healthy donor into a diseased patient with the aim of restoring a healthy gut microbiota and reshaping it to its original state (Khoruts and Sadowsky, 2016). FMT has been demonstrated to be an effective treatment for calf diarrhea (Borody and Khoruts, 2011). The gastrointestinal tract microbiota is essential for regulating host

TABLE 2 Alleviating calf diarrhea through early microbiological intervention.

Treatment	Microorganisms used	Effects	Citations
Direct feeding of probiotics	*Lactobacillus* *Bacillus* strains *Saccharomyces boulardii* non-pathogenic *Escherichia coli*	Reduced incidence of calf diarrhea	Kim et al. (2011)
Oral administration of microencapsulated *Lactobacillus*	*Lactobacillus* *Lactobacillus acidophilus*	Enhanced colonization of *Lactobacillus acidophilus* in the gut and reduced severity of diarrhea	Abu-Tarboush et al. (1996) and Khuntia and Chaudhary (2002)
Addition of *Bifidobacterium bifidum* to milk replacer	*Bifidobacterium bifidum*	Reduced diarrhea and improved weight gain	Sarkar and Mandal (2016)
Addition of *Saccharomyces cerevisiae* to milk substitutes	*Saccharomyces cerevisiae*	Promote the formation and transformation of rumen bacterial community and reduce the incidence of calf diarrhea	Krehbiel et al. (2003) and Villot et al. (2019)
Addition of *Bacillus subtilis* natto to calf feed	*Bacillus subtilis* natto	Improve the average daily weight gain and feed efficiency of calves, activate the immune system and enhance immunity	Sun et al. (2010)
Fecal microbiota transplantation	*Selenomonas* *Porphyromonadaceae*	Change intestinal microbiota and increase microbiota diversity and stability	Kim et al. (2021)
Rumen microbiota transplantation	*Succinivibrionaceae* *Prevotella*	Promote the establishment of early rumen microbiota	Bu et al. (2020)

mucosal immunity and nutrition, as well as providing resistance to pathogen colonization. Establishing a healthy microbiota is crucial for the growth and development of young animals, and when calves experience diarrhea, FMT using beneficial microbiota from healthy donors can help restore a healthy gut flora (Islam et al., 2022). Recent studies have reported that FMT is able to improve diarrhea in pre-weaned calves by modifying the intestinal microbiota and that FMT may have a potential role in improving calf growth and development (Kim et al., 2021; Table 2).

The gut microbiota has a profound impact on the development of young animals, and it has been demonstrated that changes in the gut microbiota early in life can have long-term effects on host health (Malmuthuge and Guan, 2017). Increased microbiota diversity and stability are important features of healthy calf gut microbiota development over time. Calf diarrhea can be predicted by changes in early gut microbiota to improve calf health (Ma et al., 2020). The relative abundance of *Enterobacteriaceae* bacteria in recipient calf feces at week 5 after FMT is low, suggesting that FMT may modulate gut microbiota composition early in life (Rosa et al., 2021). Studies have shown that FMT treatment in calves can decrease the occurrence of diarrhea and shift the intestinal environment of pre-weaned calves with diarrhea from an imbalanced to a symbiotic state. It was found that the intestinal microbiota of recipient calves gradually resembled that of donor calves after FMT treatment, and the relative abundance of *Porphyromonadaceae* was significantly increased, and its abundance was negatively correlated with the incidence of diarrhea, these results suggest that regulating the quantitative changes of *Porphyromonadaceae* in the calf intestine may be the key to solve diarrhea in calves (Kim et al., 2021). *Selenomonas* have also been found in successful FMT donors, suggesting that it may act as an iconic microorganism and potentially ensure donor-recipient compatibility (Islam et al., 2022). In conclusion, FMT has demonstrated effectiveness as a therapy for preventing and treating calf diarrhea through the repair of the intestinal microbiota. In addition to alleviating diarrhea symptoms, FMT may also aid in the identification of beneficial microorganisms and their functional metabolites, making it an important area for further research.

5.3. Early intervention of rumen microbiota promotes rumen development and relieves calf diarrhea

The early stages of a ruminant's life offer a window of opportunity to manipulate the rumen microbiota, which could have lasting effects on the health of adult cattle (Yáñez-Ruiz et al., 2015). Previous studies have demonstrated that repeated inoculation of adult rumen microbiota in pre-weaned calves with diarrhea can significantly reduce the frequency and duration of the condition. Analysis of the rumen microbiota showed that the rumen flora of recipient calves differed from that of the donors, with only one *Succinivibrionaceae* and five other *Prevotella* were found to have a predominance of OTUs. Therefore, rumen microbiota transplantation (RMT) could be an effective approach to preventing and reducing calf diarrhea in a herd (Bu et al., 2020; Table 2). Earlier studies have shown that individual bacteria introduced via inoculation can establish and persist in the rumen of lambs fed an aseptic diet or lambs with relatively simple rumen microbiota (Fonty et al., 1983, 1997). Early dietary intervention effectively regulated the development of rumen microbiota in dairy cows, though any long-term effect on milk production was not observed after the intervention was discontinued (Saro et al., 2018; Dill-McFarland et al., 2019). Early studies used rumen fluid or contents from adult cattle to inoculate calves have also revealed that while inoculation accelerates the colonization of rumen protozoa (Pounden and Hibbs, 1950). To further explore the effects of rumen microbiota transplantation, further studies should focus on the effects of donor-recipient matching in terms of age, species and diet. In addition, future studies should also analyze the interactions between the rumen microbiota and the host, and assess the long-term effects of early dietary intervention on milk production.

6. Mechanism of action of probiotics

6.1. Probiotics competitively inhibit colonization of intestinal pathogens

In addition to the direct effects, probiotics can also modify the intestinal environment in order to gain a competitive advantage. This can be achieved through nutritional competition, by lowering the pH through the production of inhibitory compounds, or by attaching to receptor sites in the gastrointestinal tract in order to prevent pathogen binding (Neeser et al., 2000; Dicks and Botes, 2010; Figure 3B). Certain probiotic strains have adhesion proteins on their cell surface that specifically bind to carbohydrate components of the mucous layer, such as the mannose-specific adhesion mechanism of *Lactobacillus plantarum* (Pretzer et al., 2005). Probiotics can also aggregate to form a protective barrier, thus preventing the colonization of pathogenic bacteria in the intestinal epithelium (Rolfe, 2000). In addition, the growth of pathogenic bacteria can also be inhibited by the competitive rejection of intestinal binding sites by probiotics (Mekonnen et al., 2020).

Furthermore, Probiotics can promote the production of beneficial substances, such as intestinal short-chain fatty acids (SCFAs). For example, acetic acid, which is produced by probiotic *Bifidobacterium* in the gastrointestinal tract, has been shown to reduce the risk of ETEC infection (Fukuda et al., 2011; Figure 3A). Additionally, *Lactobacillus*, such as *Lactobacillus acidophilus* and *Lactobacillus plantarum*, can metabolize complex carbohydrates, such as sugars (Walter, 2008), while *Bifidobacterium* can metabolize various plant dietary fibers using various depolymerizing enzymes (Rossi et al., 2005). The use of carbohydrates other than those used by intestinal pathogenic bacteria as a source of carbohydrates allows probiotics to expand their colonization area in the GIT, thus suppressing the spread of pathogenic bacteria.

6.2. Probiotics restore the intestinal barrier

The intestinal barrier is a complex system consisting of the mucus layer, epithelial cell and an underlying lamina propria. The physical barrier of intestinal microbiota is formed by tight junctions (TJ) multi-protein complexes. Disruption of the tight junctions increases epithelial permeability, leading to leaky gut. The gastrointestinal barrier is a critical defense mechanism for maintaining epithelial cell integrity, preventing pathogenic infections and reducing inflammation (van Zyl et al., 2020). Probiotics have been found to upregulate the synthesis of TJ proteins, such as ZO-1 and occludin, thus protecting the integrity of the intestinal barrier (La Fata et al., 2017). For example, *Lactobacillus rhamnosus* strain GG regulates the expression and distribution of ZO-1 and claudin-1 proteins, thereby preventing damage caused by Enterohemorrhagic *Escherichia coli* O157:H7 infection (Johnson-Henry et al., 2008; Figure 3C). Studies have also shown that the probiotic *Lactobacillus plantarum* ZLP001 can increase intestinal epithelial resistance to pathogens by sustaining TJ protein abundance (Wang et al., 2018). Furthermore, *in vitro* and *in vivo* studies on different cell lines and animal models have demonstrated that probiotics such as *Lactobacillus plantarum* MB452, *Lactobacillus casei*, *Lactobacillus rhamnosus* GG and *Lactobacillus reuteri* I5007 can impact trans-epithelial electrical resistance and epithelial permeability, as well as regulate the expression and distribution of TJ proteins (Eun et al., 2008; Anderson et al., 2010; Yang et al., 2015).

6.3. Probiotics regulate intestinal immunity

Probiotics have a significant impact on the host innate and adaptive immunity. They can regulate various immune cells such as dendritic cells (DCs), monocytes/macrophages, T and B lymphocytes, and improve the phagocytosis of invasive intestinal pathogens (Viaşu-Bolocan et al., 2013). Probiotics also trigger the anti-inflammatory response of the innate immune system by signaling DCs to secrete anti-inflammatory cytokines, such as IL-10 (Mirpuri et al., 2012; Figure 3D). When intestinal pathogens activate NF-κB and MAPK signal pathways, it triggers the secretion of pro-inflammatory cytokines, such as IL-8, and attract inflammatory immune cells like neutrophils to the infected site, which can lead to severe inflammation and tissue damage (Llewellyn and Foey, 2017; Figure 3D). However, studies have shown that probiotic strains can inhibit the production of pro-inflammatory cytokines. For example, *Lactobacillus casei* OLL2768 can suppress ETEC-induced pro-inflammatory responses by inhibiting NF-κB and reducing the levels of pro-inflammatory cytokines in the MAPK pathway (Finamore et al., 2014). Probiotics have been reported to enhance humoral immune responses by increasing the number of IgM, IgG and IgA secretory cells and the also stimulate non-specific immune responses by activating macrophages (Isolauri et al., 2001). Probiotic supplements are used to regulate the host immune response to potentially harmful antigens. Research studies have indicated that administering *Lactobacillus* probiotics orally can raise IgA levels in children experiencing diarrhea, which can lead to a reduction in the duration of the illness (Guarino et al., 1997; Figure 3D).

6.4. Probiotics directly regulate solute secretion and absorption

The intestinal epithelial cells play a crucial role in regulating solute levels, and disruptions in this process can lead to watery diarrhea. Cl-secretion and Na^+ transport, as well as uptake of Cl^- or HCO_3^-, are controlled by multiple lateral groups, apical channels and transporter proteins (Thiagarajah et al., 2015; Figure 3E). *Bacillus subtilis* CU1 has been shown to induce epithelial Na^+/H^+ exchange protein NHE3 expression and low levels of CFTR in mice, thereby promoting fluid absorption and reducing the risk of antibiotic-associated diarrhea (Urdaci et al., 2018). *Lactobacillus*, through its absorptive role, can also act as an antidiarrheal agent by increasing the function and expression of NHE3 (Singh et al., 2012). Probiotics, therefore, have the potential to alter intestinal electrolyte transport proteins, which are an effective mechanism to prevent antibiotic-associated diarrhea by maintaining the luminal solute concentration (Mekonnen et al., 2020).

7. Application of probiotics in calf diarrhea

7.1. *Lactobacillus*

Lactobacillus is a probiotic that inhibits the growth of pathogenic bacteria in the intestine by lowering pH and competitive attachment in the intestine (Riddell et al., 2008). Research has shown that adding *Lactobacillus* to calf diets can reduce the incidence of calf diarrhea (Abu-Tarboush et al., 1996). *Lactobacillus* also increases the total

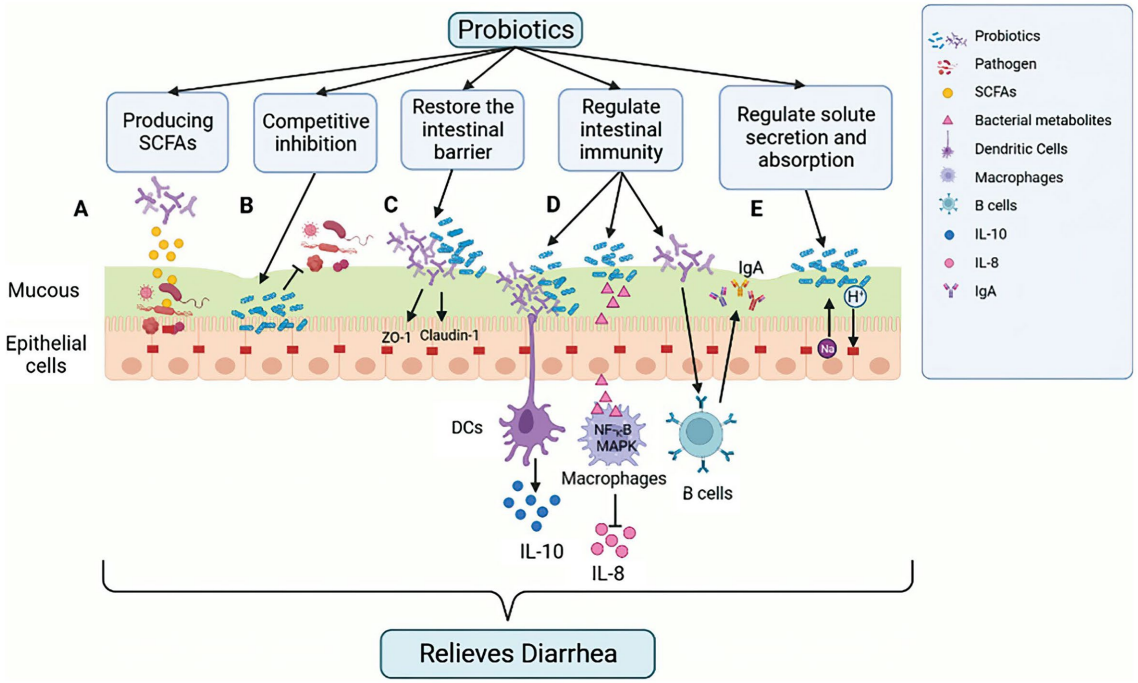

FIGURE 3
Mechanism of action of probiotics in alleviating GIT diarrhea-related inflammation in calves. Probiotics exert their effect by impacting the integrity of intestinal epithelial cells (IECs) through several mechanisms. **(A)** Production of SCFAs, lowering intestinal pH, creating a favorable environment for beneficial bacteria, and inhibiting pathogenic bacterial growth. **(B)** Competitive binding to intestinal binding sites by some probiotics, which can hinder the growth of pathogenic bacteria. **(C)** Enhancing the stability of intercellular TJ proteins, which reduces intercellular permeability of IECs to pathogens and other antigens. **(D)** Regulating the host innate and adaptive immune response, such as triggering anti-inflammatory cytokines (IL-10) secretion from DCs, inhibiting NF-κB, and reducing the levels of pro-inflammatory cytokines (IL-8) in MAPK pathway to suppress the pro-inflammatory response induced by ETEC. Probiotics can also stimulate B cells to increase the amount of IgA secreted, thus enhancing the humoral immune response and inducing an anti-inflammatory effect. **(E)** Reducing the risk of antibiotic-related diarrhea by promoting liquid absorption through the exchange of Na^+ and H^+ in epithelial cells. Figure was created in biorender (http://biorender.io).

concentration of serum immunoglobulins, suggesting that it has a positive effect on calf health (Al-Saiady, 2010). Additionally, multiple *Lactobacillus* species induced greater weight gain in less healthy calves, and calves-specific probiotic treatment reduced the incidence of diarrhea and fecal coliforms counts, as reported by health scores (Timmerman et al., 2005). Supplementation of *Lactobacillus acidophilus* in 1-day-old calves has also been found to reduce the incidence and severity of diarrhea (Khuntia and Chaudhary, 2002; Table 2). Two of the most abundant *Lactobacillus* species found in healthy calves, *Lactobacillus reuteri* and *Lactobacillus johnsonii*, are being studied as potential probiotics to improve gastrointestinal health in pre-weaned calves (Fan et al., 2021).

7.2. *Bifidobacterium*

Administering *Bifidobacterium* and *Lactobacillus* to calves during the first week after birth can have significant benefits, including increased weight, improved feed conversion rates and reduced incidence of diarrhea (Abe et al., 1995). These findings suggest that these two types of bacteria are essential for maintaining intestinal balance and preventing infections in the intestines. *Bifidobacterium*, in particular, is one of the earliest and most abundant bacteria to colonize the neonatal gut and can provide various benefits to gut, metabolic and immune health. *Bifidobacterium bifidum* has been found to synthesize extracellular polysaccharides that can inhibit the growth of pathogens and protect the host epithelial cells from invasion (Sarkar and Mandal, 2016; Table 2). Recent results on FMT between young and old mice suggest that *Bifidobacterium animalis* is associated with promoting intestinal tract and parenteral health and aging in young and older mice receiving young donor microbiotas (Sarkar and Mandal, 2016). Therefore, *Bifidobacterium* may play a crucial role in preventing calf diarrhea. Studies have revealed that *Bifidobacterium pseudocatenulatum* LI09 and *Bifidobacterium catenulatum* LI10 can potentially alleviate liver injury in mice by reducing intestinal dysbiosis and preserving intestinal barrier function. By improving intestinal barrier function, the translocation of bacteria can be reduced, leading to a downregulation of an overactive immune response, ultimately preventing liver injury (Fang et al., 2017). Furthermore, research indicates that *Bifidobacterium* can significantly antagonize *Enterobacteriaceae* and *Enterococcus* by producing acetate, potentially protecting neonates from enteropathogenic infections (Nagpal et al., 2017).

7.3. *Faecalibacterium*

Research has found that *Faecalibacterium* is a microbiota biomarker present in calf feces, which has been associated to a decrease in the incidence of diarrhea and an increase in body weight

during the first few weeks of life (Oikonomou et al., 2013). The administration of *Faecalibacterium prausnitzii* orally has been shown to be effective in reducing diarrhea incidence and related mortality in pre-weaning calves for up to 7 weeks after birth (Foditsch et al., 2015). *Faecalibacterium* is one of the major producers of butyrate in the large intestinal, butyrate enhances the integrity of the intestinal epithelial barrier. The relative abundance of *Faecalibacterium* has been found to be negatively correlated with the incidence of diarrhea in calves, suggesting that a high prevalence of *Faecalibacterium* early in the species may reduce susceptibility to intestinal infections (Oikonomou et al., 2013). Uyeno's research has shown that a high abundance of *Faecalibacterium* in calves is associated with a low incidence of diarrhea in the first 4 weeks after birth. *Faecalibacterium* has an anti-inflammatory effect due to its secreted metabolites that can inhibit the activation of NF-κB and reduce the production of IL-8 (Oikonomou et al., 2013).

7.4. *Saccharomyces cerevisiae*

Saccharomyces cerevisiae, a type of yeast, has been found to be particularly beneficial for maintaining gut health in milk-fed calves. It can reduce the abundance of *Clostridium difficile*, the main pathogen associated with infectious diarrhea in calves, and help combat other pathogenic microorganisms (Kim et al., 2011). Research has shown that administering multiple probiotic and yeast pellets to calves during diarrhea can reduce the duration of diarrhea (Renaud et al., 2019). Supplementing calves with probiotics, including *Saccharomyces cerevisiae*, before and after weaning can also promote the formation and transition of rumen bacterial communities, facilitating the transition from liquid to dry feeds (Krehbiel et al., 2003). The fermentation products of *Saccharomyces cerevisiae* have been shown to enhance rumen morphology and exert a beneficial effect on the composition of the rumen microbiota (Xiao et al., 2016). Adding *Saccharomyces cerevisiae* to milk substitutes and concentrate has been found to reduce the incidence of diarrhea in calves and to maintain a healthy gut microbiota composition, which is important for maintaining gut health (Villot et al., 2019; Table 2). These findings suggest that *Saccharomyces cerevisiae* can help calves overcome the challenging times of early life in the milk-fed calf industry.

7.5. *Bacillus subtilis*

Bacillus subtilis has been found to have multiple benefits for calves, including altering the rumen microbiota, improving digestion at weaning and decreasing the severity of diarrhea (Ushakova et al., 2013). Kim's research also supports the use of probiotics, including three strains of *Lactobacillus*, three strains of *Bacillus*, one strain of *Saccharomyces boulardii* and one strain of non-pathogenic *Escherichia coli*, for reducing the incidence of calf diarrhea when directly fed postnatally (Kim et al., 2011). Feeding the calves directly with *Bacillus subtilis* before weaning can improve the growth performance of calves, including average daily gain and feed efficiency. In addition, the *Bacillus subtilis* natto can also induce the secretion of serum IgG and Th1 cytokines in calves, including IFN-γ, This helps to activate the immune system and enhance immunity (Sun et al., 2010; Table 2). These findings suggest that *Bacillus subtilis* can be a useful probiotic for promoting calf health and reducing the risk of diarrhea.

8. Discussion and prospect

Controlling the intestinal microbiota through direct feeding of microorganisms, probiotics has been extensively researched in animal husbandry to modify rumen fermentation and prevent pathogen colonization. This approach has led to improving production and better health outcomes in animals. Among these interventions, oral probiotics have been found to be an effective means of alleviating diarrhea in calves, with numerous studies demonstrating their ability to restore dysbiosis of the intestinal microbiota. Therefore, it is important to explore the potential of various beneficial bacteria in the early development of the intestinal tract to enhance the host health. By establishing a microbiota community dominated by beneficial bacteria, the colonization of intestinal pathogens after birth may be impeded, thereby safeguarding the immune system of infant ruminants from intestinal infections.

9. Conclusion

Diarrhea is the leading cause of death in calves during the first month of life. The gut microbiota of newborn calves changes in the early postnatal period, and homeostasis of the gut microbiota ecosystem is critical for maintaining gastrointestinal function in calves until early weaning. Therefore, it is very important to establish a healthy calf gastrointestinal microbiota using early microbiota intervention methods. Supplementation with probiotics is an effective way to improve early gut health and minimize the susceptibility of calves to gut infections before weaning, thereby reducing diarrhea rates in calves and potentially affecting calf growth and development long-term effects.

Author contributions

QX and WD conceptualized the review. WD wrote this manuscript. MH, JH, YD, WS, LY, XW, LX, and QX revised the main manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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References

Abe, F., Ishibashi, N., and Shimamura, S. (1995). Effect of administration of bifidobacteria and lactic acid bacteria to newborn calves and piglets. *J. Dairy Sci.* 78, 2838–2846. doi: 10.3168/jds.S0022-0302(95)76914-4

Abu-Tarboush, H. M., Al-Saiady, M. Y., and El-Din, A. (1996). Evaluation of diet containing lactobacilli on performance. *Fec Anim. Feed Sci.* 57, 39–49. doi: 10.1016/0377-8401(95)00850-0

Aghakeshmiri, F., Azizzadeh, M., Farzaneh, N., and Gorjidooz, M. (2017). Effects of neonatal diarrhea and other conditions on subsequent productive and reproductive performance of heifer calves. *Vet. Res. Commun.* 41, 107–112. doi: 10.1007/s11259-017-9678-9

Al-Saiady, M. Y. (2010). Effect of probiotic Bacteria on immunoglobulin G concentration and other blood components of newborn calves. *J. Anim. Vet. Adv.* 9, 604–609. doi: 10.3923/javaa.2010.604.609

Ammar, S. S. M., Mokhtaria, K., Tahar, B. B., Amar, A. A., Redha, B. A., Yuva, B., et al. (2014). Prevalence of rotavirus (GARV) and coronavirus (BCoV) associated with neonatal diarrhea in calves in western Algeria. *Asian Pac. J. Trop. Biomed.* 4, S318–S322. doi: 10.12980/apjtb.4.2014c778

Anderson, R. C., Cookson, A. L., McNabb, W. C., Park, Z., McCann, M. J., Kelly, W. J., et al. (2010). *Lactobacillus plantarum* MB452 enhances the function of the intestinal barrier by increasing the expression levels of genes involved in tight junction formation. *BMC Microbiol.* 10:316. doi: 10.1186/1471-2180-10-316

Argenzio, R. A., Liacos, J. A., Levy, M. L., Meuten, D. J., Lecce, J. G., and Powell, D. W. (1990). Villous atrophy, crypt hyperplasia, cellular infiltration, and impaired glucose-Na absorption in enteric cryptosporidiosis of pigs. *Gastroenterology* 98, 1129–1140. doi: 10.1016/0016-5085(90)90325-u

Beharka, A. A., Nagaraja, T. G., Morrill, J. L., Kennedy, G. A., and Klemm, R. D. (1998). Effects of form of the diet on anatomical, microbial, and fermentative development of the rumen of neonatal calves. *J. Dairy Sci.* 81, 1946–1955. doi: 10.3168/jds.S0022-0302(98)75768-6

Borody, T. J., and Khoruts, A. (2011). Fecal microbiota transplantation and emerging applications. *Nat. Rev. Gastroenterol. Hepatol.* 9, 88–96. doi: 10.1038/nrgastro.2011.244

Bu, D., Zhang, X., Ma, L., Park, T., Wang, L., Wang, M., et al. (2020). Repeated inoculation of young calves with rumen microbiota does not significantly modulate the rumen prokaryotic microbiota consistently but decreases diarrhea. *Front. Microbiol.* 11:1403. doi: 10.3389/fmicb.2020.01403

Buret, A. G., Chin, A. C., and Scott, K. G. (2003). Infection of human and bovine epithelial cells with *Cryptosporidium andersoni* induces apoptosis and disrupts tight junctional ZO-1: effects of epidermal growth factor. *Int. J. Parasitol.* 33, 1363–1371. doi: 10.1016/s0020-7519(03)00138-3

Chen, L., Li, S., Zheng, J., Li, W., Jiang, X., Zhao, X., et al. (2018). Effects of dietary *Clostridium butyricum* supplementation on growth performance, intestinal development, and immune response of weaned piglets challenged with lipopolysaccharide. *J. Anim. Sci. Biotechnol.* 9:62. doi: 10.1186/s40104-018-0275-8

Cho, Y. I., and Yoon, K. J. (2014). An overview of calf diarrhea - infectious etiology, diagnosis, and intervention. *J. Vet. Sci.* 15, 1–17. doi: 10.4142/jvs.2014.15.1.1

Clark, M. A. (1993). Bovine coronavirus. *Br. Vet. J.* 149, 51–70. doi: 10.1016/S0007-1935(05)80210-6

Dall Agnol, A. M., Lorenzetti, E., Leme, R. A., Ladeia, W. A., Mainardi, R. M., Bernardi, A., et al. (2021). Severe outbreak of bovine neonatal diarrhea in a dairy calf rearing unit with multifactorial etiology. *Braz. J. Microbiol.* 52, 2547–2553. doi: 10.1007/s42770-021-00565-5

de Graaf, D. C., Vanopdenbosch, E., Ortega-Mora, L. M., Abbassi, H., and Peeters, J. E. (1999). A review of the importance of cryptosporidiosis in farm animals. *Int. J. Parasitol.* 29, 1269–1287. doi: 10.1016/s0020-7519(99)00076-4

Diao, Q., Zhang, R., and Fu, T. (2019). Review of strategies to promote rumen development in calves. *Animals. (Basel)* 9:490. doi: 10.3390/ani9080490

Dicks, L. M. T., and Botes, M. (2010). Probiotic lactic acid bacteria in the gastro-intestinal tract: health benefits, safety and mode of action. *Benef. Microbes.* 1, 11–29. doi: 10.3920/BM2009.0012

Dill-McFarland, K. A., Breaker, J. D., and Suen, G. (2017). Microbial succession in the gastrointestinal tract of dairy cows from 2 weeks to first lactation. *Sci. Rep.* 7:40864. doi: 10.1038/srep40864

Dill-McFarland, K. A., Weimer, P. J., Breaker, J. D., Suen, G., and Dudley, E. G. (2019). Diet influences early microbiota development in dairy calves without long-term impacts on Milk production. *Appl. Environ. Microbiol.* 85:e02141-18. doi: 10.1128/aem.02141-18

Dominguez-Bello, M. G., Costello, E. K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., et al. (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc. Natl. Acad. Sci. U. S. A.* 107, 11971–11975. doi: 10.1073/pnas.1002601107

Eun, C. S., Han, D. S., Cho, H. S., Byun, T. J., Kim, T. Y., Jeon, Y. C., et al. (2008). M1737 Lactobacillus GG prevent impaired barrier function in intestinal epithelial cells. *Gastroenterology* 134, A–408. doi: 10.1016/S0016-5085(08)61907-1

Fan, P., Kim, M., Liu, G., Zhai, Y., Liu, T., Driver, J. D., et al. (2021). The gut microbiota of newborn calves and influence of potential probiotics on reducing diarrheic disease by inhibition of pathogen colonization. *Front. Microbiol.* 12:772863. doi: 10.3389/fmicb.2021.772863

Fang, D., Shi, D., Lv, L., Gu, S., Wu, W., Chen, Y., et al. (2017). *Bifidobacterium pseudocatenulatum* LI09 and *Bifidobacterium catenulatum* LI10 attenuate D-galactosamine-induced liver injury by modifying the gut microbiota. *Sci. Rep.* 7:8770. doi: 10.1038/s41598-017-09395-8

Fentie, T., Guta, S., Mekonen, G., Temesgen, W., Melaku, A., Asefa, G., et al. (2020). Assessment of major causes of calf mortality in urban and Periurban dairy production system of Ethiopia. *Vet. Med. Int.* 2020, 3075429–3075427. doi: 10.1155/2020/3075429

Finamore, A., Roselli, M., Imbinto, A., Seeboth, J., Oswald, I. P., and Mengheri, E. (2014). *Lactobacillus amylovorus* inhibits the TLR4 inflammatory signaling triggered by enterotoxigenic *Escherichia coli* via modulation of the negative regulators and involvement of TLR2 in intestinal Caco-2 cells and pig explants. *PLoS One* 9:e94891. doi: 10.1371/journal.pone.0094891

Flint, H. J., Bayer, E. A., Rincon, M. T., Lamed, R., and White, B. A. (2008). Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. *Nat. Rev. Microbiol.* 6, 121–131. doi: 10.1038/nrmicro1817

Foditsch, C., Pereira, R. V., Ganda, E. K., Gomez, M. S., Marques, E. C., Santin, T., et al. (2015). Oral Administration of *Faecalibacterium prausnitzii* decreased the incidence of severe diarrhea and related mortality rate and increased weight gain in Preweaned dairy heifers. *PLoS One* 10:e0145485. doi: 10.1371/journal.pone.0145485

Fonty, G., Gouet, P., Jouany, J. P., and Senaud, J. (1983). Ecological factors determining establishment of cellulolytic bacteria and protozoa in the rumens of meroxenic lambs. *J. Gen. Microbiol.* 129, 213–223. doi: 10.1099/00221287-129-1-213

Fonty, G., Williams, A. G., Bonnemoy, F., Morvan, B., Withers, S. E., and Gouet, P. (1997). Effect of *Methanobrevibacter* sp MF1 inoculation on glycoside hydrolase and polysaccharide depolymerase activities, wheat straw degradation and volatile fatty acid concentrations in the rumen of gnotobiotically-reared lambs. *Anaerobe* 3, 383–389. doi: 10.1006/anae.1997.0128

Fossler, C. P., Wells, S. J., Kaneene, J. B., Ruegg, P. L., Warnick, L. D., Bender, J. B., et al. (2005). Herd-level factors associated with isolation of Salmonella in a multi-state study of conventional and organic dairy farms. *Pre. Vet. Med.* 70, 279–291. doi: 10.1016/j.prevetmed.2005.04.002

Foster, D. M., and Smith, G. W. (2009). Pathophysiology of diarrhea in calves. *Vet. Clin. North Am. Food Anim. Pract.* 25:13-+. doi: 10.1016/j.cvfa.2008.10.013

Francis, D. H., Allen, S. D., and White, R. D. (1989). Influence of bovine intestinal fluid on the expression of K99 pili by *Escherichia coli*. *Am. J. Vet. Res.* 50, 822–826.

Frank, N. A., and Kaneene, J. B. (1993). Management risk factors associated with calf diarrhea in Michigan dairy herds. *J. Dairy Sci.* 76, 1313–1323. doi: 10.3168/jds.S0022-0302(93)77462-7

Frizzo, L. S., Soto, L. P., Zbrun, M. V., Bertozzi, E., Sequeira, G., Armesto, R. R., et al. (2010). Lactic acid bacteria to improve growth performance in young calves fed milk replacer and spray-dried whey powder. *Anim. Feed. Sci. Tech.* 157, 159–167. doi: 10.1016/j.anifeedsci.2010.03.005

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

Gareau, M. G., Sherman, P. M., and Walker, W. A. (2010). Probiotics and the gut microbiota in intestinal health and disease. *Nat. Rev. Gastroenterol. Hepatol.* 7, 503–514. doi: 10.1038/nrgastro.2010.117

Gensollen, T., Iyer, S. S., Kasper, D. L., and Blumberg, R. S. (2016). How colonization by microbiota in early life shapes the immune system. *Science* 352, 539–544. doi: 10.1126/science.aad9378

Gitau, G. K., Aleri, J. W., Mbuthia, P. G., and Mulei, C. M. (2010). Causes of calf mortality in peri-urban area of Nairobi, Kenya. *Trop. Anim. Health. Prod.* 42, 1643–1647. doi: 10.1007/s11250-010-9614-2

Gomez, D. E., Arroyo, L. G., Costa, M. C., Viel, L., and Weese, J. S. (2017). Characterization of the fecal bacterial microbiota of healthy and diarrheic dairy calves. *J. Vet. Intern. Med.* 31, 928–939. doi: 10.1111/jvim.14695

Guarino, A., Canani, R. B., Spagnuolo, M. I., Albano, F., and Di Benedetto, L. (1997). Oral bacterial therapy reduces the duration of symptoms and of viral excretion in children with mild diarrhea. *J. Pediatr. Gastroenterol. Nutr.* 25, 516–519. doi: 10.1097/00005176-199711000-00005

Gulliksen, S. M., Jor, E., Lie, K. I., Hamnes, I. S., Løken, T., Åkerstedt, J., et al. (2009). Enteropathogens and risk factors for diarrhea in Norwegian dairy calves. *J. Dairy Sci.* 92, 5057–5066. doi: 10.3168/jds.2009-2080

Guzman, C. E., Bereza-Malcolm, L. T., De Groef, B., and Franks, A. E. (2015). Presence of selected methanogens, Fibrolytic Bacteria, and Proteobacteria in the gastrointestinal tract of neonatal dairy calves from birth to 72 hours. *PLoS One* 10:e0133048. doi: 10.1371/journal.pone.0133048

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). Expert consensus document: the international scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11, 506–514. doi: 10.1038/nrgastro.2014.66

Hodnik, J. J., Ježek, J., and Starič, J. (2020). Coronaviruses in cattle. *Trop. Anim. Health Prod.* 52, 2809–2816. doi: 10.1007/s11250-020-02354-y

Holt, P. S. (2000). *Host Susceptibility, Resistance and Immunity to Salmonella in Animals*. Salmonella in Domestic Animals.

Islam, J., Tanimizu, M., Shimizu, Y., Goto, Y., Ohtani, N., Sugiyama, K., et al. (2022). Development of a rational framework for the therapeutic efficacy of fecal microbiota transplantation for calf diarrhea treatment. *Microbiome* 10:31. doi: 10.1186/s40168-021-01217-4

Isolauri, E., Sütas, Y., Kankaanpää, P., Arvilommi, H., and Salminen, S. (2001). Probiotics: effects on immunity. *Am. J. Clin. Nutr.* 73, 444s–450s. doi: 10.1093/ajcn/73.2.444s

Jami, E., Israel, A., Kotser, A., and Mizrahi, I. (2013). Exploring the bovine rumen bacterial community from birth to adulthood. *ISME J.* 7, 1069–1079. doi: 10.1038/ismej.2013.2

Jang, J. Y., Kim, S., Kwon, M. S., Lee, J., Yu, D. H., Song, R. H., et al. (2019). Rotavirus-mediated alteration of gut microbiota and its correlation with physiological characteristics in neonatal calves. *J. Microbiol.* 57, 113–121. doi: 10.1007/s12275-019-8549-1

Ji, S., Jiang, T., Yan, H., Guo, C., Liu, J., Su, H., et al. (2018). Ecological restoration of antibiotic-disturbed gastrointestinal microbiota in foregut and hindgut of cows. *Front. Cell. Infect. Microbiol.* 8:79. doi: 10.3389/fcimb.2018.00079

Johnson-Henry, K. C., Donato, K. A., Shen-Tu, G., Gordanpour, M., and Sherman, P. M. (2008). *Lactobacillus rhamnosus* strain GG prevents enterohemorrhagic *Escherichia coli* O157:H7-induced changes in epithelial barrier function. *Infect. Immun.* 76, 1340–1348. doi: 10.1128/iai.00778-07

Jost, T., Lacroix, C., Braegger, C. P., and Chassard, C. (2012). New insights in gut microbiota establishment in healthy breast fed neonates. *PLoS One* 7:e44595. doi: 10.1371/journal.pone.0044595

Kayasaki, F., Okagawa, T., Konnai, S., Kohara, J., Sajiki, Y., Watari, K., et al. (2021). Direct evidence of the preventive effect of milk replacer–based probiotic feeding in calves against severe diarrhea. *Vet. Microbiol.* 254:108976. doi: 10.1016/j.vetmic.2020.108976

Kerr, C. A., Grice, D. M., Tran, C. D., Bauer, D. C., Li, D., Hendry, P., et al. (2015). Early life events influence whole-of-life metabolic health via gut microflora and gut permeability. *Crit. Rev. Microbiol.* 41, 326–340. doi: 10.3109/1040841x.2013.837863

Khoruts, A., and Sadowsky, M. J. (2016). Understanding the mechanisms of faecal microbiota transplantation. *Nat. Rev. Gastroenterol. Hepatol.* 13, 508–516. doi: 10.1038/nrgastro.2016.98

Khuntia, A., and Chaudhary, L. C. (2002). Performance of male crossbred calves as influenced by substitution of grain by wheat bran and the addition of lactic acid Bacteria to diet. *Asian-australas. J. Anim. Sci.* 15, 188–194. doi: 10.5713/ajas.2002.188

Kim, M. K., Lee, H. G., Park, J. A., Kang, S. K., and Choi, Y. J. (2011). Effect of feeding direct-fed microbial as an alternative to antibiotics for the prophylaxis of calf diarrhea in Holstein calves. *Asian-Australas. J. Anim. Sci.* 24, 643–649. doi: 10.5713/ajas.2011.10322

Kim, H. S., Whon, T. W., Sung, H., Jeong, Y.-S., Jung, E. S., Shin, N.-R., et al. (2021). Longitudinal evaluation of fecal microbiota transplantation for ameliorating calf diarrhea and improving growth performance. *Nat. Commun.* 12:161. doi: 10.1038/s41467-020-20389-5

Klein-Jöbstl, D., Iwersen, M., and Drillich, M. (2014). Farm characteristics and calf management practices on dairy farms with and without diarrhea: a case-control study to investigate risk factors for calf diarrhea. *J. Dairy Sci.* 97, 5110–5119. doi: 10.3168/jds.2013-7695

Krehbiel, C. R., Rust, S. R., Zhang, G., and Gilliland, S. E. (2003). Bacterial direct-fed microbials in ruminant diets: performance response and mode of action. *J. Anim. Sci.* 81. doi: 10.2527/2003.8114_suppl_2E120x

La Fata, G., Weber, P., and Mohajeri, M. H. (2017). Probiotics and the gut immune system: indirect regulation. *Probiotics Antimicrob. Proteins.* 10, 11–21. doi: 10.1007/s12602-017-9322-6

Li, R. W., Connor, E. E., Li, C., Baldwin Vi, R. L., and Sparks, M. E. (2012). Characterization of the rumen microbiota of pre-ruminant calves using metagenomic tools. *Environ. Microbiol.* 14, 129–139. doi: 10.1111/j.1462-2920.2011.02543.x

Llewellyn, A., and Foey, A. (2017). Probiotic modulation of innate cell pathogen sensing and signaling events. *Nutrients* 9:1156. doi: 10.3390/nu9101156

Lopez, A. J., and Heinrichs, A. J. (2022). Invited review: the importance of colostrum in the newborn dairy calf. *J. Dairy Sci.* 105, 2733–2749. doi: 10.3168/jds.2020-20114

Ma, T., Villot, C., Renaud, D., Skidmore, A., Chevaux, E., Steele, M., et al. (2020). Linking perturbations to temporal changes in diversity, stability, and compositions of neonatal calf gut microbiota: prediction of diarrhea. *ISME J.* 14, 2223–2235. doi: 10.1038/s41396-020-0678-3

Malmuthuge, N., Griebel, P. J., and Guan, L. L. (2014). Taxonomic identification of commensal Bacteria associated with the mucosa and Digesta throughout the gastrointestinal tracts of preweaned calves. *Appl. Environ. Microbiol.* 80, 2021–2028. doi: 10.1128/aem.03864-13

Malmuthuge, N., Griebel, P. J., and Guan le, L. (2015). The gut microbiome and its potential role in the development and function of newborn calf gastrointestinal tract. *Front. Vet. Sci.* 2:36. doi: 10.3389/fvets.2015.00036

Malmuthuge, N., and Guan, L. L. (2017). Understanding the gut microbiome of dairy calves: opportunities to improve early-life gut health. *J. Dairy Sci.* 100, 5996–6005. doi: 10.3168/jds.2016-12239

Malmuthuge, N., Liang, G., Griebel, P. J., and Guan, L. L. (2019). Taxonomic and functional compositions of the small intestinal microbiome in neonatal calves provide a framework for understanding early life gut health. *Appl. Environ. Microbiol.* 85:e02534-18. doi: 10.1128/aem.02534-18

Mayer, M., Abenthum, A., Matthes, J. M., Kleeberger, D., Ege, M. J., Hölzel, C., et al. (2012). Development and genetic influence of the rectal bacterial flora of newborn calves. *Vet. Microbiol.* 161, 179–185. doi: 10.1016/j.vetmic.2012.07.023

Mekonnen, S. A., Merenstein, D., Fraser, C. M., and Marco, M. L. (2020). Molecular mechanisms of probiotic prevention of antibiotic-associated diarrhea. *Curr. Opin. Biotech.* 61, 226–234. doi: 10.1016/j.copbio.2020.01.005

Mirpuri, J., Sotnikov, I., Myers, L., Denning, T. L., Yarovinsky, F., Parkos, C. A., et al. (2012). *Lactobacillus rhamnosus* (LGG) regulates IL-10 signaling in the developing murine colon through upregulation of the IL-10R2 receptor subunit. *PLoS One* 7:e51955. doi: 10.1371/journal.pone.0051955

Mosier, D. A., and Oberst, R. D. (2000). Cryptosporidiosis. A global challenge. *Ann. N. Y. Acad. Sci.* 916, 102–111. doi: 10.1111/j.1749-6632.2000.tb05279.x

Nagpal, R., Kurakawa, T., Tsuji, H., Takahashi, T., Kawashima, K., Nagata, S., et al. (2017). Evolution of gut Bifidobacterium population in healthy Japanese infants over the first three years of life: a quantitative assessment. *Sci. Rep.* 7:10097. doi: 10.1038/s41598-017-10711-5

Neeser, J. R., Granato, D., Rouvet, M., Servin, A., Teneberg, S., and Karlsson, K. A. (2000). *Lactobacillus johnsonii* La1 shares carbohydrate-binding specificities with several enteropathogenic bacteria. *Glycobiology* 10, 1193–1199. doi: 10.1093/glycob/10.11.1193

Oikonomou, G., Teixeira, A. G., Foditsch, C., Bicalho, M. L., Machado, V. S., and Bicalho, R. C. (2013). Fecal microbial diversity in pre-weaned dairy calves as described by pyrosequencing of metagenomic 16S rDNA. Associations of Faecalibacterium species with health and growth. *PLoS One* 8:e63157. doi: 10.1371/journal.pone.0063157

Pardon, B., De Bleecker, K., Hostens, M., Callens, J., Dewulf, J., and Deprez, P. (2012). Longitudinal study on morbidity and mortality in white veal calves in Belgium. *BMC Vet. Res.* 8:26. doi: 10.1186/1746-6148-8-26

Pounden, W. D., and Hibbs, J. W. (1950). The development of calves raised without Protozoa and certain other characteristic rumen microorganisms. *J. Dairy Sci.* 33, 639–644. doi: 10.3168/jds.S0022-0302(50)91948-5

Pretzer, G., Snel, J., Molenaar, D., Wiersma, A., Bron, P. A., Lambert, J., et al. (2005). Biodiversity-based identification and functional characterization of the mannose-specific adhesin of *Lactobacillus plantarum*. *J. Bacteriol.* 187, 6128–6136. doi: 10.1128/jb.187.17.6128-6136.2005

Purushe, J., Fouts, D. E., Morrison, M., White, B. A., Mackie, R. I., Coutinho, P. M., et al. (2010). Comparative genome analysis of Prevotella ruminicola and *Prevotella bryantii*: insights into their environmental niche. *Microb. Ecol.* 60, 721–729. doi: 10.1007/s00248-010-9692-8

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., et al. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 464, 59–65. doi: 10.1038/nature08821

Reis, B. P., Zhang, S., Tsolis, R. M., Bäumler, A. J., Adams, L. G., and Santos, R. L. (2003). The attenuated sop B mutant of *Salmonella enterica* serovar typhimurium has the same tissue distribution and host chemokine response as the wild type in bovine Peyer's patches. *Vet. Microbiol.* 97, 269–277. doi: 10.1016/j.vetmic.2003.09.019

Renaud, D. L., Kelton, D. F., Weese, J. S., Noble, C., and Duffield, T. F. (2019). Evaluation of a multispecies probiotic as a supportive treatment for diarrhea in dairy calves: a randomized clinical trial. *J. Dairy Sci.* 102, 4498–4505. doi: 10.3168/jds.2018-15793

Riddell, J. B., Gallegos, A. J., Harmon, D. L., and Mcleod, K. R. (2008). Addition of a Bacillus based probiotic to the diet of preruminant calves: influence on growth, health, and blood parameters. *J. Appl. Res. Vet. Med.* 8, 78–85. doi: 10.1136/inp.b5495

Rolfe, R. D. (2000). The role of probiotic cultures in the control of gastrointestinal health. *J. Nutr.* 130, 396s–402s. doi: 10.1093/jn/130.2.396S

Rolhion, N., and Chassaing, B. (2016). When pathogenic bacteria meet the intestinal microbiota. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* 371:20150504. doi: 10.1098/rstb.2015.0504

Rosa, F., Michelotti, T. C., St-Pierre, B., Trevisi, E., and Osorio, J. S. (2021). Early life fecal microbiota transplantation in neonatal dairy calves promotes growth performance and alleviates inflammation and oxidative stress during weaning. *Animals. (Basel)* 11:2704. doi: 10.3390/ani11092704

Rossi, M., Corradini, C., Amaretti, A., Nicolini, M., Pompei, A., Zanoni, S., et al. (2005). Fermentation of fructooligosaccharides and inulin by bifidobacteria: a comparative study of pure and fecal cultures. *Appl. Environ. Microbiol.* 71, 6150–6158. doi: 10.1128/aem.71.10.6150-6158.2005

Saeed, A. M., Magnuson, N. S., Gay, C. C., and Greenberg, R. N. (1986). Characterization of heat-stable enterotoxin from a hypertoxigenic *Escherichia coli* strain that is pathogenic for cattle. *Infect. Immun.* 53, 445–447. doi: 10.1128/iai.53.2.445-447.1986

Sarkar, A., and Mandal, S. (2016). Bifidobacteria-insight into clinical outcomes and mechanisms of its probiotic action. *Microbiol. Res.* 192, 159–171. doi: 10.1016/j.micres.2016.07.001

Saro, C., Hohenester, U. M., Bernard, M., Lagrée, M., Martin, C., Doreau, M., et al. (2018). Effectiveness of interventions to modulate the rumen microbiota composition and function in pre-ruminant and ruminant lambs. *Front. Microbiol.* 9:1273. doi: 10.3389/fmicb.2018.01273

Schultze, B., Wahn, K., Klenk, H. D., and Herrler, G. (1991). Isolated HE-protein from hemagglutinating encephalomyelitis virus and bovine coronavirus has receptor-destroying and receptor-binding activity. *Virology* 180, 221–228. doi: 10.1016/0042-6822(91)90026-8

Signorini, M. L., Soto, L. P., Zbrun, M. V., Sequeira, G. J., Rosmini, M. R., and Frizzo, L. S. (2012). Impact of probiotic administration on the health and fecal microbiota of young calves: a meta-analysis of randomized controlled trials of lactic acid bacteria. *Res. Vet. Sci.* 93, 250–258. doi: 10.1016/j.rvsc.2011.05.001

Singh, V., Raheja, G., Borthakur, A., Kumar, A., Gill, R. K., Alakkam, A., et al. (2012). *Lactobacillus acidophilus* upregulates intestinal NHE3 expression and function. *Am. J. Physiol-Gastr. L.* 303, G1393–G1401. doi: 10.1152/ajpgi.00345.2012

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

Sun, P., Wang, J. Q., and Zhang, H. T. (2010). Effects of *Bacillus subtilis* natto on performance and immune function of preweaning calves. *J. Dairy Sci.* 93, 5851–5855. doi: 10.3168/jds.2010-3263

Thiagarajah, J. R., Donowitz, M., and Verkman, A. S. (2015). Secretory diarrhoea: mechanisms and emerging therapies. *Nat. Rev. Gastroenterol. Hepatol.* 12, 446–457. doi: 10.1038/nrgastro.2015.111

Timmerman, H. M., Mulder, L., Everts, H., Espen, D., and Beynen, A. C. (2005). Health and growth of veal calves fed Milk replacers with or without probiotics. *J. Dairy Sci.* 88, 2154–2165. doi: 10.3168/jds.S0022-0302(05)72891-5

Torow, N., and Hornef, M. W. (2017). The neonatal window of opportunity: setting the stage for life-long host-microbial interaction and immune homeostasis. *J. Immunol.* 198, 557–563. doi: 10.4049/jimmunol.1601253

Tsolis, R. M., Adams, L. G., Ficht, T. A., and Bäumler, A. J. (1999). Contribution of *Salmonella typhimurium* virulence factors to diarrheal disease in calves. *Infect. Immun.* 67, 4879–4885. doi: 10.1128/iai.67.9.4879-4885.1999

Tzipori, S., and Ward, H. (2002). Cryptosporidiosis: biology, pathogenesis and disease. *Microbes Infect.* 4, 1047–1058. doi: 10.1016/s1286-4579(02)01629-5

Urdaci, M. C., Lefevre, M., Lafforgue, G., Cartier, C., Rodriguez, B., and Fioramonti, J. (2018). Antidiarrheal action of *Bacillus subtilis* CU1 CNCM I-2745 and *Lactobacillus plantarum* CNCM I-4547 in mice. *Front. Microbiol.* 9:1537. doi: 10.3389/fmicb.2018.01537

Urie, N. J., Lombard, J. E., Shivley, C. B., Kopral, C. A., Adams, A. E., Earleywine, T. J., et al. (2018). Preweaned heifer management on US dairy operations: part V. factors associated with morbidity and mortality in preweaned dairy heifer calves. *J. Dairy Sci.* 101, 9229–9244. doi: 10.3168/jds.2017-14019

Ushakova, N. A., Nekrasov, R. V., Meleshko, N. A., Laptev, G. Y., Ilina, L. A., Kozlova, A. A., et al. (2013). Effect of *Bacillus subtilis* on the rumen microbial community and its components exhibiting high correlation coefficients with the host nutrition, growth, and development. *Microbiology* 82, 475–481. doi: 10.1134/S0026261713040127

Uyeno, Y., Sekiguchi, Y., and Kamagata, Y. (2010). rRNA-based analysis to monitor succession of faecal bacterial communities in Holstein calves. *Lett. Appl. Microbiol.* 51, 570–577. doi: 10.1111/j.1472-765X.2010.02937.x

Uyeno, Y., Shigemori, S., and Shimosato, T. (2015). Effect of probiotics/prebiotics on cattle health and productivity. *Microbes Environ.* 30, 126–132. doi: 10.1264/jsme2.ME14176

van Zyl, W. F., Deane, S. M., and Dicks, L. M. T. (2020). Molecular insights into probiotic mechanisms of action employed against intestinal pathogenic bacteria. *Gut Microbes* 12:1831339. doi: 10.1080/19490976.2020.1831339

Viaşu-Bolocan, L., Popescu, F., and Bică, C. (2013). Probiotics and their immunomodulatory potential. *Curr. Health. Sci. J* 39, 204–209.

Villot, C., Ma, T., Renaud, D. L., Ghaffari, M. H., Gibson, D. J., Skidmore, A., et al. (2019). *Saccharomyces cerevisiae* boulardii CNCM I-1079 affects health, growth, and fecal microbiota in milk-fed veal calves. *J. Dairy Sci.* 102, 7011–7025. doi: 10.3168/jds.2018-16149

Vlasova, A. N., and Saif, L. J. (2021). Bovine coronavirus and the associated diseases. *Front. Vet. Sci.* 8:643220. doi: 10.3389/fvets.2021.643220

Walter, J. (2008). Ecological role of lactobacilli in the gastrointestinal tract: implications for fundamental and biomedical research. *Appl. Environ. Microbiol.* 74, 4985–4996. doi: 10.1128/aem.00753-08

Wang, J., Ji, H., Wang, S., Liu, H., Zhang, W., Zhang, D., et al. (2018). Probiotic *Lactobacillus plantarum* promotes intestinal barrier function by strengthening the epithelium and modulating gut microbiota. *Front. Microbiol.* 9:1953. doi: 10.3389/fmicb.2018.01953

Weaver, D. M., Tyler, J. W., VanMetre, D. C., Hostetler, D. E., and Barrington, G. M. (2000). Passive transfer of colostral immunoglobulins in calves. *J. Vet. Intern. Med.* 14, 569–577. doi: 10.1892/0891-6640(2000)014<0569:ptocii>2.3.co;2

Whitford, M. F., Forster, R. J., Beard, C. E., Gong, J., and Teather, R. M. (1998). Phylogenetic analysis of rumen bacteria by comparative sequence analysis of cloned 16S rRNA genes. *Anaerobe* 4, 153–163. doi: 10.1006/anae.1998.0155

Winder, C. B., Kelton, D. F., and Duffield, T. F. (2016). Mortality risk factors for calves entering a multi-location white veal farm in Ontario, Canada. *J. Dairy Sci.* 99, 10174–10181. doi: 10.3168/jds.2016-11345

Wu, Y., Wang, L., Luo, R., Chen, H., Nie, C., Niu, J., et al. (2021). Effect of a multispecies probiotic mixture on the growth and incidence of diarrhea, immune function, and fecal microbiota of pre-weaning dairy calves. *Front. Microbiol.* 12:681014. doi: 10.3389/fmicb.2021.681014

Xiao, J. X., Alugongo, G. M., Chung, R., Dong, S. Z., Li, S. L., Yoon, I., et al. (2016). Effects of *Saccharomyces cerevisiae* fermentation products on dairy calves: ruminal fermentation, gastrointestinal morphology, and microbial community. *J. Dairy Sci.* 99, 5401–5412. doi: 10.3168/jds.2015-10563

Yáñez-Ruiz, D. R., Abecia, L., and Newbold, C. J. (2015). Manipulating rumen microbiome and fermentation through interventions during early life: a review. *Front. Microbiol.* 6:1133. doi: 10.3389/fmicb.2015.01133

Yang, F., Wang, A., Zeng, X., Hou, C., Liu, H., and Qiao, S. (2015). *Lactobacillus reuteri* I5007 modulates tight junction protein expression in IPEC-J2 cells with LPS stimulation and in newborn piglets under normal conditions. *BMC Microbiol.* 15:32. doi: 10.1186/s12866-015-0372-1

Zeineldin, M., Aldridge, B., and Lowe, J. (2018). Dysbiosis of the fecal microbiota in feedlot cattle with hemorrhagic diarrhea. *Microb. Pathog.* 115, 123–130. doi: 10.1016/j.micpath.2017.12.059

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Isoacids supplementation improves growth performance and feed fiber digestibility associated with ruminal bacterial community in yaks

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Introduction: This study was conducted to assess the effect of mixed isoacid (MI) supplementation on fermentation characteristics, nutrient apparent digestibility, growth performance, and rumen bacterial community in yaks.

Methods: A 72-h *in vitro* fermentation experiment was performed on an ANKOM RF gas production system. MI was added to five treatments at doses of 0, 0.1%, 0.2%, 0.3%, 0.4%, and 0.5% on the dry matter (DM) basis of substrates using a total of 26 bottles (4 bottles per treatment and 2 bottles as the blank). Cumulative gas production was measured at 4, 8, 16, 24, 36, 48, and 72 h. Fermentation characteristics including pH, the concentration of volatile fatty acids (VFAs), ammonia nitrogen (NH_3-N), microbial proteins (MCP), and the disappearance rate of dry matter (DMD), neutral detergent fiber (NDFD), and acid detergent fiber (ADFD) were measured after a 72-h *in vitro* fermentation to determine an optimal MI dose. Fourteen Maiwa male yaks (180–220 kg, 3–4 years old of age) were randomly assigned to the control group (without MI, $n = 7$) and the supplemented MI group ($n = 7$, supplemented with 0.3% MI on DM basis) for the 85-d animal experiment. Growth performance, nutrient apparent digestibility, rumen fermentation parameters, and rumen bacterial diversity were measured.

Results: Supplementation with 0.3% MI achieved the greatest propionate and butyrate content, NDFD and ADFD compared with other groups ($P < 0.05$). Therefore, 0.3% was used for the animal experiment. Supplementation with 0.3% MI significantly increased the apparent digestibility of NDF and ADF ($P < 0.05$), and the average daily weight gain of yaks ($P < 0.05$) without affecting the ruminal concentration of NH_3-N, MCP, and VFAs. 0.3% MI induced rumen bacteria to form significantly different communities when compared to the control group ($P < 0.05$). g__norank_f__*Bacteroidales*_BS11_gut_group, g__norank_f__*Muribaculaceae*, g__*Veillonellaceae*_UCG-001, g__*Ruminococcus*_*gauvreauii*_group, g__norank_f__norank_o__RF39 and g__*Flexilinea* were identified as the biomarker taxa in responding to supplementation with 0.3% MI. Meanwhile, the abundance of g__*Flexilinea* and g__norank_f__norank_o__RF39 were significantly positively correlated with the NDF digestibility ($P < 0.05$).

Conclusion: In conclusion, supplementation with 0.3% MI improved the *in vitro* rumen fermentation characteristics, feed fiber digestibility, and growth

performance in yaks, which was associated with changes of the abundance of g__*Flexilinea* and g__norank_f__norank_o__RF39.

KEYWORDS

yak (*Bos grunniens*), isoacids, growth performance, fiber digestibility, ruminal bacterial community, 16S rRNA gene sequencing

1. Introduction

Yaks (*Bos grunniens*), a large ruminant, have adapted well to the high-altitude, low oxygen, and cold environment of the Qinghai-Tibet Plateau, and provide milk, meat, wool, leather, and labor for local residents (Jing et al., 2022). The adaptation of yaks to the highlands is closely related to their unique characteristics in terms of digestion, physiology, rumen fermentation, and rumen microbiota composition. It has been found that yaks have a larger heart and lung volume, a higher capacity to sense the environment, and a higher energy metabolism than cattle (Wang et al., 2011). In addition, yaks are more tolerant to roughage than cattle (Shao et al., 2010), e.g., they digest neutral detergent fiber (NDF) better than cattle and they use nitrogen more efficiently (Zhou et al., 2017, 2018). Yaks are mainly reared on natural pastures, but due to the harsh climatic conditions in the winter season, this feeding pattern cannot sustain adequate nutrient and energy intake for yaks throughout the year, resulting in a retarded growth rate and even weight loss (Long et al., 2005; Ding et al., 2012). Therefore, barn- or feedlot-feeding was introduced to conserve natural pastures, improve the growth rate, and shorten the feeding period of yaks (Kang et al., 2020; Du et al., 2021; Hu et al., 2021).

Similar to other ruminants, yaks rely on rumen microorganisms to break down dietary nutrient components. Differences in physiological characteristics and digestibility suggest that the rumen microbiome composition of yaks may differ from that of other ruminants (Xin et al., 2019). Compared with cattle, yaks show a higher abundance of *Firmicutes* in the rumen (Xue et al., 2018). A representative genus is *Clostridium*, which can degrade cellulose, xylan or pectin, and other substances in the rumen (Cholewińska et al., 2020). The ruminal bacterial community of yaks also showed better dietary fiber degradation features compared with the normal cattle or hybrid cattle-yak (Dai et al., 2021; Liu et al., 2022). This may contribute to improving the rumen fermentation characteristics of yaks, for example, yaks showed greater ruminal production of VFA but achieved lower methane emission when compared with cattle and sheep (Zhang et al., 2016). Therefore, altering the composition and structure of the bacterial community may improve the rumen fermentation characteristics, which is beneficial for the growth performance of yaks.

Isoacids, a term referring to the branched-chain volatile fatty acids (BCVFAs), namely, isovaleric acid (isovalerate), isobutyric acid (isobutyrate), and 2-methylbutyric acid (2-methybutyrate), and the straight-chain valeric acid (valerate), are the microbial metabolites of amino acids, including valine, leucine, isoleucine, and proline (Andries et al., 1987; Apajalahti et al., 2019). Previous studies have shown that the addition of isoacids to ruminant diets, alone or in combination, favors increased enzymatic activities and proliferation of ruminal fiber-degrading bacteria, and promotes crude fiber digestibility *in vivo* (Liu et al., 2014, 2020; Wang et al., 2015, 2018a; Liu C. S. et al., 2016). Furthermore, in an *in vitro* continuous fermentation model, supplementation of a mixture of BCVFAs showed improved NDF digestibility compared with the addition of individual isoacids (Roman-Garcia et al., 2021a,b) and stimulated the relative abundance of fiber-degrading bacteria, such as *Fibrobacter* and *Treponema* (Roman-Garcia et al., 2021c). Given these beneficial effects of isoacids on rumen fiber degradation, we hypothesize that supplementation with isoacids could be an effective option to promote feed fiber utilization in barn-feeding yaks. Therefore, the objective of this study was to evaluate the effects of supplementing a mixture of isoacids on growth performance, rumen fermentation characteristics, and nutrient digestibility in yaks. Considering the metabolic process of isoacids by microorganisms in the rumen, we evaluated the changes in the ruminal bacterial community in response to isoacid supplementation using 16s rRNA gene sequencing and correlated the ruminal microbiome with fermentation metabolites and fiber digestibility in yaks.

2. Material and methods

2.1. Mixed isoacids supplement

The supplemental mixture of isoacids used in this study is a commercially available feed additive product (Yangleyou, provided by Si Chuan Action Biotech Co., Ltd), which consisted of a mixture of isobutyrate, isovalerate, valerate, and 2-methylbutyrate. The effective content of mixed isoacid (MI) was 63.6 g/100 g. The liquid mixture of isoacids was absorbed using silica as the carrier in a ratio of 64:36.

2.2. *In vitro* fermentation experiment and cumulative gas production measurement

An *in vitro* fermentation experiment was conducted to evaluate the role of different supplemental levels of MI on the fiber disappearance rate and the fermentation characteristics. The treatment groups included a non-supplementation control group (CON) and five MI-supplemented groups supplemented with MI on the dry matter (DM) basis of the substrate, namely: 0.1% MI (treatment group 1, T1), 0.2% MI (treatment group 2, T2), 0.3% MI (treatment group 3, T3), 0.4% MI (treatment group 4, T4), and 0.5% MI (treatment group 5, T5). Each treatment group contained four replicates.

The *in vitro* fermentation experiment was performed using the ANKOM RF Gas Production Systems (ANKOM), which is

TABLE 1 Ingredients and chemical composition of the TMR[1].

Items	Content
Ingredient composition, g/100 g DM	
Corn	31.00
Soybean meal	13.00
Urea	0.15
Na_2SO_4	0.35
NaCl	0.35
Premix[2]	1.90
Rumen-protected lysine	0.15
Rumen-protected methionine	0.10
Yeast culture	0.50
Small peptide	0.50
Hydrogenated palm oil	2.00
Corn silage	50.00
Total	100.00
Nutrient composition, g/100 g DM[3]	
DM	95.10
EE	3.15
CP	14.31
NDF	30.13
ADF	12.16
Ga	0.76
P	0.38

TMR, Total mixed ration; DM, dry matter; NDF, neutral detergent fiber; ADF, acid detergent fiber; CP, crude protein; EE, ether extract; Ca, calcium; P, phosphorus.
The premix (vitamins and microelements) contained the following ingredients as per kg of DM: vitamin A 2 500 IU, vitamin D 550 IU, vitamin E 10 IU, copper 10 mg, iron 50 mg, manganese 40 mg, zinc 40 mg, iodine 0.5 mg, selenium 0.2 mg, and cobalt 0.2 mg.
The nutrient composition is based on all measured values.
[1]An explanation of abbreviations for Total mixed ration. [2]An explanation of the ingredients content of premix in per kg feed at dry matter basis. [3]An explanation of abbreviations for dry matter.

designed to automatically measure the kinetics of a microbial fermentation by monitoring the gas pressure within multiple modules equipped with temperature sensors and remotely recording the data in computer spreadsheets. The modules can communicate information to a computer using radio frequency (RF) transmission. Numerous variables can be operated from the computer interface such as data recording intervals and the automatic pressure release through internal valves in each module. A run was performed with a total of 24 bottles assigned to 6 groups and 2 bottles as blank. A total of 30 ml of rumen fluid, 120 ml of artificial saliva, and 1.00 g of substrate were added to a glass fermentation bottle and covered with the ANKOM module. A zero-reference module was used to measure ambient pressure. Two additional bottles incubated with rumen fluid and artificial saliva only (no substrate) were used as a blank control to correct for gas production. Briefly, fresh rumen fluid was collected from four male Maiwa yaks through a stainless-steel stomach tube attached to a rumen vacuum sampler as previously reported (Wang et al., 2016a). Yaks were fed the same regime as described in Table 1. Feed particles were discarded by filtering the liquid collection through four layers of sterile gauze. The filtered solutions were then transferred to a vacuum thermobottle, which was pre-incubated at 37°C. Carbon dioxide (CO_2) was continuously injected into the rumen fluid to maintain an anaerobic atmosphere during all of the processes. Artificial saliva solution was prepared according to the previous procedure (Menke et al., 1979) and well mixed with rumen fluid in a ratio of 1:4 while simultaneously streaming with CO_2. The fermentation bottles and modules were stabilized at 39°C for 1 h, then added with substrates and 150 ml of the mixing liquids, and incubated at 39°C, 110 rpm/min for 72 h. Real-time absolute gas pressure and temperature of each module were recorded every 15 min during the 72 h of fermentation. The cumulative gas production at 4, 8, 16, 24, 48, and 72 h was converted according to the ANKOM manual using the following equation:

$$V_x = V_j \times P_{psi} \times 0.068\,004\,084$$

where V_x = gas production volume, V_j = volume of space above the liquid surface inside the bottle, and P_{psi} = gas pressure, recorded by the ANKOM GPM software (1 psi≈ 6.89 kPa).

2.3. *In vitro* rumen fermentation characteristics and nutrient disappearance rate

The *in vitro* fermentation experiment was terminated in an ice-water bath after 72 h. The pH value of each fermentation fluid was immediately measured using a pH meter. The fermentation fluid was collected to determine the rumen fermentation characteristics, including the concentration of NH_3-N, MCP, and VFAs. Briefly, the fermentation fluid was centrifuged at 3,000 rpm for 10 min, and an aliquot of the supernatant was used to determine the concentration of NH_3-N using the micro-Kjeldahl method (AOAC, 2001). Another aliquot of the supernatant was transferred to a 10-ml centrifuge tube, ultrasonicated (ultrasonic probe = 2 mm, 350 W, repeated 3 times, 30 s each time, and interval = 30 s), and centrifuged at 3,000 rpm at 4°C for 10 min. Briefly, 1 ml of the supernatant was centrifuged at 10,000 rpm at 4°C for 20 min. The precipitate was rinsed twice with 1 ml of saline and then re-suspended with 1 ml of distilled water to determine the concentration of MCP according to the manufacturer's instructions of the BCA kits (Beijing Solarbio Science & Technology Co. Ltd, Beijing, China).

The concentration of VFAs in the fermentation fluid was measured according to the method described by Wang et al. (2014). Briefly, 1.0 ml of the supernatant was acidified with 200 μL metaphosphoric acid (25%) overnight at 4°C. The samples were analyzed on a gas chromatograph (7890B, Agilent Technology Inc., Santa Clara, CA) with a chromatographic column (HP INNOwax - 19091N, 30 m long, 0.32 mm ID, 0.50 m film) according to the manufacturer's protocol. The VFAs content was determined by comparison with the linear retention times of known standards (Shanghai Anpel Experimental Technology Co., LTD, Shanghai, China).

DM, NDF, and acid detergent fiber (ADF) degradability (DMD, NDFD, and ADFD) of the fermentation residue were measured after 72 h of incubation. The DM content was determined according to the AOAC method (AOAC, 2001), and the contents of NDF and ADF were analyzed using a fiber analyzer (automatic fiber determination analyzer, Gernardt F12) as described by Van Soest et al. (1991). Finally, they were calculated using the following equations:

$$DMD = \frac{(\text{DM of substrate} - \text{DM of residue}) \times 100}{\text{DM of substrate}}$$

$$NDFD = \frac{(\text{NDF of substrate} - \text{NDF of residue}) \times 100}{\text{NDF of substrate}}$$

$$ADFD = \frac{(\text{ADF of substrate} - \text{ADF of residue}) \times 100}{\text{ADF of substrate}}$$

2.4. Animals, management, and sample collection

All animal experimental procedures were approved by the Animal Care and Use Committee of Southwest Minzu University (approval number P20210510-2). The animal experiment was conducted at Seda Niuduoduo Yak Breeding Co. LTD (Ganzi, China, 30°055′ N and 101°969′ E). A total of fourteen Maiwa male yaks (body weight of 180–220 kg and 3–4 years of age) were randomly divided into two groups ($n = 7$ per group), tied and fed individually, and provided with a basal diet (control group; CON) or the basal diet supplemented with 0.3% MI. The Total Mixed Ration (TMR) diet was formulated according to the Feeding Standards of Beef Cattle of China (NY/T 815-2004, Ministry of Agriculture of the People's Republic of China, 2004). The ingredients and nutrient compositions are listed in Table 1. During the 95-day experimental period, the yaks had free access to water and were offered diets *ad libitum* twice a day (at 08:00–09:00 and 17:30–18:00). After 10-days of acclimatization, average dry matter intake (ADMI) was recorded before morning feeding. Animal body weight (BW) was recorded at the beginning and the end of the experiment to calculate the average daily gain (ADG) and feed conversion ratio (FCR).

Feed samples were collected once every 2 weeks and stored at −20°C for nutritional evaluation. Total fecal samples were collected three times daily and pooled on the last 3 days of the experiment. In total, 200 g of total fecal sample was collected from each yak, sampled, and stored at −20°C until analysis. Briefly, the contents of DM, crude protein (CP), acid insoluble ash (AIA), and ether extract (EE), in the diet and fecal samples, were analyzed according to the standard methods described by AOAC (2001). Calcium (Ca) content was measured by EDTA complexometric titration, and total phosphorus (P) content was determined using a spectrophotometer (the People's Republic of China National Standard, 2002a,b). NDF and ADF were analyzed as described in 2.3. AIA was used as an internal marker to evaluate the apparent digestibility of nutrients, which was calculated using the following equation:

$$\text{Nutrient apparent digestibility} = 100 \times (1 - \frac{R_{AIA}}{M_{AIA}} \times \frac{M_n}{R_n})$$

where Rn = nutrient content in the feed, Mn = nutrient content in the feces, R_{AIA} = hydrochloric acid insoluble ash content in the feed, and M_{AIA} =hydrochloric acid insoluble ash content in the feces (Cheng et al., 2014).

Rumen fluid was collected through a stainless-steel stomach tube at the end of the experiment after overnight fasting. The first two tubes of ruminal fluid were discarded to avoid saliva contamination. The rumen fluid was then divided into two aliquots. One aliquot (5 ml) was transferred to a cryogenic vial and immediately frozen in liquid nitrogen for 16S rRNA sequencing. The other aliquot (30 ml) was transferred to a centrifuge tube and stored at −80°C for chemical determination of VFAs, NH_3-N, and MCP concentrations using the same methods described in Section 2.2. Blood samples were collected from the jugular vein of yaks and anticoagulated with heparin sodium. Plasma was collected after centrifugation at 4,000 rpm for 10 min and stored at −80°C for further analysis of biochemical and hormonal parameters.

2.5. Rumen fluid microbial DNA extraction, 16S rRNA gene amplification, and bioinformatics analysis

Total microbial genomic DNA from the rumen fluid was extracted using a FastDNA® Spin Kit for soil (MP Biomedicals, USA), according to the standard operating procedures. The concentration, quality, and completeness of the extracted DNA were determined using a spectrophotometer (NanoDrop2000, Thermo Fisher, USA) and 1.0% agarose gel electrophoresis (Biowest Agarose, Biowest, Spain), and then the DNA was stored at −80°C for further use. The hypervariable region V3–V4 of the bacterial 16S rRNA gene was amplified with primer pairs 338F (5′ ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) using an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA; Liu Q. et al., 2016). A 20-μL reaction system contained 4 μL of 5 × Fast Pfu buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of Fast Pfu polymerase, 10 ng of template DNA, and ddH_2O to a final volume of 20 μL. The temperature program was performed as follows: 95°C for 3 min, followed by 27 cycles of denaturation at 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, a single extension at 72°C for 10 min, and ending at 4°C. Purification and quantification of the amplification product were performed using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Axygen, USA) and the Quantus™ Fluorometer (Promega, USA).

The amplicons were sequenced on an Illumina MiSeq PE300 platform (Illumina, San Diego, CA, USA) at Majorbio (Shanghai, China). Raw sequencing reads were de-multiplexed using an in-house Perl script, quality-filtered using FASTP (v 0.19.6; Chen et al., 2018), and merged using FLASH (v1.2.11; Magoc and Salzberg, 2011). Although chloroplast DNA was not removed during the quality control process, this had a negligible effect on the analysis results. Paired-end reads were merged into a sequence with a minimum overlap length of 10 bp and a maximum mismatch ratio of 0.2 according to the overlapping relationships. The optimized sequences were clustered into operational taxonomic units (OTUs)

based on a similarity >97% by using UPARSE 11 (Stackebrandt and Goebel, 1994; Edgar, 2013). To minimize the effect of sequencing depth on the bacterial diversity measure, the number of 16S rRNA gene sequences from each sample was rarefied to 32095, which still resulted in an average Good's coverage of 99.06 ± 0.044%.

The data were analyzed on the online platform of Majorbio Cloud Platform (www.majorbio.com) according to the description found in the study by Ren et al. (2022). The Mothur software programs (version v.1.30.2; Schloss et al., 2009) were applied to calculate the alpha diversity of the bacterial community, including the Sobs, Shannon, Simpson, and Chao indices. Principal coordinates analysis (PCoA) was used to determine the similarity among the microbial communities in different samples based on the Bray-Curtis distance metric using the Vegan v2.5-3 package of R (version 3.3.1). The bacterial community composition and sample-to-taxa relationship were visualized using Circos-0.67-7 (http://circos.ca/). The most discriminative abundant bacterial taxa (from phyla to genera) between groups were identified using a linear discriminant analysis (LDA) effect size (LEfSe; LDA score>3, $P < 0.05$; Segata et al., 2011). The association between the biomarker taxa and the phenotypic parameters was analyzed using Spearman's correlation and visualized using the Pheatmap package of R (version 3.3.1).

2.6. Statistical analysis

Data were analyzed using the SPSS (Statistical software package, version 20.0, IBM, USA). A two-way ANOVA used a general linear model (GLM) to examine the effect of treatment on *in vitro* gas production. Duncan's method was used for multiple comparisons. A one-way ANOVA was used to analyze other parameters after data normality was tested using the Shapiro-Wilk test, and polynomial contrasts were generated using a curve estimation regression analysis. The data from the animal experiment were analyzed via the student's *t*-test. The student's *t*-test was used to determine the differential bacterial taxa. Significance was declared at $P \leq 0.05$, and a significant trend was considered when $0.05 < P \leq 0.10$.

2.7. Data availability

The raw sequencing reads of the bacterial 16S rRNA amplicon for all of the ruminal fluid samples are deposited in the genome sequence archive (GSA) database under the BioProject accession number PRJCA016021.

3. Results

3.1. Supplementation with mixed isoacids promoted feed fiber disappearance rate and VFAs production *in vitro*

The addition of different levels of MI did not affect the cumulative *in vitro* gas production volumes, and no interaction between incubation time and treatment was observed (Supplementary Figure 1). Nevertheless, the inclusion of MI strongly affected the NDFD and ADFD ($P = 0.002$ and $P = 0.004$; Table 2). With increasing supplementation, the NDFD and ADFD showed significant quadratic trends ($P < 0.01$). Compared with the control and other supplementation levels, the supplementation with 0.3% MI achieved the greatest NDFD and ADFD ($P < 0.05$).

The effects of supplementing MI on the *in vitro* fermentation characteristics are presented in Table 3. The pH value of the fermentation fluid was strongly affected by MI ($P < 0.001$). When the addition levels were higher than 0.3%, the pH value of the fermentation fluid was significantly lower than that of the control group ($P < 0.05$). The addition of MI showed a quadratic effect on the concentration of NH_3-N ($P = 0.021$), but not on the concentration of MCP ($P = 0.685$). Propionate and butyrate production displayed a clear quadratic effect in response to MI supplementation ($P < 0.05$). Supplementation with 0.3% MI achieved the highest levels of these two volatile metabolites compared with the control and other supplementation groups ($P < 0.05$). Moreover, the concentrations of total VFAs (TVFA) tended to show a quadratic increase tendency ($P = 0.055$). No marked differences were detected for the production of acetate, valerate, and the ratio of acetate to propionate among the groups ($P > 0.05$). No significant difference was observed in the molar percentage of any of these VFAs ($P > 0.05$).

3.2. Supplementation with mixed isoacids improved the growth performance and apparent digestibility of nutrients without affecting plasma biochemical and hormonal parameters in yaks

As shown in Table 4, supplementation with 0.3% MI significantly increased the ADG and ADMI when compared with the control ($P<0.05$), but did not affect the FCR ($P > 0.05$). Yaks exhibited greater apparent digestibility of NDF and ADF in response to supplementation with 0.3% MI ($P < 0.05$). There was no significant difference in the apparent digestibility of EE, CP, Ca, and P between the treatments ($P > 0.05$). Meanwhile, supplementation with 0.3% MI had no effect on the concentration of plasma biochemical parameters and hormones (Supplementary Table 1), suggesting the nutrition metabolism of yaks themselves may not attribute to the growth-promoting effect of MI.

3.3. Supplementation with mixed isoacids tended to influence the ruminal fermentation characteristics in yaks

The concentrations of NH_3-N, MCP, and VFAs in the rumen fluid of yaks are shown in Table 5. No significant difference was observed in NH_3-N, MCP, and VFAs between the two groups ($P > 0.05$). Among the VFAs, the concentrations of acetate, propionate, and butyrate tended to decrease in response to the supplementation with 0.3% MI ($0.05 < P < 0.1$).

TABLE 2 Effects of mixed isoacids supplementation on DMD, NDFD, and ADFD of the *in vitro* fermentation experiment.

Items[1]	Groups						SEM	*P*-value[2]		
	CON	T1	T2	T3	T4	T5		Treatment	Linear	Quadratic
DMD	79.77	83.33	83.82	80.88	79.19	79.05	1.763	0.277	0.233	0.129
NDFD	53.14^{bc}	58.51^{bc}	62.33^{ab}	71.49^{a}	52.54^{bc}	52.35^{bc}	3.037	0.002	0.741	0.007
ADFD	31.65^{c}	37.26^{bc}	44.01^{b}	53.88^{a}	41.66^{bc}	41.49^{bc}	3.219	0.004	0.051	0.002

DMD, dry matter degradability; NDFD, neutral detergent fiber degradability; ADFD, acid detergent fiber degradability.
CON: control group (no mixed isoacids added); T1: treatment 1 (0.1% mixed isoacids added); T2: treatment 2 (0.2% mixed isoacids added); T3: treatment 3 (0.3% mixed isoacids added); T4: treatment 4 (0.4% mixed isoacids added); and T5: treatment 5 (0.5% mixed isoacids added).
Different superscripts indicated significant differences within a row ($P < 0.05$).
[1] An explanation of DMD, NDFD and ADFD which were showed in the table. [2] An explanation that about *P*-value for significant differences.

TABLE 3 Effects of mixed isoacids supplementation on ruminal fermentation characteristics of the *in vitro* fermentation experiment.

Items[1]	Groups[2]						SEM	*P*-value[3]		
	CON	T1	T2	T3	T4	T5		Treatment	Linear	Quadratic
pH	6.93^{a}	6.92^{a}	6.96^{a}	6.74^{b}	6.75^{b}	6.77^{b}	0.014	<0.001	<0.001	<0.001
NH_3-N (mg/dL)	8.39	8.43	8.46	8.34	8.03	7.97	0.156	0.154	0.013	0.021
MCP (mg/mL)	0.38	0.42	0.42	0.46	0.40	0.43	0.037	0.685	0.367	0.381
VFA concentration, mmol/L										
Propionate	6.09^{b}	7.94^{ab}	8.35^{a}	8.49^{a}	6.24^{b}	6.82^{ab}	0.587	0.026	0.829	0.027
Butyrate	4.82^{b}	5.44^{ab}	5.30^{ab}	6.20^{a}	4.30^{b}	4.40^{b}	0.404	0.033	0.266	0.045
Acetate	22.38	27.17	26.70	31.25	22.17	22.75	2.764	0.182	0.737	0.116
Valerate	0.96	1.11	1.22	0.93	0.95	0.96	0.247	0.948	0.693	0.818
Ratio of acetate to propionate	3.68	3.46	3.25	3.63	3.54	3.34	0.292	0.890	0.644	0.881
TVFA	34.65	42.47	42.20	48.14	34.08	35.35	3.614	0.072	0.660	0.055
VFA molar proportion (mol/100 mol)										
Propionate	17.81	18.72	19.87	17.80	18.58	19.62	1.238	0.773	0.513	0.809
Butyrate	13.91	12.87	12.56	13.15	12.55	12.51	0.584	0.524	0.128	0.244
Acetate	64.32	63.80	63.18	64.45	64.78	63.85	1.585	0.984	0.879	0.981
Valerate	2.89	2.69	2.93	2.24	2.96	2.92	0.828	0.988	0.963	0.929

NH_3-N, ammonia nitrogen; MCP, microbial protein; VFA, volatile fatty acids; TVFA, total volatile fatty acids.
CON: control group (no mixed isoacids added); T1:treatment 1 (0.1% mixed isoacids added); T2:treatment 2 (add 0.2% mixed isoacids added); T3:treatment 3 (add 0.3% mixed isoacids added); T4:treatment 4 (add 0.4% mixed isoacids added); and T5:treatment 5 (add 0.5% mixed isoacids added).
Different superscripts showed significant differences ($P < 0.05$).
[1] An explanation of abbreviations for some items in the table, such as MCP, NH_3-N and VFA. [2] An explanation of abbreviations for the groups, such as CON and 0.3% MI. [3] An explanation that about *P*-value for significant differences.

3.4. Supplementation with mixed isoacids stimulated the formation of distinct ruminal bacterial communities in yaks

Illumina Miseq 16S rRNA gene sequencing obtained 48,307 ± 2,147 high-quality trimmed sequences per sample from 14 rumen fluid samples, which belonged to 2,612 OTUs, with an average sequence length of 418 bp (Supplementary Table 2). The information on barcodes from 14 rumen fluid samples is provided in Supplementary Table 2. A total of 1,937 OTUs (74.16%) were commonly shared by the control and 0.3% MI groups, while 276 OTUs (10.57%) were specific to the control group and 399 OTUs (15.28%) were specific to the 0.3% MI group (Supplementary Figure 2). The composition of the ruminal bacterial community was visualized using the Circos plot (Supplementary Figure 3). Both groups displayed similar bacterial composition at the phylum level (Supplementary Figure 3A), family level (Supplementary Figure 3B), and genus level (Supplementary Figure 3C).

The rarefaction curves based on Sobs indices reached the saturation stage with the increasing number of sequenced reads (Figure 1A), suggesting that the majority of rumen bacterial members were captured from rumen fluid samples in this experiment. However, the bacterial community richness (Chao index, Figure 1B) and diversity (Shannon and Simpson indices, Figures 1C, D) did not differ between the control and 0.3% MI groups ($P>0.05$). Principal coordinates analysis (PCoA) based on the Bray-Curtis distance metric showed that supplementation

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TABLE 4 Effects of mixed isoacids supplementation on the growth performance and apparent digestibility of nutrients in yaks.

Items[1]	Groups[2]		SEM	*P*-value[2]
	CON	0.3% MI		
Growth performance				
IBW (kg)	207.07	194.14	8.372	0.296
FBW (kg)	277.64	284.29	12.940	0.725
ADMI (kg/d)	6.10^{b}	6.72^{a}	0.170	0.012
ADG (kg)	0.83^{b}	1.06^{a}	0.066	0.037
FCR	7.76	6.41	0.482	0.110
Apparent digestibility of nutrient %				
NDF	70.86^{b}	78.22^{a}	1.938	0.024
ADF	62.03^{b}	71.59^{a}	2.744	0.042
CP	80.73	82.36	1.429	0.439
EE	88.92	88.93	1.354	0.995
Ca	49.38	52.22	3.952	0.619
P	76.22	71.20	2.779	0.293

IBW, initial body weight; FBW, final body weight; ADMI, average dry matter intake; ADG, average daily gain; FCR, feed conversion rate; NDF, neutral detergent fiber; ADF, acid detergent fiber; CP, crude protein; EE, ether extract; Ca, calcium; P, phosphorus.
CON, the basal diet; 0.3% MI, 0.3% mixed isoacids added to the basal diet on a dry matter basis.
Different superscribed letters showed significant differences ($P < 0.05$).
[1]An explanation of abbreviations for some items in the table, such as IBW, FBW, ADMI, ADG, FCR, NDF, ADF, CP, EE Ca and P. [2]An explanation of abbreviations for the groups, such as CON and 0.3% MI. [3]An explanation that about *P*-value for significant differences.

TABLE 5 Effects of mixed isoacids supplementation on ruminal fermentation characteristics in yaks.

Items[1]	Groups[2]		SEM	*P*-value[3]
	CON	0.3% MI		
NH_3-N, mg/dL	13.13	14.39	1.016	0.423
MCP, mg/mL	0.85	0.85	0.079	0.979
VFA concentration, mmol/L				
Acetate	31.94	25.37	1.979	0.072
Propionate	6.96	5.51	0.529	0.079
Butyrate	3.89	3.03	0.280	0.056
Valerate	0.27	0.27	0.052	0.961
Isovalerate	1.37	1.41	0.293	0.824
Isobutyrate	1.04	1.07	0.171	0.765
Ratio of acetate to propionate	4.58	4.74	0.563	0.646
TVFA	45.89	36.67	7.183	0.064
VFA proportion, mol/100 mol				
Propionate	15.31	14.88	1.334	0.577
Butyrate	8.63	8.22	1.091	0.513
Acetate	69.90	69.37	2.680	0.716
Isovalerate	3.16	3.86	0.993	0.211
Valerate	0.63	0.74	0.163	0.221
Isovalerate	2.38	2.94	0.559	0.084

NH_3-N, ammonia nitrogen; MCP, microbial protein; VFA, volatile fatty acids; TVFA, total volatile fatty acids.
CON, the basal diet; 0.3% MI, 0.3% mixed isoacids added to the basal diet on a dry matter basis.
[1]An explanation of abbreviations for some items in the table, such as MCP, NH_3-N and VFA. [2]An explanation of abbreviations for the groups, such as CON and 0.3% MI. [3]An explanation that about *P*-value for significant differences.

with 0.3% MI induced the rumen bacteria to form significantly different communities when compared with the control group (PERMANOVA, $R^2 = 0.1098$, $P = 0.022$, Figure 2A). A similarities analysis showed that the difference in bacterial communities between the groups was greater than the difference within a group (ANOSIM, $R^2 = 0.2765$, $P = 0.004$, Figure 2B).

The most discriminative taxa responding to supplementation with 0.3% MI was identified using the linear discriminant analysis (LDA) effect size (LEfSe). Fifteen differentially abundant taxa from phylum to genus level were discovered as high-dimensional biomarkers for separating rumen bacterial communities between the two groups (Figure 3A). Nine of these taxa were higher, and six were lower in the 0.3% MI group than in the CON group (Figure 3A). Dietary supplementation with 0.3% MI showed a significantly higher abundance of f__*Bacteroidales*_BS11_gut_group, g__norank_f__*Bacteroidales*_BS11_gut_group, g__norank_f__*Muribaculaceae*, f__*Muribaculaceae*, g__*Veillonellaceae*_UCG-001, g__*Ruminococcus_gauvreauii*_group, g__norank_f__norank_o__RF39, f__norank_o__RF39, and o__RF39 ($P<0.05$). In the CON group group, f__F082, g__norank_f__F082, f__*Ruminococcaceae*, p__*Actinobacteriota*, f__*Eubacterium_coprostanoligenes*_group, and g__norank_f__*Eubacterium_coprostanoligenes*_group were significantly enriched, from the family to the genus level (Figures 3A, B, $P < 0.05$). Besides, a Student's *t*-test was performed to detect all differentially abundant taxa in the top 100 (Supplementary Table 3).

3.5. Apparent digestibility of NDF and ADF were positively associated with ruminal differentially abundant taxa

Differentially abundant taxa at different levels, identified by the Student's *t*-test, were selected for the correlation analysis with apparent digestibility of nutrients. Spearman's correlation indicated that g__*Flexilinea* and g__norank_f__norank_o__RF39 were significantly positively correlated with the NDF digestibility ($P<0.05$); and g__norank_f__norank_o__RF39 was significantly positively correlated with the ADF digestibility ($P<0.05$). In addition, g__*Ornithinimicrobium* was significantly negatively correlated with the ADF and NDF digestibility ($P < 0.05$, Figure 4).

4. Discussion

Improving the fiber utilization by yaks is important for highland yak farming in winter when forage is severely scarce (Dong et al., 2006). The rumen is well known as a natural bioreactor for its high efficiency of fiber degradation by rumen microbiota (Wang et al., 2020). Isoacids are a batch of amino acid-derived

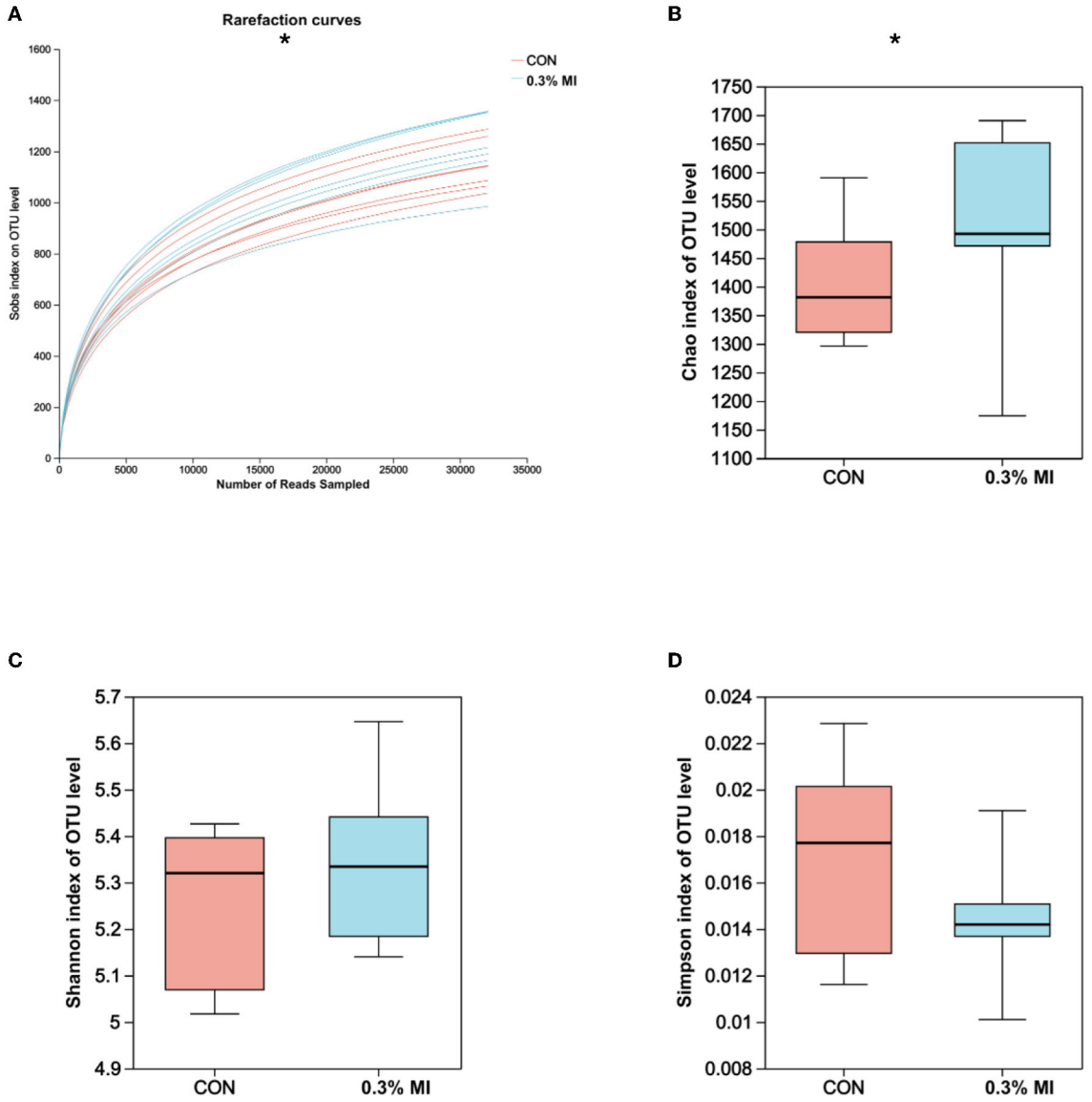

FIGURE 1
Effects of mixed isoacids supplementation on rumen bacterial richness and diversity in yaks. **(A)** Rarefaction curve based on Sobs indices; **(B)** Boxplot of Chao indices; **(C)** Boxplot of Shannon indices; **(D)** Boxplot of Simpson indices. CON, the basal diet; 0.3% MI, 0.3% mixed isoacids added to the basal diet on a dry matter basis. Significance was tested using the independent two-group Wilcoxon rank-sum tests. $^{*}P < 0.05$, $n = 7$ per group.

metabolites in the rumen that act as a nutrient factor for rumen fiber-degrading bacteria, thereby facilitating VFAs production and fiber utilization (Liu et al., 2009, 2014; Roman-Garcia et al., 2021a,c). Concentrations of butyrate and propionate, as well as the degradation of NDF and ADF, tended to increase and then decrease with increasing dose of MI in this study. These results are consistent with those of Wang et al. (2012), who obtained results from an *in vitro* fermentation assay supplemented with 2-methylbutyrate, suggesting that a higher dose of MI may dampen the *in vitro* fermentation characteristics. The supplementation level of 0.3% could be an optimal dose, as it achieved the highest propionate and butyrate content, as well as NDFD and ADFD, indicating that the effect of MI on rumen fermentation parameters in yaks is related to its supplementation level.

Supplementation with isoacids alone or in combination could improve feed fiber digestibility and average daily gain in beef cattle and dairy calves (Wang et al., 2012; Liu C. S. et al., 2016; Liu et al., 2018). Improved digestibility of NDF and NDF responsive to MI makes more fermentation metabolites, such as VFAs, available in the rumen. For example, Wang et al. (2018b) found that supplementation with 16.8 g of 2-methylbutyrate per steer per day increased the molar ratio of effectively degradable NDF and rumen acetate. Similar results were observed in this study, suggesting that supplementation with 0.3% MI could significantly improve the apparent digestibility of NDF and ADF, thereby increasing the ADG and ADMI in yaks. However, ruminal production of VFA was not affected by the addition of MI, which may be due to factors such as diet composition, rumen microbial species,

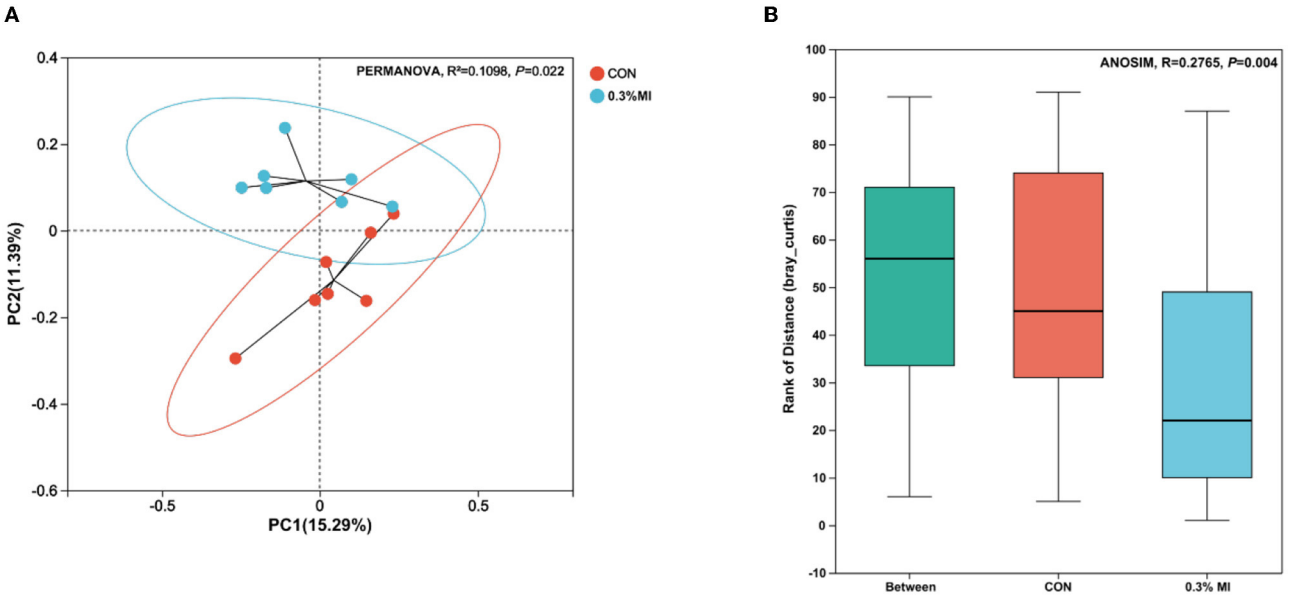

FIGURE 2
Effects of mixed isoacids supplementation on rumen bacterial community similarity. **(A)** Principal coordinates analysis (PCoA) plot based on the Bray-Curtis distance metric. Difference between clusters is measured by the permutational multivariate analysis of variance (PERMANOVA). **(B)** Distances box plot depicted the analysis of similarities (ANOSIM) between the CON group and the 0.3% MI group. CON, the basal diet; 0.3% MI, 0.3% mixed isoacids added to the basal diet on a dry matter basis.

pen temperature, and the amount or composition of MI (Wang et al., 2016a,b). NH_3-N is a product of the fermentation and degradation of dietary proteins and is an essential component for microbial synthesis of MCP in the rumen (Garcia-Gonzalez et al., 2010; Thao et al., 2014; Lv et al., 2020). However, no difference in the response of NH_3-N concentration to MI was observed in this study, which may be related to the amount of water consumed by the yaks or the amount or composition of the MI supplement.

After a long period of natural selection, the yak rumen microbiota has evolved a higher capacity for fiber degradation (Ma et al., 2020). Thus, the yak rumen may harbor a unique microbiome for efficient conversion of feed fiber. Isoacids improve the digestibility of feed fiber, mainly by increasing the activity of cellulase and cellulose-producing bacteria in the rumen. For example, Liu C. S. et al. (2016) reported that supplementation with 6.0 g of isovalerate per calf per day increased the activities of caboxymethylcellulase, cellobiase, xylanase and pectinase and the relative amounts of *Butyrivibrio fibrisolvens, Ruminococcus albus, Fibrobacter succinogenes,* and *Ruminococcus flavefaciens* in dairy calves at 90 days of age. Similarly, Wang et al. (2019) found that supplementation with 6 g of isobutyrate per calf per day increased the activities of cellobiase, xylanase, pectinase, b-amylase protease, and CMCase in post-weaned calves and the population of *B. fibrisolvens* in pre- and post-weaned calves. Rather than looking at changes in the rumen microbiota as a whole, these studies have focused on changes in the abundance of specific microbes. In this study, beta-diversity by PCoA showed that supplementation with 0.3% MI induced the rumen bacteria to form significantly different communities, indicating that 0.3% MI significantly altered the rumen microbial structure, which may be related to the fact that the microbial 16S rRNA gene was less diverse in the yak rumen than that in the bovine rumen (An et al., 2005). This resulted in a significant effect of 0.3% MI on some uncultured fiber-degrading bacteria in the yak rumen and ultimately caused a shift in the structure of the rumen microbiota.

The LEfSe analysis showed that some biomarker taxa such as norank_f__*Bacteroidales*_BS11_gut_group, norank_f__*Muribaculaceae*, *Veillonella- ceae*_UCG-001, and *Ruminococcus_gauvreauii*_group were found in 0.3% of the MI group. The *Bacteroidales* belong to the phyla of *Bacteroidetes*, which act mainly on steroids, polysaccharides, and bile acids, which contribute to the absorption of polysaccharides and proteins by the body (Backhed et al., 2005). Therefore, we can speculate that the norank_f__*Bacteroidales*_BS11_gut_group may be a fiber-degrading bacterium. One study found that norank_f__*Muribaculaceae* was positively correlated with milk yield (Sun et al., 2019), and some studies found that isoacids can increase milk production in dairy cows (Otterby et al., 1990). The higher relative expression of norank_f__*Muribaculaceae* in the 0.3% MI group suggests that 0.3% MI may increase milk production in yaks, but no studies have been reported on this aspect. In addition, *Muribaculaceae* can also degrade carbohydrates (He et al., 2022), which corresponds to a higher apparent digestibility of NDF and ADF in the 0.3% MI group than in the control group, suggesting that 0.3% MI can promote the growth of rumen fibrinolytic bacteria in yaks, which is consistent with other studies (Moharrery and Das, 2001; Firkins, 2010; Liu et al., 2014; Wang et al., 2015). *Veillonellaceae*_UCG-001 belong

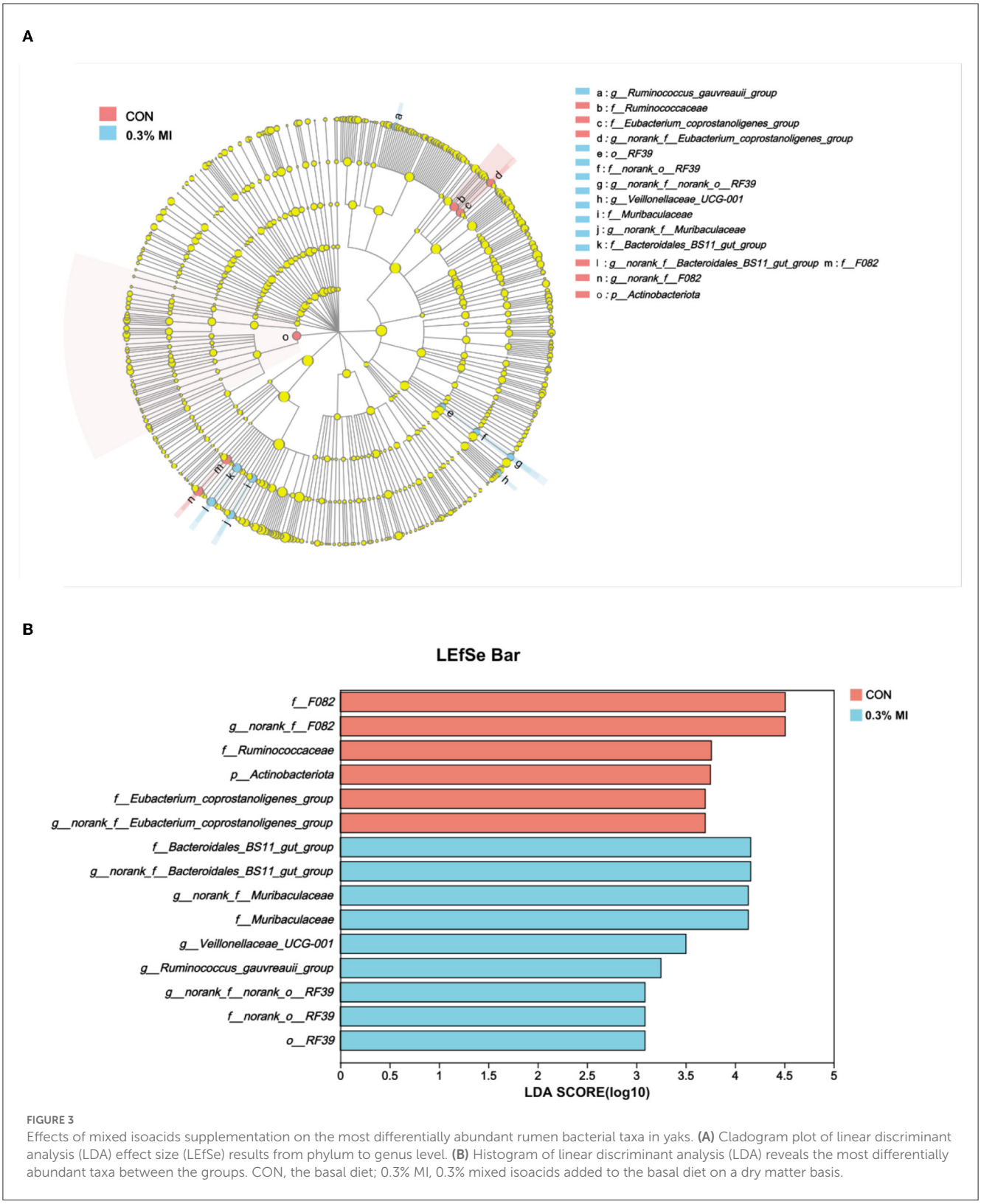

FIGURE 3
Effects of mixed isoacids supplementation on the most differentially abundant rumen bacterial taxa in yaks. **(A)** Cladogram plot of linear discriminant analysis (LDA) effect size (LEfSe) results from phylum to genus level. **(B)** Histogram of linear discriminant analysis (LDA) reveals the most differentially abundant taxa between the groups. CON, the basal diet; 0.3% MI, 0.3% mixed isoacids added to the basal diet on a dry matter basis.

to the phylum *Firmicutes*, which contains many fibrolytic bacteria (Chen et al., 2022). According to a remarkably higher apparent digestibility of NDF and ADF in the 0.3% MI group than that in the control group. It is speculated that *Veillonellaceae*_UCG-001 may also be a type of fibrolytic bacteria in the yak rumen. *Ruminococcus* also belongs to the phylum *Firmicutes*, which can use cellulose and hemicellulose as substrates to produce VFA (Liu et al., 2019). The *in vitro* tests in this study showed a significant increase in

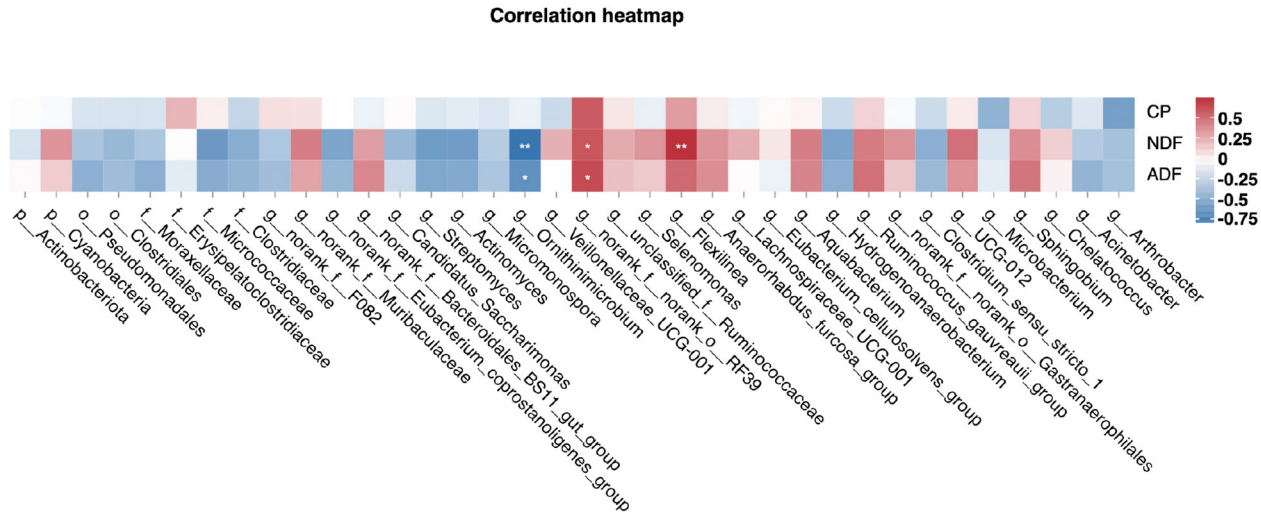

FIGURE 4
The correlations of feed digestibility with the bacterial community from phylum to genus level. The heatmap shows the absolute value of Spearman's correlation $R > 0.1$. CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber. Asterisk indicates the significance of Spearman's correlation, $^*P < 0.05$, $^{**}P < 0.01$, $n = 7$ per group.

propionate acid and butyrate in the 0.3% MI group and a trend toward higher TVFA, which may be related to the increased relative abundance of the *Ruminococcus_gauvreauii_group*. In addition, supplementation with 0.3% MI reduced the relative abundance of norank_f__F082 by 49.98%, which was reported to be negatively correlated with feed fiber concentration and digestibility in yaks (Yi et al., 2022), suggesting that supplementation with MI may increase the available fiber content and digestibility in the rumen of yaks.

The correlation analysis showed that g__*Flexilinea* and g__norank_f__norank_o__RF39 were positively correlated with the apparent digestibility of NDF. g__*Flexilinea* was reported as a filamentous strictly anaerobic Gram-negative bacterium which could digest all kinds of carbohydrates (Wang et al., 2021). In addition, one study has reported that the abundance of g__norank_f__norank_o__RF39 was increased with the increase of NDF digestibility (Liu et al., 2023). We speculate that supplementation with 0.3% MI significantly improved the apparent digestibility of feed fiber, which was associated with the change in the abundance of g__*Flexilinea* and g__norank_f__norank_o__RF39.

5. Conclusion

The optimum dose of MI was found to be 0.3% based on the *in vitro* experiment, which significantly reduced ruminal pH, increased NDFD, ADFD, propionate, and butyrate concentrations, and tended to increase the content of TVFA. Supplementation with 0.3% MI significantly improved the apparent digestibility of feed fiber and altered the ruminal bacterial diversity, which was positively correlated with the abundance of g__*Flexilinea* and g__norank_f__norank_o__RF39.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI - Genome Sequence Archive (GSA) database, and the BioProject accession number is PRJCA016021.

Ethics statement

The animal study was reviewed and approved by Animal Care and Use Committee of Southwest Minzu University.

Author contributions

YG and ZP contributed to conception and design of the study. FJ and XM performed the *in vitro* fermentation experiment. FJ, YY, ZH, YL, and AH conducted the animal experiment. YG and FJ performed the statistical analysis and wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

YL was employed by Si Chuan Action Biotech Co., Ltd.

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1175880/full#supplementary-material

References

An, D., Dong, X., and Dong, Z. (2005). Prokaryote diversity in the rumen of yak (*Bos grunniens*) and Jinnan cattle (*Bos taurus*) estimated by 16s rDNA homology analyses. *Anaerobe*. 11, 207–215. doi: 10.1016/j.anaerobe.2005.02.001

Andries, J. I., Buysse, F. X., Brabander, D., and Cottyn, B. G. (1987). Isoacids in ruminant nutrition: their role in ruminal and intermediary metabolism and possible influences on performances — a review. *Anim. Feed. Sci. Tech*. 18, 169–180. doi: 10.1016/0377-8401(87)90069-1

AOAC (2001). *Official Methods of Analysis*. Gaithersburg: Association of Official Analytical Chemists, Washington, DC.

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

Backhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., and Gordon, J. I. (2005). Host-bacterial mutualism in the human intestine. *Science*. 307, 1915–1920. doi: 10.1126/science.1104816

Chen, S. F., Zhou, Y. Q., Chen, Y. R., and Gu, J. (2018). Fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics*. 34, 884–890. doi: 10.1093/bioinformatics/bty560

Chen, X. D., Yan, F., Liu, T., Zhang, Y. L., Li, X. Y., and Wang, M. Y., et al. (2022). Ruminal microbiota determines the high-fiber utilization of ruminants: evidence from the ruminal microbiota transplant. *Microbiol spectr*. 10, e00446–e00422. doi: 10.1128/spectrum.00446-22

Cheng, J. B., Bu, D. P., Wang, J. Q., Sun, X. Z., Pan, L., and Zhou, L. Y., et al. (2014). Effects of rumen-protected γ-aminobutyric acid on performance and nutrient digestibility in heat-stressed dairy cows. *J. Dairy Sci.* 97, 5599–5607. doi: 10.3168/jds.2013-6797

Cholewińska, P., Czyż, K., Nowakowski, P., and Wyrostek, A. (2020). The microbiome of the digestive system of ruminants – a review. *Anim. Health Res. Rev*. 21, 3–14. doi: 10.1017/S1466252319000069

Dai, Q. D., Ma, J., Cao, G., Hu, R., Zhu, Y. X., and Li, G. Y., et al. (2021). Comparative study of growth performance, nutrient digestibility, and ruminal and fecal bacterial community between yaks and cattle-yaks raised by stall-feeding. *AMB Express*. 11, 98. doi: 10.1186/s13568-021-01259-9

Ding, X. Z., Guo, X., Yan, P., Liang, C. N., Bao, P. J., and Chu, M. (2012). Seasonal and nutrients intake regulation of lipoprotein lipase (LPL) activity in grazing yak (*Bos grunniens*) in the alpine regions around Qinghai Lake. *Livest. Sci.* 143, 29–34. doi: 10.1016/j.livsci.2011.08.004

Dong, Q. M., Zhao, X. Q., Ma, Y. S., Xu, S. X., and Li, Q. Y. (2006). Live-weight gain, apparent digestibility, and economic benefits of yaks fed different diets during winter on the Tibetan Plateau. *Livest. Sci.* 101, 199–207. doi: 10.1016/j.livprodsci.2005.11.009

Du, M., Yang, C., Liang, Z. Y., Zhang, J. B., Yang, Y. Y., and Ahmad, A. A., et al. (2021). Dietary energy levels affect carbohydrate metabolism-related bacteria and improve meat quality in the longissimus thoracis muscle of yak (*Bos grunniens*). *Front. Vet. Sci.* 8, 718036. doi: 10.3389/fvets.2021.718036

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

Firkins, J. L. (2010). Reconsidering rumen microbial consortia to enhance feed efficiency and reduce environmental impact of ruminant livestock production systems. *Rev. Bra. Zootecn*. 39, 445–457. doi: 10.1590/S1516-35982010001300049

Garcia-Gonzalez, R., Gonzalez, J. S., and Lopez, S. (2010). Decrease of ruminal methane production in rusitec fermenters through the addition of plant material from rhubarb (*Rheum spp*.) and alder buckthorn (*Frangula alnus*). *J. Dairy Sci.* 93, 3755–3763. doi: 10.3168/jds.2010-3107

He, Q. D., Guo, J. J., Zhang, Q., Yau, Y. M., Yu, Y., and Zhong, Z. H., et al. (2022). Effects of electroacupuncture on the gut microbiome in cisplatin-induced premature ovarian failure mice. *Evid.-based Compl. Alt.* 2022, 9352833. doi: 10.1155/2022/9352833

Hu, C. S., Ding, L. M., Jiang, C. X., Ma, C. F., Liu, B. T., and Li, D. L., et al. (2021). Effects of management, dietary intake, and genotype on rumen morphology, fermentation, and microbiota, and on meat quality in yaks and cattle. *Front Nutr*. 8, 755255. doi: 10.3389/fnut.2021.755255

Jing, X., Ding, L., Zhou, J., Huang, X., Degen, A., and Long, R. (2022). The adaptive strategies of yaks to live in the Asian highlands. *Anim Nutr*. 9, 249–258. doi: 10.1016/j.aninu.2022.02.002

Kang, K., Ma, J., Wang, H., Wang, Z., Peng, Q., and Hu, R., et al. (2020). High-energy diet improves growth performance, meat quality and gene expression related to intramuscular fat deposition in finishing yaks raised by barn feeding. *Vet. Med. Sci.* 6, 755–765. doi: 10.1002/vms3.306

Liu, C. S., Zhao, D. F., Ma, W. J., Guo, Y. D., Wang, A. J., and Wang, Q. L., et al. (2016). Denitrifying sulfide removal process on high-salinity wastewaters in the presence of *Halomonas* sp. *Appl. Microbiol. Biot.* 100, 1421–1426. doi: 10.1007/s00253-015-7039-6

Liu, Q., Wang, C., Guo, G., Huo, W. J., Zhang, Y. L., and Pei, C. X., et al. (2018). Effects of branched-chain volatile fatty acids supplementation on growth performance, ruminal fermentation, nutrient digestibility, hepatic lipid content and gene expression of dairy calves. *Anim. Feed Sci. Tech.* 237, 27–34. doi: 10.1016/j.anifeedsci.2018.01.006

Liu, Q., Wang, C., Pei, C. X., Li, H. Y., Wang, Y. X., and Zhang, S. L., et al. (2014). Effects of isovalerate supplementation on microbial status and rumen enzyme profile in steers fed on corn stover based diet. *Livest. Sci.* 161, 60–68. doi: 10.1016/j.livsci.2013.12.034

Liu, Q., Wang, C., Yang, W. Z., Zhang, B., Yang, X. M., and He, D. C., et al. (2009). Effects of isobutyrate on rumen fermentation, lactation performance and plasma characteristics in dairy cows. *Anim. Feed Sci. Tech.* 154, 58–67. doi: 10.1016/j.anifeedsci.2009.08.001

Liu, Q., Wang, C., Zhang, Y. L., Pei, C. X., Zhang, S. L., and Wang, Y. X., et al. (2016). Effects of isovalerate supplementation on growth performance and ruminal fermentation in pre- and post-weaning dairy calves. *J. Agr. Sci.* 154, 1499–1508. doi: 10.1017/S0021859616000630

Liu, S. Q., Shah, A. M., Yuan, M., Kang, K., Wang, Z. S., and Wang, L. Z., et al. (2022). Effects of dry yeast supplementation on growth performance, rumen fermentation characteristics, slaughter performance and microbial communities in beef cattle. *Anim. Biotechnol.* 33, 1150–1160. doi: 10.1080/10495398.2021.1878204

Liu, Y. F., Hu, J. M., Li, M. M., and Zhao, G. Y., et al. (2023). Effects of taurine on rumen fermentation, nutrient digestion, rumen bacterial community

and metabolomics and nitrogen metabolism in beef steers. *J. Sci. Food. Agr.* doi: 10.1002/jsfa.12474

Liu, Y. R., Wang, C., Liu, Q., Guo, G., Huo, W. J., and Zhang, Y. L., et al. (2020). Effects of branched-chain volatile fatty acids and fibrolytic enzyme on rumen development in pre- and post-weaned Holstein dairy calves. *Anim. Biotechnol.* 31, 512–519. doi: 10.1080/10495398.2019.1633340

Liu, Y. Z., Chen, X., Zhao, W., Lang, M., Zhang, X. F., and Wang, T., et al. (2019). Effects of yeast culture supplementation and the ratio of non-structural carbohydrate to fat on rumen fermentation parameters and bacterial-community composition in sheep. *Anim. Feed Sci. Tech.* 249, 62–75. doi: 10.1016/j.anifeedsci.2019.02.003

Long, R. J., Dong, S. K., Wei, X. H., and Pu, X. P. (2005). The effect of supplementary feeds on the bodyweight of yaks in cold season. *Livest. Prod. Sci.* 93, 197–204. doi: 10.1016/j.livprodsci.2004.08.016

Lv, F., Wang, X. J., Pang, X., and Liu, G. H. (2020). Effects of supplementary feeding on the rumen morphology and bacterial diversity in lambs. *PeerJ*. 8, 9353. doi: 10.7717/peerj.9353

Ma, J. A., Zhu, Y. X., Wang, Z. S., Yu, X., Hu, R., and Wang, X. Y., et al. (2020). Comparing the bacterial community in the gastrointestinal tracts between growth-retarded and normal yaks on the Qinghai-Tibetan Plateau. *Front. Microbiol.* 11, 600516. doi: 10.3389/fmicb.2020.600516

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

Menke, K. H., Raab, L., Salewski, A., Steingass, H., Fritz, D., and Schneider, W. (1979). The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor *in vitro*. *J. Agr. Sci.* 93, 217–222. doi: 10.1017/S0021859600086305

Ministry of Agriculture of the People's Republic of China (2004). *NY/T 815–2004 Beef Cattle Raising Standard*. Beijing: China Agricultural Press.

Moharrery, A., and Das, T. K. (2001). Correlation between microbial enzyme activities in the rumen fluid of sheep under different treatments. *Reprod. Nutr. Dev.* 41, 513–529. doi: 10.1051/rnd:2001106

Otterby, D. E., Johnson, D. G., Towns, R., Cook, R. M., Erdman, R. A., Van Horn, H. H., et al. (1990). Dose response of dairy cows to ammonium salts of volatile fatty acids. *J. Dairy Sci.* 73, 2168–2178. doi: 10.3168/jds.S0022-0302(90)78897-2

People's Republic of China National Standard (2002a). *GB/T 6436–2002 Determination of Total Phosphorus in Feed - Spectrophotometric Method*. Beijing: China Standard Press.

People's Republic of China National Standard (2002b). *GB/T 6437–2002 Determination of Total Phosphorus in Feed - Spectrophotometric Method*. Beijing: China Standard Press.

Ren, Y., Yu, G., Shi, C., Liu, L., Guo, Q., and Han, C., et al. (2022). Majorbio Cloud: a one-stop, comprehensive bioinformatic platform for multiomics analyses. *iMeta*. 1, e12. doi: 10.1002/imt2.12

Roman-Garcia, Y., Denton, B. L., Mitchell, K. E., Lee, C., Socha, M. T., and Firkins, J. L. (2021a). Conditions stimulating neutral detergent fiber degradation by dosing branched-chain volatile fatty acids. I: Comparison with branched-chain amino acids and forage source in ruminal batch cultures. *J. Dairy Sci.* 104, 6739–6755. doi: 10.3168/jds.2020-20054

Roman-Garcia, Y., Mitchell, K. E., Denton, B. L., Lee, C., Socha, M. T., and Wenner, B. A., et al. (2021b). Conditions stimulating neutral detergent fiber degradation by dosing branched-chain volatile fatty acids. II: Relation with solid passage rate and pH on neutral detergent fiber degradation and microbial function in continuous culture. *J. Dairy Sci.* 104, 9853–9867. doi: 10.3168/jds.2021-20335

Roman-Garcia, Y., Mitchell, K. E., Lee, C., Socha, M. T., Park, T., and Wenner, B. A., et al. (2021c). Conditions stimulating neutral detergent fiber degradation by dosing branched-chain volatile fatty acids. III: Relation with solid passage rate and ph on prokaryotic fatty acid profile and community in continuous culture. *J. Dairy Sci.* 104, 9868–9885. doi: 10.3168/jds.2021-20336

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., and Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microb.* 75, 7537–7541. doi: 10.1128/AEM.01541-09

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

Shao, B., Long, R., Ding, Y., Wang, J., Ding, L., and Wang, H. (2010). Morphological adaptations of yak (*Bos grunniens*) tongue to the foraging environment of the Qinghai-Tibetan Plateau. *J. Anim. Sci.* 88, 2594–2603. doi: 10.2527/jas.2009-2398

Stackebrandt, E., and Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacterio.* 44, 846–849. doi: 10.1099/00207713-44-4-846

Sun, Z. Q., Yu, Z., and Wang, B. (2019). *Perilla frutescens* leaf alters the rumen microbial community of lactating dairy cows. *Microorganisms*. 7, 562. doi: 10.3390/microorganisms 7110562

Thao, N. T., Wanapat, M., Cherdthong, A., and Kang, S. (2014). Effects of eucalyptus crude oils supplementation on rumen fermentation, microorganism and nutrient digestibility in swamp buffaloes. *Asian Austral. J. Anim.* 27, 46–54. doi: 10.5713/ajas.2013.13301

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

Wang, C., Liu, Q., Guo, G., Huo, W. J., Pei, C. X., and Zhang, S. L., et al. (2018a). Effects of concentrate-to-forage ratios and 2-methylbutyrate supplementation on ruminal fermentation, bacteria abundance and urinary excretion of purine derivatives in Chinese Simmental steers. *J. Anim. Physiol. An. N.* 102, 901–909. doi: 10.1111/jpn.12915

Wang, C., Liu, Q., Guo, G., Huo, W. J., Pei, C. X., and Zhang, S. L., et al. (2018b). Effects of dietary protein levels and 2-methylbutyrate on ruminal fermentation, nutrient degradability, bacterial populations and urinary purine derivatives in Simmental steers. *J. Anim. Physiol. An. N.* 102, 611–619. doi: 10.1111/jpn.12797

Wang, C., Liu, Q., Guo, G., Huo, W. J., Wang, Y. X., and Zhang, Y. L., et al. (2019). Effects of fibrolytic enzymes and isobutyrate on ruminal fermentation, microbial enzyme activity and cellulolytic bacteria in pre- and post-weaning dairy calves. *Anim. Prod. Sci.* 59, 471–478. doi: 10.1071/AN17270

Wang, C., Liu, Q., Pei, C. X., Li, H. Y., Wang, Y. X., and Wang, H., et al. (2012). Effects of 2-methylbutyrate on rumen fermentation, ruminal enzyme activities, urinary excretion of purine derivatives and feed digestibility in steers. *Livest. Sci.* 145, 160–166. doi: 10.1016/j.livsci.2012.01.013

Wang, C., Liu, Q., Zhang, Y. L., Pei, C. X., Zhang, S. L., and Wang, Y. X., et al. (2015). Effects of isobutyrate supplementation on ruminal microflora, rumen enzyme activities and methane emissions in Simmental steers. *J. Anim. Physiol. An. N.* 99, 123–131. doi: 10.1111/jpn.12191

Wang, H., Long, R., Liang, J. B., Guo, X., Ding, L., and Shang, Z. (2011). Comparison of nitrogen metabolism in yak (*Bos grunniens*) and indigenous cattle (*Bos taurus*) on the Qinghai-Tibetan Plateau. *Asian Austral. J. Anim.* 24, 766–773. doi: 10.5713/ajas.2011.10350

Wang, L. J., Zhang, G. N., Li, Y., and Zhang, Y. G. (2020). Effects of high forage/concentrate diet on volatile fatty acid production and the microorganisms involved in VFA production in cow rumen. *Animals*. 10, 223. doi: 10.3390/ani10020223

Wang, M., Sun, X. Z., Janssen, P. H., Tang, S. X., and Tan, Z. L. (2014). Responses of methane production and fermentation pathways to the increased dissolved hydrogen concentration generated by eight substrates in *in vitro* ruminal cultures. *Anim. Feed Sci. Tech.* 194, 1–11. doi: 10.1016/j.anifeedsci.2014.04.012

Wang, M., Wang, R., Janssen, P. H., Zhang, X. M., Sun, X. Z., and Pacheco, D., et al. (2016a). Sampling procedure for the measurement of dissolved hydrogen and volatile fatty acids in the rumen of dairy cows. *J. Anim. Sci.* 94, 1159–1169. doi: 10.2527/jas.2015-9658

Wang, M., Wang, R., Xie, T. Y., Janssen, P. H., Sun, X. Z., and Beauchemin, K. A., et al. (2016b). Shifts in rumen fermentation and microbiota are associated with dissolved ruminal hydrogen concentrations in lactating dairy cows fed different types of carbohydrates. *J. Nutr.* 146, 1714–1721. doi: 10.3945/jn.116.232462

Wang, Z., Yang, D. S., Li, X. Y., Yu, L. Y., Zhang, P. H., and He, H. J., et al. (2021). Modulation of rumen fermentation and microbial community through increasing dietary cation–anion difference in Chinese Holstein dairy cows under heat stress conditions. *J. Appl. Microbiol.* 130, 722–735. doi: 10.1111/jam.14812

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

Xue, D., Chen, H., Luo, X., Guan, J., He, Y., and Zhao, X. (2018). Microbial diversity in the rumen, reticulum, omasum, and abomasum of yak on a rapid fattening regime in an agro-pastoral transition zone. *J. Microbiol.* 56, 734–743. doi: 10.1007/s12275-018-8133-0

Yi, S. M., Dai, D. W., Wu, H., Chai, S. T., Liu, S. J., and Meng, Q. X., et al. (2022). Dietary concentrate-to-forage ratio affects rumen bacterial community composition and metabolome of yaks. *Front Nutr*. 9, 1459. doi: 10.3389/fnut.2022.927206

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

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

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

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Polyclonal antibodies inhibit growth of key cellulolytic rumen bacterial species

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Antibodies targeting specific bacterial species could allow for modification of the rumen microbial population to enhance rumen fermentation. However, there is limited knowledge of targeted antibody effects on rumen bacteria. Therefore, our objective was to develop efficacious polyclonal antibodies to inhibit the growth of targeted cellulolytic bacteria from the rumen. Egg-derived, polyclonal antibodies were developed against pure cultures of *Ruminococcus albus* 7 (**anti-RA7**), *Ruminococcus albus* 8 (**anti-RA8**), and *Fibrobacter succinogenes* S85 (**anti-FS85**). Antibodies were added to a cellobiose-containing growth medium for each of the three targeted species. Antibody efficacy was determined via inoculation time (0 h and 4 h) and dose response. Antibody doses included: 0 (**CON**), 1.3×10^{-4} (**LO**), 0.013 (**MD**), and 1.3 (**HI**) mg antibody per ml of medium. Each targeted species inoculated at 0 h with HI of their respective antibody had decreased ($P < 0.01$) final optical density and total acetate concentration after a 52 h growth period when compared with CON or LO. Live/dead stains of *R. albus* 7 and *F. succinogenes* S85 dosed at 0 h with HI of their respective antibody indicated a decrease ($\geq$ 96%; $P < 0.05$) in live bacterial cells during the mid-log phase compared with CON or LO. Addition of HI of anti-FS85 at 0 h in *F. succinogenes* S85 cultures reduced ($P < 0.01$) total substrate disappearance over 52 h by at least 48% when compared with CON or LO. Cross-reactivity was assessed by adding HI at 0 h to non-targeted bacterial species. Addition of anti-RA8 or anti-RA7 to *F. succinogenes* S85 cultures did not affect ($P \geq 0.45$) total acetate accumulation after 52 h incubation, indicating that antibodies have less of an inhibitory effect on non-target strains. Addition of anti-FS85 to non-cellulolytic strains did not affect ($P \geq 0.89$) OD, substrate disappearance, or total VFA concentrations, providing further evidence of specificity against fiber-degrading bacteria. Western blotting with anti-FS85 indicated selective binding to *F. succinogenes* S85 proteins. Identification by LC-MS/MS of 8 selected protein spots indicated 7 were outer membrane proteins. Overall, polyclonal antibodies were more efficacious at inhibiting the growth of targeted cellulolytic bacteria than non-targeted bacteria. Validated polyclonal antibodies could serve as an effective approach to modify rumen bacterial populations.

KEYWORDS

rumen fermentation, cellulolytic bacteria, *Fibrobacter succinogenes*, polyclonal antibodies, *Ruminococcus albus*

1. Introduction

The rumen ecosystem contains a consortium of microbes that collectively degrade plant biomass and enable ruminants to convert indigestible plant material into digestible food products for human consumption. Feed additives are an important nutritional tool that can modify rumen microbial populations to enhance substrate degradation and rumen fermentation. Among these additives, ionophores and antibiotics can be used to inhibit specific rumen bacteria resulting in improved feed efficiency and animal performance (Callaway et al., 2003). However, the use of antibiotics in beef and dairy production has raised concerns about the emergence of antibiotic-resistant microorganisms (Sharma et al., 2018).

An alternative approach to modulating the rumen microbial population is the application of targeted avian-derived immunoglobulin (IgY). Immunizing laying hens against a target antigen such as bacteria or viruses produces polyclonal egg yolk antibodies that can be easily extracted and administered orally. Unlike monoclonal antibodies, IgY is relatively heat-stable and more resistant to acid digestion and proteolysis (Shimuzu et al., 1988). Oral administration of polyclonal antibodies has been effective against bovine rotavirus and coronavirus, as well as *Salmonella*, *Staphylococcus*, and *Pseudomonas* (Mine and Kovacs-Nolan, 2002). Polyclonal antibodies generated toward *Streptococcus bovis* and *Fusobacterium necrophorum* dosed in the rumen decreased the abundance of targeted bacteria as measured by most probable number (MPN) viable cell counts (DiLorenzo et al., 2006, 2008) but had varying effects on dry matter intake, volatile fatty acid concentrations, and rumen pH (Marino et al., 2011; Silva et al., 2022a,b). Avian antibodies have also been investigated to reduce methane produced by ruminal archaea (Cook et al., 2008).

The application of this technology could allow for modification of the rumen microbial population to improve fermentation and animal performance. However, the exact mechanisms through which IgY inhibits bacterial activity have not been determined, and there is limited knowledge of targeted IgY application on rumen bacteria in pure culture. A better understanding of dose and time response along with inhibition efficacy and cross-reactivity to non-targeted rumen bacteria strains is needed before polyclonal antibodies can be used as a reliable tool to alter rumen bacterial populations. Cellulolytic bacteria use adherent degradation systems using a variety of cell-surface proteins to utilize substrates in the rumen (Miron et al., 2001). Thus, they serve as suitable model organisms to provide proof-of-concept for polyclonal antibody efficacy as they have many unique target antigens on the cell surface to distinguish mechanisms and extent of cross-reactivity. Therefore, the objective of this study was to develop efficacious polyclonal antibodies and validate their ability to inhibit the growth of targeted cellulolytic bacteria in pure culture.

2. Materials and methods

2.1. Bacteria

Ruminococcus albus 7, *Ruminococcus albus* 8, *Fibrobacter succinogenes* S85, *Streptococcus bovis* JB1, *Prevotella bryantii* B_14, and *Megasphaera elsdenii* T81 were obtained from the culture collection of Dr. Roderick Mackie, Department of Animal Sciences at the University of Illinois. Pure cultures were routinely transferred in defined media (Supplementary Table S1), with cellobiose or starch as the sole growth substrate (0.4% w/v). Cells from overnight grown cultures ($OD_{600} = 0.8$) were used in all growth experiments. Balch tubes containing 9.0 ml of anaerobically prepared medium (Bryant, 1972) were inoculated with 1.0 ml of each respective culture in triplicate.

2.2. Antibody preparation

Ruminococcus albus 7, *Ruminococcus albus* 8, and *Fibrobacter succinogenes* S85 cells were grown overnight in 10 ml cultures. Cells were inactivated by the addition of 10% formalin and heat treatment at 65°C for 60 min. Cells were pelleted and washed two consecutive times with sodium phosphate-buffered saline (PBS). The washed pellets were suspended in 10 ml of PBS. Polyclonal antibodies were commercially produced (GeneTel Laboratories LLC, Madison, WI). Two hens were immunized with inactivated cells from each bacteria. The first injection was subcutaneous, and the three subsequent injections were intramuscular. All injections included Freund's adjuvant to illicit an immune response. Avian-derived IgY antibodies (13 mg/ml) were, then, extracted from pooled egg yolks (approximately 20 eggs) produced by each hen.

2.3. Substrate disappearance

The phenol–sulfuric acid procedure (Wood and Bhat, 1988) was used to determine substrate disappearance. The sample (300 µl) was centrifuged at 20,000 × g for 20 min and mixed with 5% phenol (300 µl) and concentrated sulfuric acid (1.5 ml). Samples were incubated at room temperature for 30 min before reading absorbance at 490 nm. The absorbance values were calculated as glucose equivalents using a standard linear graph (0–100 mg).

2.4. Volatile fatty acid assay

Concentrations of volatile fatty acids were determined by gas chromatography using an adapted method by Erwin et al. (1961). In brief, samples were acidified with 2 N HCl and centrifuged at 20,000 × g for 20 min. The supernatant was divided into two tubes, and 250 µl of 25% m-phosphoric acid solution was added to each. Samples were frozen overnight, thawed, and centrifuged at 20,000 × g for 5 min. The supernatant was transferred to gas chromatography vials and analyzed for acetate concentration (mM) in cellulolytic bacteria growth assays or acetate, butyrate, propionate, valerate, isobutyrate, and isovalerate concentrations in non-cellulolytic bacteria growth assays (mM). Volatile fatty acid concentrations were used to characterize fermentation across all strains. Additional end products (succinate, formate, ethanol, and H_2) were not measured.

2.5. Live/dead stain

To measure the viability of each culture, samples were assayed at 12 h with a live/dead stain (LIVE/DEAD BacLight Bacterial Viability Kit). Samples were prepared for readings on a fluorescence microplate, according to the manufacturer's instructions. In brief, 100 μl of dye mixture was added to 100 μl of bacterial suspension, thoroughly mixed, and incubated in the dark for 15 min. Fluorescence intensity was measured at 530 nm and 630 nm to produce a live/dead ratio. Suspensions containing a range of percentages of live cells (100%, 90%, 50%, 10%, and 0%) were used to prepare the standard curves for each strain (relationship between % live bacteria and live/dead ratio).

2.6. Scanning electron microscopy

To observe cell surface morphology, samples were taken at h 3 for scanning electron microscopy imaging (Microscopy Suite, Beckman Institute, University of Illinois). Cells were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde. Fixed cells were washed with 0.1 mol/L of sodium cacodylate buffer for 10 min, dehydrated in an ethanol series, and dried by the critical point method. Cells were, then, affixed to aluminum SEM stubs with double-stick carbon tape and sputter-coated with 7 nm of gold-palladium. Samples were examined and imaged on a Field-Emission Environmental Scanning Electron Microscope (FEI Quanta FEG 450 ESEM).

2.7. 2D electrophoresis and Western blot

Two-dimensional electrophoresis was performed according to the carrier ampholine method of isoelectric focusing (Kendrick et al., 2020) by Kendrick Labs, Inc. (Madison, WI). In brief, isoelectric focusing was carried out in a glass tube using 2.0% pH 3-10 Isodalt Servalytes (Serva, Heidelberg, Germany) for 9,600 volt-hrs. After equilibration for 10 min in Buffer 'O' (10% glycerol, 50 mM DTT, 2.3% SDS, and 0.0625 M tris, pH 6.8), each tube gel was sealed to the top of a stacking gel that overlaid a 10% acrylamide slab gel (0.75 mm thick). Sodium dodecyl-sulfate (SDS) gel electrophoresis was carried out for 4 h at 15 mA/gel. The gel was dried between sheets of cellophane paper with the acid at the edge to the left.

After slab gel electrophoresis, the gel for blotting was placed in a transfer buffer and transblotted onto a polyvinylidene fluoride (PVDF) membrane overnight. The PVDF membrane was destained in 100% methanol, rinsed briefly in Tween-Tris-buffered saline (TTBS), and blocked for 2 h in 5% non-fat dried milk (NFDM). The blot was, then, incubated in chicken polyclonal antibody (diluted 1:2,000,000 in 2% NFDM TTBS) overnight and rinsed 3 x 10 min in TTBS. The blot was, then, placed in secondary antibody (KPL anti-Chicken Ig-HRP, LGC SeraCare, United States) (diluted 1:5,000 in 2% NFDM TTBS) for 2 h, rinsed as above, treated with ECL (Thermo Fisher Scientific, United States), and exposed to X-ray film (GE Amersham Hyperfilm ECL).

2.8. Mass spectrometry

Liquid chromatography with tandem mass spectrometry (LS-MS/MS) analyses were conducted at the Protein Sciences laboratory of the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. Gel spots were first destained with 50% acetonitrile (ACN) and then dehydrated with ACN. Next, the gel spots were rehydrated in 50 mM triethylammonium bicarbonate buffer (TEAB) containing 500 ng of proteomics-grade trypsin (Pierce, Thermo Fisher Scientific, United States). The proteins were digested at 55°C for 30 min using a Discover microwave reactor (CEM Corporation, United States), and the resulting peptides were extracted from the gel pieces with 5% formic acid (FA) in 50% ACN. The peptides were dried and desalted with SDB-XC StageTips prior to LC-MS analysis.

The LC-MS analyses were performed with an UltiMate 3000 rsnLC connected to a Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific, United States). The peptides were separated at a flow rate of 300 nL/min on a 25 cm Acclaim PepMap 100 C18 column (Thermo Fisher Scientific, United States) with a 45-min gradient from 5% B to 50% B, where mobile phase A was 0.1% FA and mobile phase B was 0.1% FA in 80% ACN. The mass spectrometer was operated in the positive mode with full MS scans collected at 120k resolution; the top 15 precursors from each MS1 scan were selected for higher energy collisional dissociation (HCD), and the MS2 scans were acquired at 30k resolution. The raw data were searched with Mascot Distiller v2.8.3.0 and Mascot server v.2.8.2 (Matrix Science) against the NCBI *Fibrobacter succinogenes* S85 proteome (9,320 entries; downloaded on January 2023). Search settings included a maximum of two missed cleavages, a peptide mass tolerance of 10 ppm, a fragment mass tolerance of 0.02 Da, and variable modifications of acetylation on protein N-termini and oxidation of methionine residues. High-scoring peptides corresponded to those above the default significance threshold in MASCOT ($P < 0.05$, peptide score >60). Nucleotide sequences were searched through the National Center for Biotechnology Information (NCBI), to determine similarities among proteins annotated as hypothetical.

2.9. Determination of antibody efficacy and cross-reactivity

Growth assays were conducted to test the efficacy of anti-RA7, anti-RA8, and anti-FS85 against cell cultures of *R. albus* 7, *R. albus* 8, and *F. succinogenes* S85. Antibody efficacy was evaluated via an inoculation time (0 h and 4 h) and dose-response. Antibody doses included: 0 (**CON**), 1.3×10^{-4} (**LO**), 0.013 (**MD**), and 1.3 (**HI**) mg antibody per 1 ml of medium. Doses were determined by executing log-fold dilutions of starting material (13 mg/ml). Inoculation time was evaluated to determine the efficacy of each antibody dose at different cell abundances and growth rates. To test for antibody cross-reactivity, the highest antibody dose (1.3 mg/ml) of all three antibody treatments was added to non-target cultures of each strain at 0 h. Growth was determined by optical density (600 nm) measurements over 52 h. Samples were collected at 6, 12, 18, 24, and 52 h for determination of substrate disappearance

and at 12, 24, and 52 h for determination of volatile fatty acid concentration. Samples were collected at 12 h for the live/dead stain assay. Samples were collected at 3 h for SEM imaging.

Additional growth assays were conducted to test for cross-reactivity of anti-FS85 antibodies against cell cultures of the non-target strains *Streptococcus bovis* JB1, *Prevotella bryantii* B_14, and *Megaspahera elsdenii* T81. The highest antibody dose (1.3 mg/ml) of anti-FS85 was added to non-target cultures at 0 h to determine cross-reactivity. Growth was determined by optical density (600 nm) measurements over 24 h. Samples were collected at 4, 8, 12, 16, and 24 h for determination of substrate disappearance and volatile fatty acid concentration. To identify antibody binding-specificity of anti-FS85 to *F. succinogenes* S85, two-dimensional electrophoresis and Western blot analyses were performed by Kendrick Labs, INC. (Madison, WI).

2.10. Statistical analysis

The MIXED procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC) was used for all statistical analyses. Repeated measures were used to analyze optical density, substrate disappearance, and VFA with fixed effects of treatment, time, and the interaction of treatment and time. Compound symmetry was used as the covariance structure after consideration of the fit statistics. Tukey's test was used for *post hoc* pair-wise comparisons. Significance was declared at $P \leq 0.05$, and tendencies were discussed at $0.05 < P < 0.10$.

3. Results

3.1. Determination of antibody efficacy on targeted strains

3.1.1. Fibrobacter succinogenes S85

A treatment × time interaction was observed ($P < 0.001$; Figure 1A) for optical density of *F. succinogenes* S85. Over 9–36 h, addition of HI at 0 h and 4 h decreased OD compared with all other treatments. At 52 h, the addition of HI at 0 h and 4 h decreased OD by 78% and 57%, respectively, when compared with CON. A treatment effect was also observed ($P < 0.001$). Addition of HI at 0 h and 4 h decreased OD compared with CON. Addition of MD at 4 h or LO at 0 h did not affect OD compared with CON. A treatment × time interaction was observed ($P < 0.001$; Figure 1D) for residual cellobiose over 52 h of incubation. The remaining cellobiose at 52 h was greatest in HI at 0 h (2.43 mg/ml), intermediate in HI at 4 h (1.50 mg/ml), and least in all remaining treatments (≤ 0.70 mg/ml).

A treatment effect was observed ($P = 0.01$; Figure 2A) for the percentage of live bacterial cells at 12 h. Addition of HI at 0 h decreased live cells by 98% compared with CON. However, CON and addition of LO at 4 h were similar ($P = 0.99$). There were no differences in the percentage of live bacterial cells between LO at 0 h, MD at 0 h and 4 h, and HI at 4 h. Compared with all other treatments, HI at 0 h had the least ($P < 0.05$) amount of live bacterial cells (2.5%). Total acetate concentrations produced by *F. succinogenes* S85 were affected by treatment ($P = 0.003$; Figure 2D). Acetate was greatest in CON (12.4 mM) and least in HI at 0 h (8.5 mM) and 4 h (9.5 mM). Addition of LO or MD at 0 h or 4 h resulted in similar acetate concentrations compared with CON.

3.1.2. Ruminococcus albus 8

A treatment × time interaction was observed ($P < 0.001$; Figure 1B) for optical density of *R. albus* 8. At 24 h, the addition of LO, MD, and HI at 0 h decreased OD by 19, 43, and 90%, respectively, compared with CON. By 52 h, HI at 0 h and 4 h treatments had increased OD compared with CON. Between 36 and 52 h, CON decreased by 20% and HI increased by at least 60%. An overall treatment effect was also observed ($P < 0.001$). Addition of LO at 4 h, MD at 0 h and 4 h, or HI at 0 h and 4 h decreased OD compared with CON or LO at 0 h. Addition of LO, MD, and HI at 4 h decreased OD by 27, 37, and 63%, respectively, compared with CON. A tendency for a treatment × time interaction ($P = 0.09$; Figure 1E) was observed for residual cellobiose over 52 h of incubation. At h 18, HI at 0 h had greater residual cellobiose compared with LO at 0 h and 4 h. At 52 h, all treatments were similar. A treatment effect was also observed ($P = 0.01$). Overall, remaining cellobiose was greatest in HI at 0 h (2.04 mg/ml), least in MD at 4 h (1.02 mg/ml), and LO at 4 h (0.95 mg/ml) and intermediate in all remaining treatments (1.55–1.21 mg/ml).

A treatment effect was observed ($P = 0.004$; Figure 2B) for the percentage of live *R. albus* 8 cells at 12 h. Live percentage of cells was greatest in CON, intermediate in LO, and least in all other treatments. CON and addition of LO at 0 h or 4 h were similar. Addition of MD or HI at 0 h or 4 h decreased live cell percentage compared with CON. Addition of HI at 0 h decreased live cells by 50% compared with CON. No treatment effect ($P = 0.14$; Figure 2E) was observed for the total acetate concentration of *R. albus* 8. Addition of HI, LO, or MD at 0 h or 4 h did not affect acetate production compared with CON.

3.1.3. Ruminococcus albus 7

A treatment × time interaction was observed ($P < 0.001$; Figure 1C) for optical density of *R. albus* 7. At 10–24 h, OD of CON and LO at 0 h or 4 h remained greater compared with all other treatments. At 36 h, all treatments were similar ($P > 0.99$). A treatment effect was also observed ($P < 0.001$) with CON and LO having the greatest OD overall and HI and MD having the least. A treatment × time interaction was observed ($P < 0.001$; Figure 1F) for residual cellobiose over 52 h incubation. Overall, remaining cellobiose was greatest in HI at 0 h (3.35 mg/ml), least in CON (2.56 mg/ml) and LO at 0 h (2.37 mg/ml) and 4 h (2.40 mg/ml), and intermediate in all remaining treatments (3.04–2.86 mg/ml).

A treatment effect was observed ($P < 0.001$; Figure 2C) for the percentage of live *R. albus* 7 cells at 12 h. Addition of MD or HI at 0 h or 4 h decreased live bacterial cell percentage compared with CON or LO at 0 h or 4 h. Addition of MD at 0 h or 4 h decreased cells by 84% and 79%, respectively, compared with CON. Addition of HI at 0 h or 4 h decreased cells by 100% and 99%, respectively, compared with CON. There was a treatment effect ($P < 0.001$; Figure 2F) for *R. albus* 7 acetate concentrations. Addition of MD or HI at 0 h or 4 h decreased acetate concentrations compared with CON or LO at 0 h or 4 h. Addition of HI or MD at 0 h decreased acetate by 19% compared with CON. Addition of HI or MD at

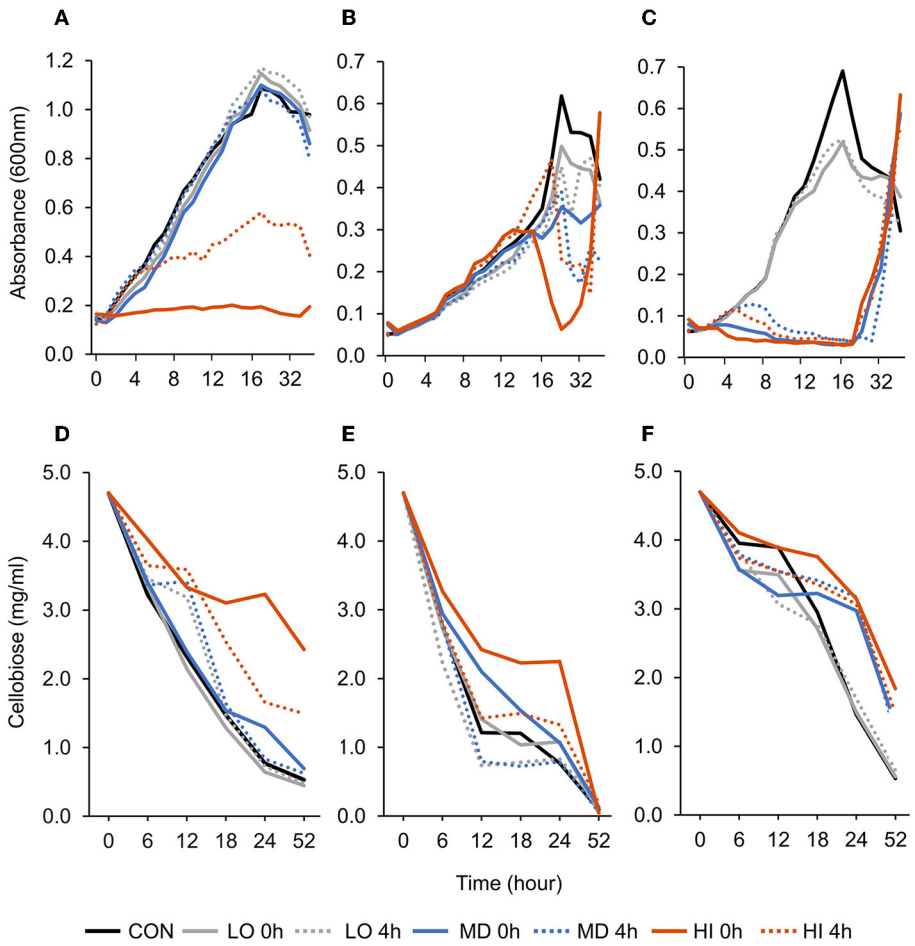

FIGURE 1
Effect of targeted antibody treatment on optical density and residual cellobiose. **(A–C)** Optical density (600nm). **(A)**—*F. succinogenes*; a treatment × time interaction was observed ($P < 0.001$; SEM = 0.028) **(B)**—*R. albus* 8; a treatment × time interaction was observed ($P < 0.001$; SEM = 0.041). **(C)**—*R. albus* 7; a treatment × time interaction was observed ($P < 0.001$; SEM = 0.038). **(D–F)** Residual cellobiose (mg/ml). **(D)**—*F. succinogenes*; a treatment × time interaction was observed ($P < 0.001$; SEM = 0.136). **(E)**—*R. albus* 8; a tendency for a treatment × time interaction was observed ($P = 0.09$; SEM = 0.275). **(F)**—*R. albus* 7; a treatment × time interaction was observed ($P < 0.001$; SEM = 0.149). Treatment; CON = 0, LO = 1.3×10^{-4}, MD = 0.013, HI = 1.3 mg/ml dosed at 0 h or 4 h.

4 h decreased acetate by 19% and 21%, respectively, compared with CON.

3.2. Determination of antibody cross-reactivity

3.2.1. Fibrobacter succinogenes S85

A treatment × time interaction was observed ($P < 0.001$; Figure 3A) for optical density of *F. succinogenes* S85. At 24–52 h, treatments were similar. At 11–18 h, the addition of anti-RA8 decreased OD compared with CON and anti-RA7. Additionally, a treatment effect was observed ($P < 0.001$) for optical density of cells with CON being the greatest (0.47 OD), addition of anti-RA7 being intermediate (0.42 OD), and addition of anti-RA8 being the least (0.22 OD). A treatment × time interaction ($P = 0.003$; Figure 3D) was observed for residual cellobiose. At 52 h, the remaining cellobiose was similar ($P = 1$) among treatments. A treatment effect was also observed ($P < 0.001$). Addition of anti-RA8 increased remaining cellobiose compared with anti-RA7 or CON. Addition of anti-RA7 did not affect substrate disappearance in the growth medium over time compared with CON.

A treatment effect was observed ($P < 0.001$; Figure 4A) for the percentage of live *F. succinogenes* S85 cells at 12 h with CON being the greatest (86%), anti-RA7 being intermediate (44%), and anti-RA8 being the least (0%). Addition of anti-RA7 decreased live bacterial cells by 49% compared with CON. Addition of anti-RA8 decreased live bacterial cells by 100% compared with CON. No treatment effect ($P = 0.17$; Figure 4D) was observed for total acetate concentrations. Addition of anti-RA7 or anti-RA8 did not affect the overall acetate production compared with CON. Scanning electron microscopy images taken 3 h after the addition of anti-FS85 show more cell-to-cell aggregation and disruption of the cell surface when compared with control cells and the addition of anti-RA7 or anti-RA8 (Figure 5). Extracellular polymeric substance (EPS) was seen in greater amounts on the surface of cells treated with the targeted anti-FS85 compared with the non-targeted antibodies.

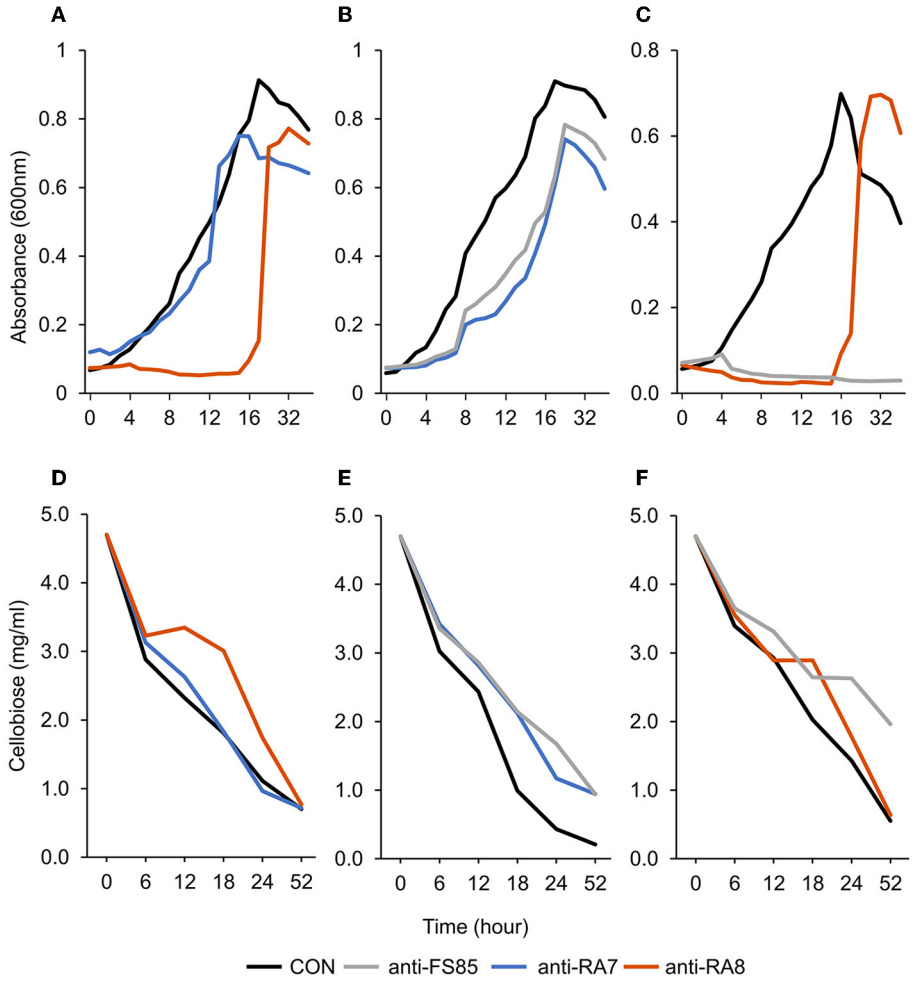

FIGURE 2
Effect of non-targeted antibody treatment on optical density and residual cellobiose. **(A–C)** Optical density (600 nm). **(A)**—*F. succinogenes*; a treatment × time interaction was observed ($P < 0.001$; SEM = 0.049) **(B)**—*R. albus* 8; a treatment × time interaction was observed ($P < 0.001$; SEM = 0.009). **(C)**—*R. albus* 7; a treatment × time interaction was observed ($P < 0.001$; SEM = 0.008). **(D–F)** Residual cellobiose (mg/ml). **(D)**—*F. succinogenes*; a treatment × time interaction was observed ($P = 0.003$; SEM = 0.138). **(E)**—*R. albus* 8; a treatment × time interaction was observed ($P = 0.03$; SEM = 0.145). **(F)**—*R. albus* 7; a treatment × time interaction was observed ($P < 0.001$; SEM = 0.112). Treatment; CON = 0 mg/ml, anti-FS85 = 1.3 mg/ml of *F. succinogenes* antibody, anti-RA7 = 1.3 mg/ml of *R. albus* 7 antibody, anti-RA8 = 1.3 mg/ml of *R. albus* 8 antibody.

3.2.2. Ruminococcus albus 8

A treatment × time interaction was observed ($P < 0.001$; Figure 3B) for optical density of *R. albus* 8. At 0–4 h, treatments were similar. Over 4–9 h, OD decreased in anti-FS85 and anti-RA7 treatments compared with CON. At 10–15 h, the addition of anti-RA7 decreased OD when compared with anti-FS85 or CON. Additionally, a treatment effect was observed ($P < 0.001$) for optical density of cells with CON being the greatest (0.52 OD), anti-FS85 being intermediate (0.37 OD), and anti-RA7 being the least (0.32 OD). A treatment × time interaction ($P = 0.03$; Figure 3E) was observed for residual cellobiose over 52 h incubation. At 6 and 12 h, treatments were similar. At 52 h, there was a tendency ($P \leq 0.07$) for increased cellobiose remaining in anti-FS85 and anti-RA7 compared with CON. A treatment effect was also observed ($P < 0.001$) with anti-FS85 or anti-RA7 having increased the total remaining cellobiose compared with CON.

A treatment effect was observed ($P < 0.001$; Figure 4B) for the percentage of live bacterial *R. albus* 8 cells at 12 h with CON being the greatest (100%), anti-RA7 being intermediate (54%), and anti-FS85 being the least (16%). Addition of anti-RA7 decreased live bacterial cells by 46% compared with CON. Addition of anti-FS85 decreased live bacterial cells by 84% compared with CON. A treatment effect was observed ($P = 0.001$; Figure 4E) for total *R. albus* 8 acetate concentrations with CON being greater compared with anti-RA7 and anti-FS85. Overall, anti-RA7 decreased acetate by 30% compared with CON. Addition of anti-FS85 decreased acetate by 25% compared with CON. Scanning electron microscopy images taken 3 h after the addition of anti-RA8 show more cell-to-cell aggregation when compared with control and the addition of anti-RA7 or anti-FS85 (Supplementary Figure S1). Cells with anti-RA8 and anti-FS85 show EPS on the surface of cells.

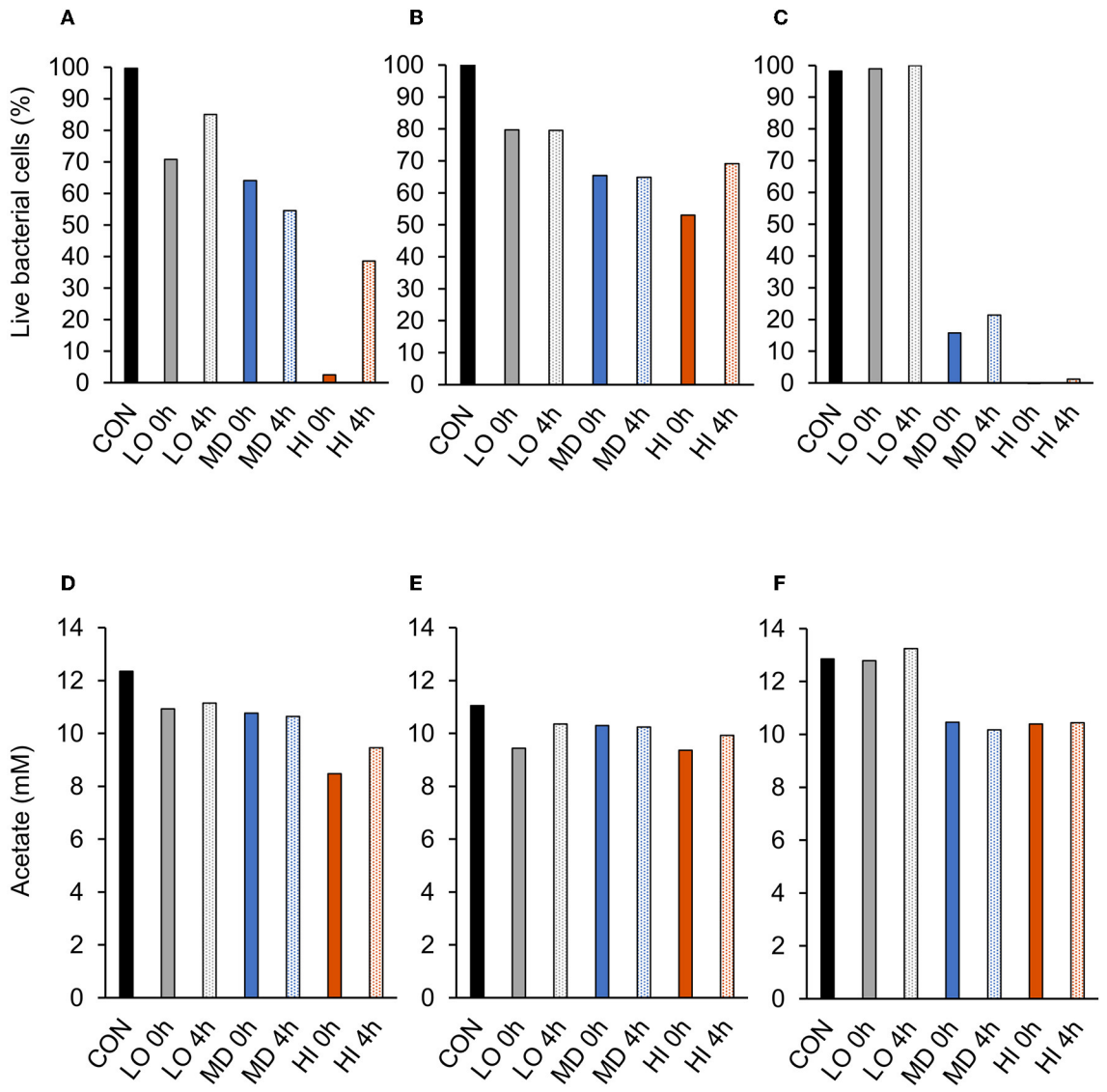

FIGURE 3
Effect of targeted antibody treatment on live bacterial cells and acetate production. **(A–C)** Live bacterial cells (%) at mid-log phase. **(A)**—*F. succinogenes*; a treatment effect was observed ($P = 0.01$; SEM = 14.64) **(B)**—*R. albus* 8; a treatment effect was observed ($P = 0.004$; SEM = 7.10). **(C)**—*R. albus* 7; a treatment effect was observed ($P < 0.001$; SEM = 5.91). **(D–F)** Total acetate (mM). **(D)**—*F. succinogenes*; a treatment effect was observed ($P = 0.003$; SEM = 0.953). **(E)**—*R. albus* 8; no treatment effect was observed ($P = 0.14$; SEM = 0.656). **(F)**—*R. albus* 7; a treatment effect was observed ($P < 0.001$; SEM = 0.463). Treatment; CON = 0, LO = 1.3×10^{-4}, MD = 0.013, HI = 1.3 mg/ml dosed at 0 h or 4 h.

3.2.3. Ruminococcus albus 7

A treatment × time interaction was observed ($P < 0.001$; Figure 3C) for optical density of *R. albus* 7. At 4–18 h, the addition of anti-RA8 decreased OD compared with CON. However, at 24–52 h, the addition of anti-RA8 increased OD compared with CON. Additionally, a treatment effect was observed ($P < 0.001$) for optical density of cells with CON being the greatest (0.35 OD), anti-RA8 being intermediate (0.18 OD), and anti-FS85 being the least (0.05 OD). A treatment × time interaction was observed ($P < 0.001$; Figure 3F) for residual cellobiose over 52 h incubation. At 18 h, the addition of anti-RA8 and anti-FS85 increased the remaining cellobiose compared with CON. At 6, 12, 24, and 52 h, CON and anti-RA8 had similar remaining cellobiose concentrations. At 18 h, the addition of anti-RA8 and anti-FS85 increased the remaining cellobiose by at least 30% when compared with CON. A treatment effect ($P < 0.001$) was observed for residual cellobiose remaining with CON being the greatest (2.07 mg/ml), anti-RA8 being intermediate (2.35 mg/ml), and anti-FS85 being the least (2.84 mg/ml).

A treatment effect was observed ($P < 0.001$; Figure 4C) for the percentage of live *R. albus* 7 cells at 12 h with CON being the greatest (95%), anti-FS85 being intermediate (33%), and anti-RA8 being the least (6%). Addition of anti-FS85 decreased live bacterial cells by 65% when compared with CON. Addition of anti-RA8 decreased live bacterial cells by 94% when compared with CON. A treatment effect ($P < 0.001$; Figure 4F) was observed for total *R. albus* 7 acetate concentrations. Overall, anti-RA8 decreased total acetate by 32% when compared with CON. Addition of anti-FS85

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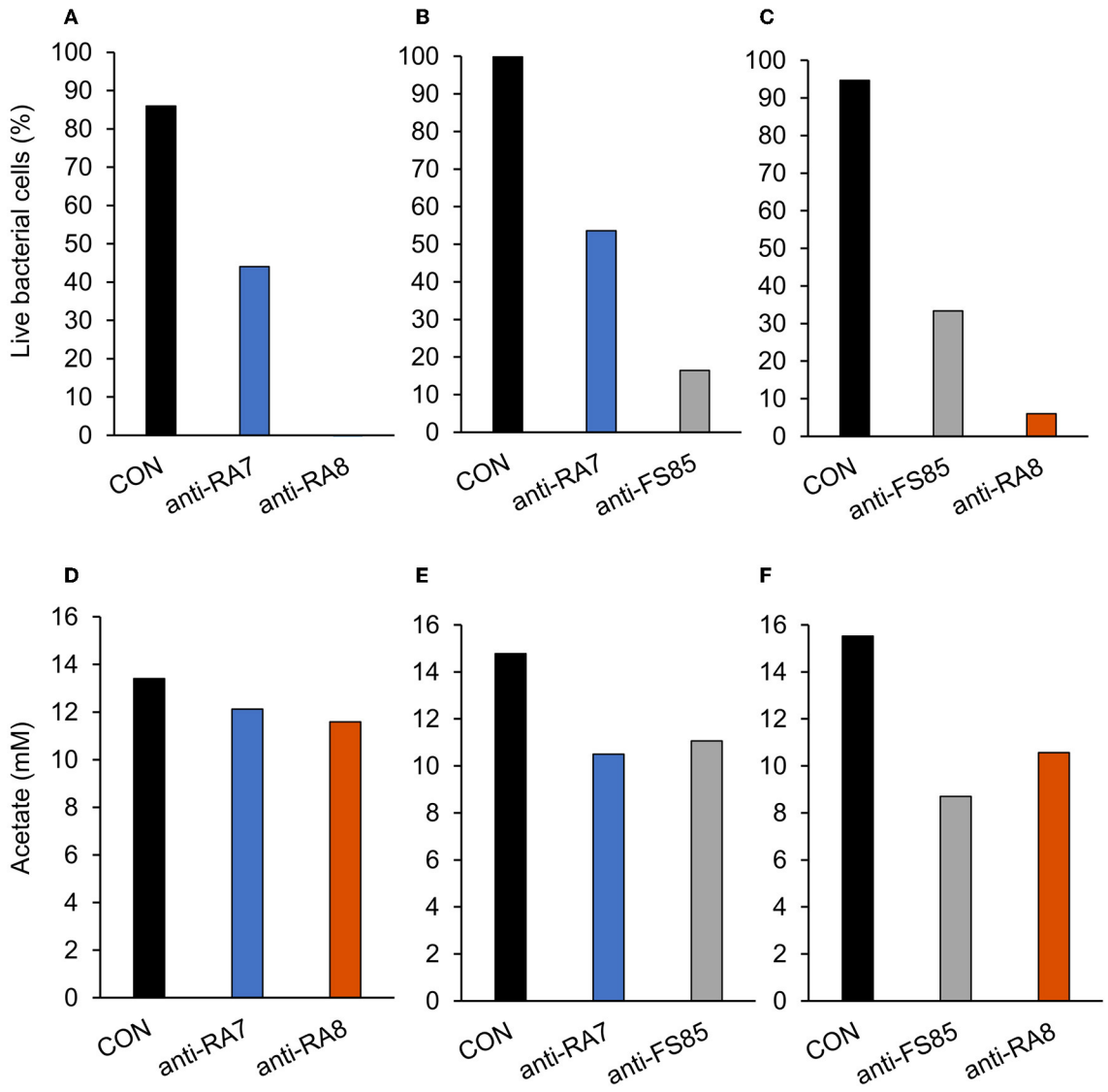

FIGURE 4
Effect of non-targeted antibody treatment on live bacterial cells and acetate production **(A–C)**; live bacterial cells (%) at mid-log phase. **(A)**—*F. succinogenes*; a treatment effect was observed ($P < 0.001$; SEM = 4.47) **(B)**—*R. albus* 8; a treatment effect was observed ($P < 0.001$; SEM = 5.27). **(C)**—*R. albus* 7; a treatment effect was observed ($P < 0.001$; SEM = 3.89). **(D–F)** R Total acetate (mM). **(D)**—*F. succinogenes*; no treatment effect was observed ($P = 0.17$; SEM = 0.781). **(E)**—*R. albus* 8; a treatment effect was observed ($P = 0.001$; SEM = 0.712). **(F)**—*R. albus* 7; a treatment effect was observed ($P < 0.001$; SEM = 0.574). Treatment; CON = 0 mg/ml, anti-FS85 = 1.3 mg/ml of *F. succinogenes* antibody, anti-RA7 = 1.3 mg/ml of *R. albus* 7 antibody, anti-RA8 = 1.3 mg/ml of *R. albus 8* antibody.

decreased total acetate by 44% compared with CON. Scanning electron microscopy images taken 3h after the addition of anti-RA7 and anti-FS85, show more cell surface EPS compared with the addition of anti-RA8 (Supplementary Figure S2).

3.3. Evaluation of anti-FS85 cross-reactivity and antibody-binding specificity

A treatment × time interaction was observed ($P < 0.001$; Figure 6A) for optical densities of *F. succinogenes*, *S. bovis* JB1, *P. bryantii* B_14, and *M. elsdenii* T81. At 3–24 h, addition of anti-FS85 decreased optical density of *F. succinogenes*. However, over 1–24 h, the addition of anti-FS85 to *S. bovis* or *M. elsdenii* did not affect optical density compared with their respective controls. Addition of anti-FS85 to *P. bryantii* decreased optical density at 7 h, but between 8 h and 24 h, optical density was similar to *P. bryantii* control.

A treatment × time interaction was observed ($P < 0.001$; Figure 6B) for residual cellobiose over 24 h incubation. At 8, 12, 16, and 24 h, the addition of anti-FS85 increased the remaining cellobiose in *F. succinogenes* cultures. At 24 h, remaining cellobiose concentrations were similar among *S. bovis* JB1, *M. elsdenii* T81, *P. bryantii* B_14, and their respective cultures treated with anti-FS85. A treatment effect was observed ($P < 0.001$; Figure 6C) for total volatile fatty acid concentrations. Addition of anti-FS85 did not affect VFA concentrations in *P. bryantii*, *S. bovis*, or *M. elsdenii* cultures. However, the addition of

anti-FS85 to *F. succinogenes* decreased total VFA concentration by 37%.

Whole-cell lysate of *F. succinogenes* was subjected to two-dimensional electrophoresis (Figure 7A) to visualize patterns of the cell protein. The final 2D slab gel was transferred to a PVDF membrane, and a Western blot was performed. The spots were visualized on an ECL film after 3 min of X-ray exposure (Figure 7B). Protein patterns on the 2D gel differ from the protein patterns visualized on the ECL film suggesting selective antibody binding.

A total of 10 protein spots were identified on the 2D gel and selected for LC-MS/MS analysis. In total, 9 of the 10 proteins had Mascot scores > 60 (Table 1), indicating successful protein identification. Proteins 1–8 were visualized on ECL film and selected to determine a subset of proteins with anti-FS85 binding-specificity. Proteins 9 and 10 were selected as controls to determine the profile of proteins that were not bound by anti-FS85. Of the eight proteins that bound to anti-FS85, seven were identified as outer membrane proteins. Control protein spots 9 and 10 were identified as cytoplasmic proteins.

4. Discussion

4.1. Determination of antibody efficacy on targeted strain

The use of egg-derived, targeted polyclonal antibodies has been shown to inhibit a variety of intestinal pathogens such as bovine and human rotaviruses, bovine coronavirus, *Salmonella*, *Staphylococcus,* and *Pseudomonas* (Mine and Kovacs-Nolan, 2002) as well as growth of *S. bovis* and *F. necrophorum* in the rumen (DiLorenzo et al., 2006). Additionally, targeted polyclonal antibodies used *in vitro*, inhibited the growth of lipolytic bacteria (Edwards et al., 2017) and several pathogenic bacteria such as *Escherichia coli*, *Salmonella enteritidis,* and *Helicobacter pylori* (Lee et al., 2002; Sunwoo et al., 2002; Solhi et al., 2017). However, there is limited knowledge of targeted IgY application on rumen bacteria, and a targeted rumen antibody approach has not been validated for efficacy and cross-reactivity in pure culture. The ability to inhibit the growth of specific rumen bacteria could be used to modulate the rumen toward enhanced fermentation patterns.

In the present study, the addition of the highest dose (HI; 1.3 mg/ml) of each antibody into cultures of their targeted strain had the greatest inhibitory effect on OD, substrate utilization, and live bacterial cell percentage when compared with the intermediate (MD; 0.13 mg/ml) or low (LO; 1.3×10^{-4} mg/ml) doses. Addition of the HI dose at 0 h decreased total optical density by 74% for *F. succinogenes* S85, 30% for *R. albus* 8, and 68% for *R. albus* 7. The inhibitory effect of HI and MD in *R. albus* 7 persisted for 32 h before those treatments began to increase in OD. This suggests that the mechanism for inhibition may be limited to the initial growth phase when cell counts are low. Bacterial agglutination is a potential mechanism that could reduce OD measurements but not viable cell counts (Tsubokura et al., 2003). Other *in vitro* studies have suggested IgY can cause an

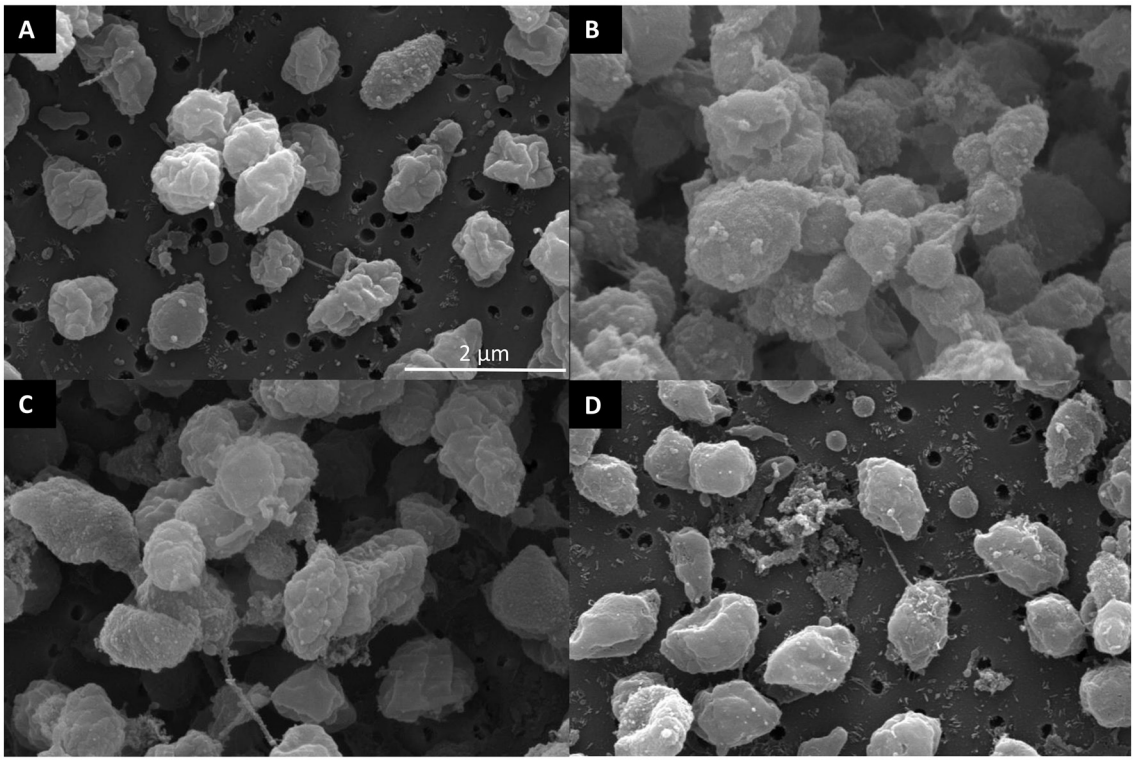

FIGURE 5
Scanning electron micrographs of surface morphology after antibody treatment of *F. succinogenes* S85. **(A)** CON—no antibody **(B)** 1.3 mg/ml of anti-FS85 **(C)** 1.3 mg/ml of anti-RA7 **(D)** 1.3 mg/ml of anti-RA8.

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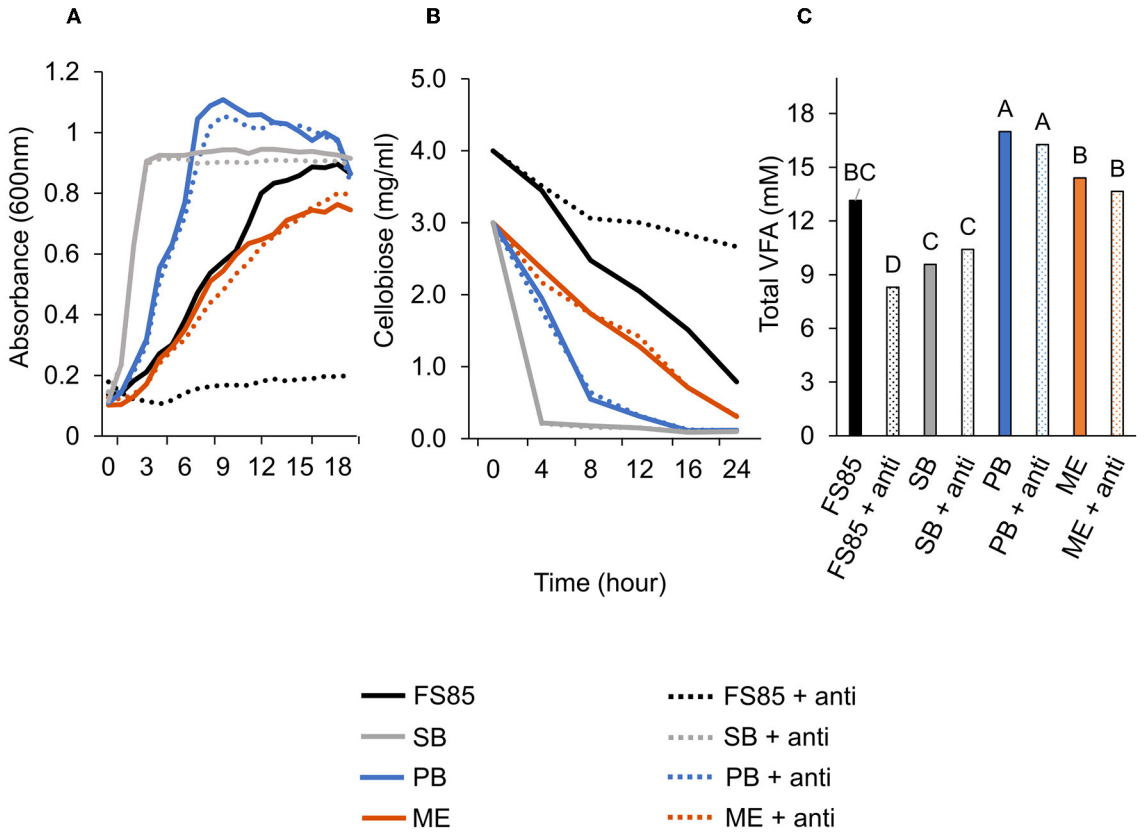

FIGURE 6
Effect of anti-FS85 antibody treatment on non-cellulolytic bacteria. **(A)**—optical density (600nm) a treatment × time interaction was observed ($P < 0.001$; SEM = 0.012). **(B)**—residual cellobiose (mg/ml) a treatment × time interaction was observed ($P < 0.001$; SEM = 0.070). **(C)**—total volatile fatty acids (mM) a treatment effect was observed ($P < 0.001$; SEM = 0.695). Treatment; FS85 = *F. succinogenes*, SB = *S. bovis* JB1, PB = *P. bryantii* $B_1$4, ME = *M. elsdenii* T81, + anti = 1.3mg/ml of anti-FS85. Means with unlike labels differ ($P \leq 0.05$).

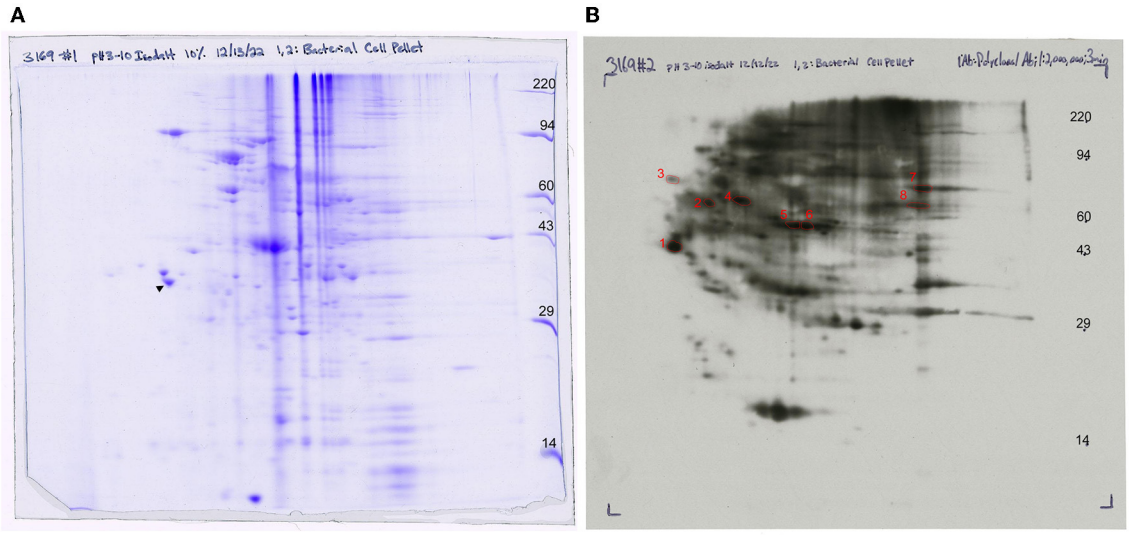

FIGURE 7
Western blot analysis of anti-FS85 antibody treatment **(A)** 2D SDS PAGE and **(B)** 2D Western blot ECL detection with selected protein spots.

TABLE 1 Proteins identified by LC-MS/MS from 2D gel spots.

Protein #	Gene ID[a]	Mascot score[b]	emPAI[c]	Annotation
1	ADL26237.1	90	0.3	Hypothetical protein FSU_1693
2	ADL24646.1	36	0.07	Conserved domain protein
3	ADL26485.1	85	0.06	Segregation and condensation protein A
4	ADL26888.1	196	0.47	Putative lipoprotein
5	ADL25872.1	1182	4.28	Putative carboxyl-terminal protease
6	ADL24825.1	510	2.22	Outer membrane efflux protein
7	ADL25985.1	874	3.17	Hypothetical protein FSU_0659
8	ADL27203.1	5828	320.98	Translation elongation factor Ts
9	ADL27311.1	397	0.56	Putative saccharopine dehydrogenase
10	ADL25838.1	3019	34.16	Glyceraldehyde 3-phosphate dehydrogenase A

[a]National Center for Biotechnology Information (NCBI). Bethesda, MD.
[b]Reflects combined scores of all observed mass spectra that can be matched to amino acid sequence within that protein (Matrix Science).
[c]$10^{PAI}-1$, where PAI (Protein Abundance Index) denotes the ratio of observed to observable peptides (Ishihama et al., 2005).

adherence blockade that may impair growth-related functions (Jin et al., 1998; Lee et al., 2002). Addition of anti-RA8 at all doses had no effect on growth until 18 h. However, OD of *R. albus* 8 CON remained below 0.35 until 18 h, suggesting slow cell growth may have decreased the opportunity to observe a response to antibody inclusion.

Substrate disappearance was measured to determine the amount of cellobiose remaining throughout the 52 h incubation period. The medium contained 4 mg/ml of cellobiose at the beginning of the experiment. The total remaining cellobiose was greatest ($\geq$ 2.04 mg/ml) with the addition of HI at 0 h. At the end of the incubation period, the addition of HI at 0 h or 4 h resulted in increased remaining cellobiose in *F. succinogenes* S85 and *R. albus* 7 compared with CON. This decreased substrate utilization corresponds with decreased growth measured by OD. In contrast, at 52 h, *R. albus* 8 treatments did not differ and had depleted cellobiose ($\leq$ 0.18 mg/ml). At 24 h, 2.25 mg/ml of cellobiose remained in HI at 0 h in *R. albus* 8-treated cells, indicating most of the substrate utilization for this treatment occurred between 24 h and 52 h. This data also correspond with OD as the addition of HI at 0 h, increased from 0.06 to 0.58 OD between 24 h and 52 h.

Addition of the HI dose (1.3 mg/ml) at 0 h decreased live bacterial cells by 98% for *F. succinogenes* S85, 50% for *R. albus* 8, and 100% for *R. albus* 7. In a similar study, the use of a lower dose (0.5 mg/ml) of egg-derived *Propionibacterium avidum* antibody reduced the lipolytic activity of target *P. avidum* by 59% (Edwards et al., 2017). Addition of 2.5 ml of an *S. bovis*-targeted polyclonal antibody to the feed of ruminally cannulated steers decreased ($P < 0.05$) ruminal MPN enumeration counts of *S. bovis* by 80% (DiLorenzo et al., 2006). Overall, these data suggest that anti-FS85 and anti-RA7 have a greater inhibitory effect on the growth of their targeted strain compared with anti-RA8. However, 1.3 mg/ml of antibody added at 0 h will decrease the growth of each targeted strain over a 52-h incubation period.

4.2. Determination of antibody cross-reactivity

Whole-cell preparations of polyclonal antibodies can recognize and bind to many different epitopes of a single antigen. Therefore, cross-reactivity is more likely to occur with polyclonal antibodies than with monoclonal antibodies, which detect a single epitope on the antigen (Frank, 2002). In the present study, antibodies were capable of reducing the growth activity of strains that they were not specifically generated against.

In *F. succinogenes* S85, optical density decreased by 11% in anti-RA7 compared with 54% in anti-RA8 treatments. However, OD was similar in all treatments at 24–52 h. Therefore, *F. succinogenes* S85 cells treated with non-targeted antibodies had greater max OD (0.68) compared with cells treated with targeted antibodies (0.20), suggesting targeted antibodies have greater inhibition efficacy than non-targeted antibodies. At 12 h, live bacterial cells were decreased by 49% with the addition of anti-RA7 and by 100% with the addition of anti-RA8. This data corresponds with the OD at 12 h with CON being the greatest (0.50), anti-RA7 being intermediate (0.40), and anti-RA8 being the least (0.10). However, by 18 h, anti-RA8-treated cells were similar compared with CON, indicating inhibitory effects had decreased by this stage. A similar cross-reactivity response revealed that the addition of 0.5 mg/ml of *P. avidum* polyclonal antibody decreased the lipolytic activity of *P. acnes* by 70% and *B. fibrisolvens* H17c by 49% (Edwards et al., 2017). Scanning electron images show more cell surface disruption with the addition of anti-FS85 compared with anti-RA7 or anti-RA8. Similarly, transmission electron microscopy images of *E. coli* O157:H7 incubated with targeted antibodies resulted in morphological changes and IgY binding around the cell surface (Sunwoo et al., 2002). Overall, these data suggest that the addition of anti-RA8 had a greater inhibitory effect on growth characteristics of *F. succinogenes* S85 than anti-RA7. Additionally, anti-RA7 had lesser inhibitory effects on growth characteristics than HI of anti-FS85.

In *R. albus* 8, optical density decreased by 30% in anti-FS85 and 38% in anti-RA7. Throughout the 52 h incubation period, CON remained the greatest in OD, anti-FS85 was intermediate, and anti-RA7 was the least. The max OD reached by anti-RA7 was 0.74 at 24 h. In experiment 1, the max OD reached by HI at 0 h was 0.58 at 52 h. Substrate disappearance was decreased with the addition of anti-RA7 and anti-FS85 compared with control. However, by 52 h, all treatments had similar remaining cellobiose concentrations. Overall, acetate concentrations were greater in CON compared with anti-FS85 and anti-RA7. At 24 h, anti-FS85 and CON had similar acetate concentrations. These data suggest that both antibodies had an inhibitory growth effect on *R. albus* 8. Similarly, a study aimed to evaluate the inhibitory effects of IgY polyclonal antibodies generated against four strains of *H. pylori* showed a cross-strain inhibitory effect, reducing the growth of non-targeted strains by 29–86% (Solhi et al., 2017).

In *R. albus* 7, optical density decreased by 49% in anti-RA8 and 87% in anti-F85. This percentage of decrease in anti-FS85 is greater than the percentage of decrease in HI at 0 h of anti-RA7 (68%). Interestingly, the anti-RA7 antibody only decreased *F. succinogenes* S85 cells by 11%, suggesting anti-FS85 had a greater inhibitory effect on OD toward *R. albus* 7 than anti-RA7 had on *F. succinogenes* S85. Substrate disappearance was decreased with the addition of anti-RA8 and anti-FS85. At 52 h, CON and anti-RA8 had similar cellobiose remaining. Additionally, live bacterial cell percentage and total acetate were decreased with the addition of anti-RA8 and anti-FS85. Overall, *R. albus* 7 showed the greatest cross-reactivity to non-targeted antibodies with anti-FS85 having a greater inhibitory effect than anti-RA8. Additionally, anti-FS85 and anti-RA7 have similar inhibitory effects on *R. albus* 7 when added at the highest dose. Similar to *H. pylori*, these cellulolytic bacteria may have similar antigens that each antibody could interact with, causing this cross-reactivity effect (Solhi et al., 2017). In contrast, polyclonal antibodies generated against *E.coli* O157:H7 had very little cross-reactivity among other members of the family Enterobacteriaceae (Sunwoo et al., 2002).

4.3. Evaluation of anti-FS85 cross-reactivity and antibody-binding specificity

Overall, anti-FS85 displayed greater inhibitory effects on the growth of *F. succinogenes* compared with anti-RA8 and anti-RA7 but was still able to inhibit the growth of the other two cellulolytic strains, *R. albus* 7 and *R. albus* 8. Further evaluation of this antibody was conducted to explore its effects on non-cellulolytic rumen bacteria. *S. bovis* JB1, *P. bryantii* $B_1$4, and *M. elsdenii* T81 were dosed with 1.3 mg/ml of anti-FS85. Overall, OD, substrate disappearance, and total VFA were not affected by the addition of anti-FS85. As expected, *F. succinogenes* had decreased OD, decreased VFA concentrations, and increased residual cellobiose when dosed with 1.3 mg/ml of anti-FS85. These results indicate that anti-FS85 has greater binding specificity to *F. succinogenes* when compared with *S. bovis* JB1, *P. bryantii* $B_1$4, and *M. elsdenii* T81, suggesting that the epitope regions targeted by anti-FS85 are not located on these non-cellulolytic rumen bacteria strains.

A 2D Western blot was performed to further evaluate antibody-binding specificity. The Western blotting process relies on the primary antibody's specificity, to recognize and bind to its target antigen, and selectivity, to bind to its target antigen in the presence of other proteins (Pillai-Kastoori et al., 2020). As expected, anti-FS85 was able to recognize and bind to multiple epitopes but did not bind to all proteins separated on the 2D gel. This suggests that anti-FS85 has both specificity and selectivity toward its target antigens.

To identify these epitope regions, eight protein spots were excised and analyzed through LC-MS/MS. Protein 1 was annotated as hypothetical protein FSU_0659 and was most likely associated with pilus assembly. This protein shares motifs with type IV pilus assembly proteins and is clustered with genes annotated as pilus assembly proteins (FSU_0658, FSU_0659, FSU_0660, FSU_0661, and FSU_0662). Type IV pili are filaments located on the cell surface of bacteria and have been shown to play a role in cellulose adhesion in *R. albus* and *R. flavefaciens* (Vodovnik et al., 2013). Protein 2 was annotated as a conserved domain protein identified from outer membrane proteins in *F. succinogenes* S85 (Jun et al., 2007). Protein 3 was annotated as FSU_1323, a segregation and condensation protein A that forms part of the condensin complex necessary for chromosomal partition during cell division. The condensin complex pulls DNA away from the mid-cell into both cell halves (Dervyn et al., 2004). Protein 4 was annotated as a putative lipoprotein, which is a hydrophilic protein anchored to the cell membrane (Wilson and Bernstein, 2016). Similarly, *F. succinogenes* outer membrane proteins were identified from cells grown on cellulose and glucose (Jun et al., 2007), and four of the identified proteins were putative lipoproteins. Protein 5 was identified as a putative-carboxyl-terminal protease (CtpA), which functions in post-translational protein processing (Satoh and Yamamoto, 2007). These proteins have been identified on the outer membrane of *Borrelia burgdorferi* (Östberg et al., 2004). Protein 6 was annotated as an outer membrane efflux protein and functions as a channel to allow the export of substrates in Gram-negative bacteria (Johnson and Church, 1999). The most abundant protein, with an emPAI score of 320, was annotated as a translation elongation factor Ts, which functions alongside EF-Tu to assist in protein synthesis (Burnett et al., 2013). EF-Tu has been found on the cell surface of Gram-positive and Gram-negative bacteria (Granato et al., 2004). The control proteins that did not bind to anti-FS85 were annotated as a putative saccharopine dehydrogenase (protein 9) and a glyceraldehyde 3-phosphate dehydrogenase (protein 10). These proteins are located in the cytoplasm. Of the eight selected proteins that did bind to anti-FS85, seven were associated with the outer membrane, and SEM images indicated that anti-FS85 caused cell surface disruption to *F. succinogenes*. This suggests that the anti-FS85 antibody is binding or interacting with cell surface proteins. This may prevent the function of OM proteins or disrupt cellular components through signal transduction, leading to cell death (Wade and O'Toole, 2010). Thus, developing polyclonal antibodies from heat-inactivated whole-cell lysates may be an effective way to target outer membrane proteins of specific bacteria to inhibit growth. Future studies should address antibody-binding specificity toward Gram-positive bacterial cells as they lack an outer membrane layer and toward non-targeted strains that display high levels of cross-reactivity.

5. Conclusion

Overall, polyclonal antibodies generated against key cellulolytic rumen bacterial strains inhibited the growth of targeted strains in monoculture. Additionally, antibodies were capable of reducing the growth activity of strains that they were not specifically generated against. However, these inhibitory effects on growth characteristics of non-targeted strains are less impactful to the overall growth of cells over a 52-h incubation period. This suggests that while there is cross-reactivity, there may be a greater specificity for antibodies generated against their target strain. Additionally, polyclonal antibodies generated against whole bacterial cell lysates appear to target outer membrane proteins. This method of antibody generation could be used in future studies to inhibit the growth of specific bacteria to modify rumen microbial populations toward improved fermentation patterns and increased animal performance. Determining the affinity for targeted and non-targeted strains in mixed cultures can further validate antibody selectivity and efficacy.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

ST contributed to acquisition and analysis of data and wrote the first draft of the manuscript. All authors contributed to the design of the study, manuscript revision, and approved the submitted version.

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Conflict of interest

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

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Supplementary material

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References

Bryant, M. P. (1972). Commentary on the Hungate technique for culture of anaerobic bacteria. *Am. J. Clin. Nutr.* 25, 1324–1328. doi: 10.1093/ajcn/25.12.1324

Burnett, B. J., Altman, R. B., Ferrao, R., Alejo, J. L., Kaur, N., Kanji, J., et al. (2013). Elongation factor Ts directly facilitates the formation and disassembly of the Escherichia coli elongation factor Tu·GTP·aminoacyl-tRNA ternary complex. *J. Biol. Chem.* 288, 13917–13928. doi: 10.1074/jbc.M113.460014

Callaway, T. R., Edrington, T. S., Rychlik, J. L., Genovese, K. J., Poole, T. L., Jung, Y. S., et al. (2003). Ionophores: Their use as ruminant growth promotants and impact on food safety. *Curr Issues Intest Microbiol* v. 4, 43–51.

Cook, S. R., Maiti, P. K., Chaves, A. V., Benchaar, C., Beauchemin, K. A., and McAllister, T. A. (2008). Avian (IgY) anti-methanogen antibodies for reducing ruminal methane production: in vitro assessment of their effects. *Aust. J. Exp. Agric.* 48, 260–264. doi: 10.1071/EA07249

Dervyn, E., Noirot-Gros, M.-F., Mervelet, P., McGovern, S., Ehrlich, S. D., Polard, P., et al. (2004). The bacterial condensin/cohesin-like protein complex acts in DNA repair and regulation of gene expression. *Mol. Microbiol.* 51, 1629–1640. doi: 10.1111/j.1365-2958.2003.03951.x

DiLorenzo, N., Dahlen, C. R., Diez-Gonzalez, F., Lamb, G. C., Larson, J. E., and DiCostanzo, A. (2008). Effects of feeding polyclonal antibody preparations on rumen fermentation patterns, performance, and carcass characteristics of feedlot steers. *J. Anim. Sci.* 86, 3023–3032. doi: 10.2527/jas.2008-0859

DiLorenzo, N., Diez-Gonzalez, F., and DiCostanzo, A. (2006). Effects of feeding polyclonal antibody preparations on ruminal bacterial populations and ruminal pH of steers fed high-grain diets. *J. Anim. Sci.* 84, 2178–2185. doi: 10.2527/jas.2005-489

Edwards, H. D., Shelver, W. L., Choi, S., Nisbet, D. J., Krueger, N. A., Anderson, R. C., et al. (2017). Immunogenic inhibition of prominent ruminal bacteria as a means to reduce lipolysis and biohydrogenation activity in vitro. *Food Chem.* 218, 372–377. doi: 10.1016/j.foodchem.2016.09.052

Erwin, E. S., Marco, G. J., and Emery, E. M. (1961). Volatile fatty acid analyses of blood and rumen fluid by gas chromatography. *J. Dairy Sci.* 44, 1768–1771. doi: 10.3168/jds.S0022-0302(61)89956-6

Frank, S. A. (2002). Specificity and cross-reactivity. In: *Immunology and Evolution of Infectious Disease*. Princeton, NY: Princeton University Press.

Granato, D., Bergonzelli, G. E., Pridmore, R. D., Marvin, L., Rouvet, M., and Corthésy-Theulaz, I. E. (2004). Cell surface-associated elongation factor Tu mediates the attachment of *Lactobacillus johnsonii* NCC533 (La1) to human intestinal cells and mucins. *Infect. Immun.* 72, 2160–2169. doi: 10.1128/IAI.72.4.2160-2169.2004

Ishihama, Y., Oda, Y., Tabata, T., Sato, T., Nagasu, T., Rappsilber, J., et al. (2005). Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. *Mol. Cell Proteomics.* 4, 1265–1272. doi: 10.1074/mcp.M500061-MCP200

Jin, L. Z., Baidoo, S. K., Marquardt, R. R., and Frohlich, A. A. (1998). In vitro inhibition of adhesion of enterotoxigenic *Escherichia coli* K88 to piglet intestinal mucus by egg-yolk antibodies. *FEMS Immunol. Med. Microbiol.* 21, 313–321. doi: 10.1111/j.1574-695X.1998.tb01179.x

Johnson, J. M., and Church, G. M. (1999). Alignment and structure prediction of divergent protein families: periplasmic and outer membrane proteins of bacterial efflux pumps. *J. Mol. Biol.* 287, 695–715. doi: 10.1006/jmbi.1999.2630

Jun, H.-S., Qi, M., Gong, J., Egbosimba, E. E., and Forsberg, C. W. (2007). Outer membrane proteins of *Fibrobacter succinogenes* with potential roles in adhesion to cellulose and in cellulose digestion. *J. Bacteriol.* 189, 6806–6815. doi: 10.1128/JB.00560-07

Kendrick, N., Powers, G., Johansen, J., Hoelter, M., Koll, A., Carlson, S., et al. (2020). Preparation of a phosphotyrosine-protein standard for use in semiquantitative

western blotting with enhanced chemiluminescence. *PLoS ONE* 15, e0234645. doi: 10.1371/journal.pone.0234645

Lee, E. N., Sunwoo, H. H., Menninen, K., and Sim, J. S. (2002). In vitro studies of chicken egg yolk antibody (IgY) against *Salmonella enteritidis* and *Salmonella typhimurium*. *Poult. Sci.* 81, 632–641. doi: 10.1093/ps/81.5.632

Marino, C. T., Otero, W. G., Rodrigues, P. H. M., DiCostanzo, A., Millen, D. D., Pacheco, R. L. D., et al. (2011). Effects of adding polyclonal antibody preparations on ruminal fermentation patterns and digestibility of cows fed different energy sources. *J. Anim. Sci.* 89, 3228–3235. doi: 10.2527/jas.2010-3062

Mine, Y., and Kovacs-Nolan, J. (2002). Chicken egg yolk antibodies as therapeutics in enteric infectious disease: a review. *J. Med. Food* 5, 159–169. doi: 10.1089/10966200260398198

Miron, J., Ben-Ghedalia, D., and Morrison, M. (2001). Invited review: adhesion mechanisms of rumen cellulolytic bacteria. *J. Dairy Sci.* 84, 1294–1309. doi: 10.3168/jds.S0022-0302(01)70159-2

Östberg, Y., Carroll, J. A., Pinne, M., Krum, J. G., Rosa, P., and Bergström, S. (2004). Pleiotropic effects of inactivating a carboxyl-terminal protease, CtpA, in *Borrelia burgdorferi*. *J. Bacteriol.* 186, 2074–2084. doi: 10.1128/JB.186.7.2074-2084.2004

Pillai-Kastoori, L., Heaton, S., Shiflett, S. D., Roberts, A. C., Solache, A., and Schutz-Geschwender, A. R. (2020). Antibody validation for Western blot: by the user, for the user. *J. Biol. Chem.* 295, 926–939. doi: 10.1016/S0021-9258(17)49905-4

Satoh, K., and Yamamoto, Y. (2007). The carboxyl-terminal processing of precursor D1 protein of the photosystem II reaction center. *Photosyn. Res.* 94, 203–215. doi: 10.1007/s11120-007-9191-z

Sharma, C., Rokana, N., Chandra, M., Singh, B. P., Gulhane, R. D., Gill, J. P. S., et al. (2018). Antimicrobial resistance: its surveillance, impact, and alternative management strategies in dairy animals. *Front. Vet. Sci.* 4, 237. doi: 10.3389/fvets.2017.00237

Shimuzu, M., Fitzsimmons, R. C., and Nakai, S. (1988). Anti-E. *coli* immunoglobulin Y isolated from egg yolk of immunized chickens as a potential food ingredient. *J. Food. Sci.* 53, 1360–1368. doi: 10.1111/j.1365-2621.1988.tb09277.x

Silva, G. M., Podversich, F., Schulmeister, T. M., Santos, E. R. S., Sanford, C., Siqueira, M. C. B., et al. (2022a). Polyclonal antibody preparations from avian origin as a feed additive to beef cattle: ruminal fermentation during the step-up transition diets. *Transl. Anim. Sci.* 6, txac070. doi: 10.1093/tas/txac070

Silva, G. M., Schulmeister, T. M., Podversich, F., Tarnonsky, F., Garcia-Ascolani, M. E., and DiLorenzo, N. (2022b). Impacts of polyclonal antibody preparations from avian origin on nutrient digestibility and performance of backgrounding beef cattle. *Transl. Anim. Sci.* 6, txac016. doi: 10.1093/tas/txac016

Solhi, R., Alebouyeh, M., Khafri, A., Rezaeifard, M., and Aminian, M. (2017). In vitro evaluation of cross-strain inhibitory effects of IgY polyclonal antibody against *H. pylori*. *Microb. Pathog.* 110, 682–687. doi: 10.1016/j.micpath.2017.03.025

Sunwoo, H. H., Lee, E. N., Menninen, K., Suresh, M. R., and Sim, J. S. (2002). Growth inhibitory effect of chicken egg yolk antibody (IgY) on *Escherichia coli* O157:H7. *J. Food Sci.* 67, 1486–1494. doi: 10.1111/j.1365-2621.2002.tb10310.x

Tsubokura, K., Berndston, E., Bogstedt, A., Kaijser, B., Kim, M., Ozeki, M., et al. (2003). Oral administration of antibodies as prophylaxis and therapy in *Campylobacter jejuni* -infected chickens. *Clin. Exp. Immunol.* 108, 451–455. doi: 10.1046/j.1365-2249.1997.3901288.x

Vodovnik, M., Duncan, S. H., Reid, M. D., Cantlay, L., Turner, K., Parkhill, J., et al. (2013). Expression of cellulosome components and type IV pili within the extracellular proteome of *Ruminococcus flavefaciens* 007. *PLoS ONE* 8, e65333. doi: 10.1371/annotation/fed83700-d3cd-428e-ae52-e60524c97529

Wade, W. F., and O'Toole, G. A. (2010). Antibodies and immune effectors: shaping Gram-negative bacterial phenotypes. *Trends Microbiol.* 18, 234–239. doi: 10.1016/j.tim.2010.03.001

Wilson, M. M., and Bernstein, H. D. (2016). Surface-exposed lipoproteins: an emerging secretion phenomenon in Gram-negative bacteria. *Trends Microbiol.* 24, 198–208. doi: 10.1016/j.tim.2015.11.006

Wood, T. M., and Bhat, K. M. (1988). Methods for measuring cellulase activities. *Meth. Enzymol.* 160, 87–112. doi: 10.1016/0076-6879(88)60109-1

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Effects of different forage proportions in fermented total mixed ration on muscle fatty acid profile and rumen microbiota in lambs

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Objective: The objectives of this study were to evaluate the effects of different forage proportions in the fermented total mixed ration (FTMR) on growth performance, muscle fatty acid profile, and rumen microbiota of lambs.

Methods: Thirty 6-month-old small tail Han sheep × Ujumqin lambs with initial body weight (BW) of 27.8 ± 0.90 kg were selected for the test and divided into two groups of 15 sheep in each treatment (three pens per treatment and five lambs per pen) according to the principle of homogeneity. Two isoenergetic and isonitrogenous diets were formulated according to the NRC. The diet treatments were designed as (1) OH treatment containing 25% alfalfa hay and 35% oat hay, and (2) AH treatment containing 35% alfalfa hay with 25% oat hay. The forage-to-concentrate ratio for both diets was 65: 35 (DM basis). Three replicates were randomly selected from each treatment to determine growth performance, fatty acid profile and rumen bacterial communities in lambs.

Results: Results revealed no statistically significant ($p > 0.05$) differences in dry matter intake and average daily gain between the two diet groups. Cholesterol and intramuscular fat were significantly ($p > 0.05$) higher in the AH group, while no statistically significant difference ($p > 0.05$) was found in pH24 value. The muscle fatty acid compositions of lambs were obviously ($p < 0.05$) influenced by the diet treatments. Compared with the OH group, the C16:1, C17:0, and C20:3n6 contents were higher ($p < 0.05$) in the AH group, whereas the content of C18:1n9c, C20:1, C18:3n3, and C22:6n3 was obviously ($p < 0.05$) increased in the OH group. The monounsaturated fatty acid (MUFA) contents were significantly higher in the OH group, whereas no significant differences ($p > 0.05$) were detected in saturated fatty acid (SFA) and polyunsaturated fatty acid (PUFA) contents among the two diet treatments. Bacterial composition was generally separated into two clusters based on principal coordinate analysis, and the OH group had a higher Shannon index. The relative abundance at the genes level of the *Rikenellaceae_RC9_gut_group* was obviously ($p < 0.05$) increased in the AH group and the relative abundances of *Prevotella_1*, *Fibrobacter*, and *Bacteroidales_UCG_001_unclassified* were obviously ($p < 0.05$) enriched in the OH group. Integrated correlation analysis also underscored a possible link between the muscle fatty acid compositions and significantly altered rumen microbiota.

Conclusion: Overall, oat-based roughage in FTMR could promote a beneficial lipid pattern in the Longissimus lumborum muscles of lambs. These findings provide a potential insight into diet effects on fatty acid profile and the rumen microbiome of lambs, which may help make decisions regarding feeding.

KEYWORDS

oat, alfalfa, fermented total mixed ration, meat quality, rumen microbiota

Introduction

Alfalfa (*Medicago sativa* L.) is a promising forage used for animals worldwide due to its strong adaptability and good nutritional value (Zhao et al., 2021). However, the higher cost of alfalfa might affect economic efficiency (Wang et al., 2021; Chávez et al., 2022). Thus, a high-priority task is to find suitable forages to replace alfalfa hay in order to meet the needs of the herbivorous animal industry.

Oat (*Avena sativa* L.) has high protein and digestible fractions, can grow well in the cooler temperatures of spring and fall, and is an important part of feedstuff for ruminants (Harper et al., 2017; Jia et al., 2021). Previous research has demonstrated that oat hay has been widely used due to its advantages of higher apparent digestibility of fiber, which can balance the stable state of the rumen internal environment in animals (An et al., 2020). Zou et al. (2018) demonstrated that partially replacing alfalfa hay with oat hay in total mixed rations (TMR) could promote nitrogen utilization. However, TMR is associated with a high level of aerobic deterioration and needs to be freshly prepared prior to each use, which limits its use in some farms due to labor shortages (Du et al., 2022). Previous research has demonstrated that fermented total mixed ration (FTMR) could be used as a diet in animal production (Kumagai et al., 1983). In addition, FTMR could improve total tract apparent digestibility while decreasing fecal N excretion, which enhances economic benefits and reduces environmental pollution (Zhang et al., 2020). Furthermore, Zhao et al. (2020) confirmed that bamboo (*Bambusoideae*) as a roughage source of FTMR had no adverse effect on fermentation quality while improving aerobic stability.

The rumen serves as a natural bioreactor and is occupied by a highly diverse and dense microbiota population consisting of bacteria, fungi, archaea, and protozoa (Carballo et al., 2021). The rumen is responsible for the ruminant's ability to convert fibrous plant materials and indigestible plant mass into energy and protein (Carrasco et al., 2017). The rumen microbial population ferments feedstuffs and converts fibrous-rich plant materials into microbial proteins. This unique microbial ecosystem provides ~70% of the daily energy requirement for ruminant needs and improves the growth and performance of the host (Krause et al., 2020; Du et al., 2022). Additionally, rumen bacteria are involved in biological hydrogenation and isomerization *in vivo*, which may eventually affect muscle fatty acid (FA) deposition by changing the FA composition of the rumen digesta (Enjalbert et al., 2017). The fatty acid composition of meat is closely related to human health benefits, and the excessive consumption of saturated fatty acid (SFA) will increase the risk of cardiovascular disease (Abuelfatah et al., 2016). A previous study suggested that replacing SFA with polyunsaturated fatty acid (PUFA) may be beneficial to human health, which can be achieved by altering rumen microbial metabolism (Zhu et al., 2022). In turn, the host provides an anaerobic and substrate-rich environment for microbes to thrive (Ellison et al., 2017). Generally, bacteria are the most important players among rumen microorganisms during feed biopolymer degradation and fermentation (Welkie et al., 2010). However, the composition and distribution of rumen microflora are affected by many factors, especially diet, season, environmental temperature, and the development and growth stage of the host animals (Cui et al., 2022). Previous research showed that dietary composition was the main factor affecting rumen microbial compositions and functions (Liu et al., 2022). Li et al. (2020) provided evidence that the rumen microbial community and their functions could be dramatically affected by different diets and further affect the metabolism of fatty acids by ruminal microorganism biohydrogenation in the muscle of animals. Studies have shown that the 20% changes in feed efficiency could be explained by the rumen microbiome (Liu et al., 2022). Therefore, a greater understanding of the functions of rumen microbiota is necessary to investigate the mechanisms involved in the effects of dietary composition on the formation and improvement of the quality of meat produced by animals. However, data on the influences of different forage proportions in FTMR on the fatty acid profile and ruminal microbiota are still lacking.

We hypothesized that increasing the proportion of oat hay in FTMR would provide sufficient physical active fiber to enhance rumen functions and the meat quality of lambs. Therefore, this study aimed to evaluate the effects of forage proportion in FTMR on muscle fatty acid composition and the rumen microbiota of lambs.

Materials and methods

Animals, diets, and experimental design

The study protocol selected for this experiment was based on the Institutional Guidelines for Animal Experiments and the Regulations for the Administration of Affairs Concerning Experimental Animals of the College of Grassland, Resources, and Environment, Inner Mongolia Agricultural University, Hohhot, China. All the experimental protocols carried out in this study were approved by the Animal Care Committee of Inner Mongolia Agricultural University. The feeding experiment was carried out at

TABLE 1 Growth performance of lambs feed fermented total mixed rations with different ratios of alfalfa and oat.

Items	OH	AH	SEM	*P*-value
Initial BW (kg)	27.67	28.00	0.366	0.875
Final BW (kg)	41.00	42.51	0.569	0.267
ADG (g/day)	222.22	241.83	5.119	0.181
DMI (kg/day)	1.10	1.16	0.017	0.210

BW, body weight; ADG, average daily gain; DMI, dry matter intake; SEM, standard error of the mean. Means within the same rows with different letters are significantly different ($p < 0.05$). SEM, standard error of means. OH, high oat percentages group; AH, high alfalfa percentage group.

Inner Mongolia Hongpeng Technology Co. Ltd (Balin Left Banner, Chifeng, China). A total of 30 small-tail Han sheep × Ujumqin sheep crossed uncastrated male lambs weighing ∼27.8 ± 0.90 kg were randomly allocated to the two diet groups, with 15 sheep in each treatment (three pens per treatment and five lambs per pen) according to the principle of homogeneity. The pens were separated into five single stalls (0.8 × 1.3 m). Two isoenergetic and isonitrogenous diets were formulated according to the NRC (NRC, 2007; Nasehi et al., 2018). The forage-to-concentrate ratio for both diets was 65:35 (DM basis) to meet the essential energetic and nutritional requirements of the experimental lambs. The gross energy (GE), energy in feces (FE), energy in urine (UE), and energy in gaseous products of digestion (Eg) were analyzed using an oxygen and nitrogen analyzer (IKA Works GmbH and Co., Staufen, Germany). Metabolizable energy (ME) was estimated following the procedure reported by Freer et al. (2007), and the formula was as follows: ME = GE – FE – UE – Eg. The composition and nutrition of the diets are listed in Supplementary Table 1. The FTMR was prepared with alfalfa hay, oat hay, corn stover, natural forage, and concentrate mixtures provided by Chaoyue Feed Co. Ltd. The experimental treatments were designed as (1) OH containing 25% alfalfa hay with 35% oat hay and (2) AH containing 35% alfalfa hay with 25% oat hay. The diets were prepared as follows. The forage samples were chopped into ∼1–2 cm and mixed according to the above ratios, and then adjusted for moisture to 500 g/kg using a water sprayer after additives had been dissolved in water. The additives were compound bacterial agents (containing *bacillus subtilis* R2 and *bacillus subtilis* N10) purchased from Hebei Zhong bang Biotechnology Co. Ltd. (Strong brand, Hebei, China) and were added in proportion to 1 g/kg of fresh TMR. FTMR was placed in fermentation bags (55 × 85 cm) equipped with a one-way exhaust valve, compacted, and sealed at the mouth of the bag, and all samples were stored indoors (∼15°C) for 60 days. The experiment was performed over 75 days, with the first 15 days for adaptation. During the adaptation period, the animals also received their respective test diets. The lambs were fed twice daily (7: 00 h a.m. and 5: 00 h p.m.), and no more than 10% of refusals were allowed. The lambs were provided with free access to drinking water and fed *ad libitum* on their respective FTMR diets.

Sample collection and processing

The weight of feed delivered and refused was registered every day to evaluate the dry matter intake (DMI). The weight of all lambs was assessed after their adaptation period and every 2 weeks thereafter. The initial body weight and final body weight were measured to estimate the average daily gain (ADG). Following the experimental tests, the experimental lambs were provided with free access to drinking water and fasted for 12 h, then stunned electrically, and exsanguinated to death by severing the jugular vein at a butchery. The slaughter procedures were performed according to operating procedures of livestock and poultry slaughtering for sheep and goat (NY/T 3469-2019, Ministry of Agriculture, China) (Ministry of Agriculture, 2019). The muscle samples of *Longissimus lumborum* were isolated from the right side of the carcass to determine pH value, cooking loss rate, and dripping loss rate, and the part of the isolated sample was immediately stored at −20°C in a freezer until further assay of chemical composition, meat quality, and fatty acid profile. In addition, rumen fluid samples of all lambs were collected immediately after slaughtering, and six ruminal fluid samples were randomly selected from each group for further measurement. Approximately 50 ml of rumen fluid was gathered from each lamb by straining the rumen content with four layers of cheesecloth. The rumen fluid samples were immediately frozen in liquid nitrogen and then stored in a cryogenic refrigerator at −80°C for 16S rRNA sequencing analysis.

Feed composition analysis

Feed samples were dried for 48 h in a convection oven at 60 to 65°C until constant weight to calculate dry matter content (DM) and then ground with a sample mill (1 mm screen) for further analysis of crude protein (CP), organic matter (OM), neutral detergent fiber (NDF), acid detergent fiber (ADF), and ash. DM, CP, OM, and ash were analyzed following the AOAC (2005) standard procedures (925.04, 954.01, 920.39, and 942.05, respectively). ADF and NDF contents were measured based on the methods previously published by Van Soest et al. (1991). Ten grams of FTMR samples were randomly selected and further mixed with 90 mL sterilized saline and homogenized in a blender for 2 min to extract the fermentation broth. After that, the bacterial solution was diluted from 10^{-1} to 10^{-5} to count the number of microorganisms. The amount of LAB and aerobic bacteria was calculated with the MRS medium base (MRS) medium and nutrient agar, respectively. The pH value of the FTMR extract was measured immediately with a calibrated glass electrode pH meter (STARTED 100/B, OHAUS, Shanghai, China). The concentrations of organic acids were characterized based on high-performance liquid chromatography (HPLC) following the method reported by You et al. (2021). Ammonia nitrogen (NH_3-N) was assayed by the phenol-hypochlorite procedure according to the methods of Broderick and Kang (1980).

Meat quality analysis

Association of Official Analytical Chemists methods (AOAC, 2005) were used to assess the contents of moisture, protein, cholesterol, intramuscular fat, and ash. The pH_{24} value of the *longissimus lumborum* muscle samples was estimated after 24 h

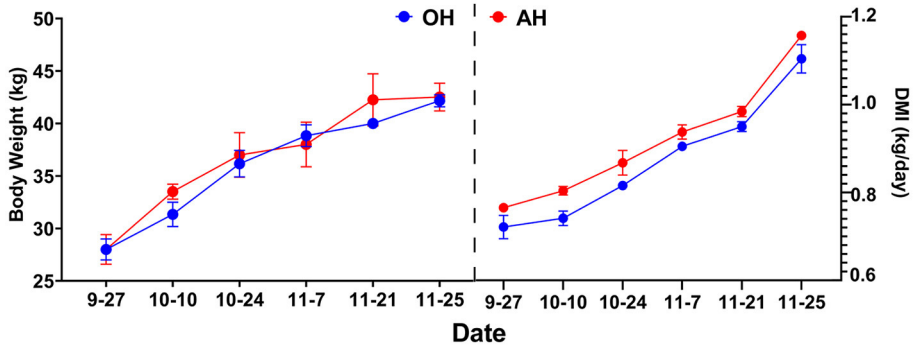

FIGURE 1
Dynamic changes of ADG and DMI every 2 weeks among different treatments. DMI, dry matter intake; OH, high oat percentages group; AH, high alfalfa percentage group.

TABLE 2 Meat quality of lambs feed fermented total mixed rations with different ratios of alfalfa and oat.

Items	OH	AH	SEM	*P*-value
pH_{24h}	5.56	5.55	0.033	0.907
Dripping loss rate (%)	4.73	3.31	0.296	<0.001
Cooking loss rate (%)	34.34	33.77	0.329	0.492
Moisture (%)	76.17	75.57	0.250	0.324
Protein (%)	21.00	20.50	0.142	0.111
Cholesterol (mg/100 g)	35.60	47.10	2.406	0.001
Intramuscular fat (%)	1.83	3.60	0.363	<0.001
Ash (%)	0.85	0.76	0.020	0.019

Means within the same rows with different letters are significantly different ($p < 0.05$). SEM, standard error of means; OH, high oat percentages group; AH, high alfalfa percentage group.

(stored at 4°C) by a pre-calibrated portable pH meter equipped with a glass electrode shaped to easily penetrate meat (STARTED 100/B; Ohaus, Shanghai, China). The cooking loss rate of the *longissimus lumborum* muscle samples was calculated by taking the difference between the initial uncooked weight and the final weight of the sample after cooking following the recommendations of the American Meat Science Association (Seman et al., 2017). The samples were stored in inflated plastic bags at 4°C for 24 h. The dripping loss rate was represented by the ratio of weight loss (Honikel, 1998). The fatty acids of *longissimus lumborum* samples were tested by gas chromatography (GC) of fatty acid methyl esters (FAMEs). The GC was equipped with a fused silica capillary column (crosslinking bonded stationary phase containing 50% propyl, 60 mm × 0.25 mm × 0.25 μm), split injector, and flame ionization detector fitted with a Galaxie Chromatography Data System (Version 1.0 software). The oven was programmed as follows: initial temperature of 130°C for 1 min, which was first increased to 170°C at 6.5°C/min, then increased to 215°C at 2.75°C/min and held at that temperature for 12 min, and finally, the temperature was further increased to 230°C at 4°C/min and held at that temperature for 3 min. The injector and detector temperatures were 270 and 280°C, respectively. Helium was used as a carrier gas at the column flow of 0.8 ml/min and a split ratio of 50:1. The methylation of fatty acids in meat samples was carried out based on the procedure of AOAC (2005). The compositions of muscle saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA) were obtained from individual fatty acid percentages.

Bacterial DNA extraction, polymerase chain reaction amplification, and 16s rRNA sequencing

The microbial DNA of six rumen fluid samples was isolated using E.Z.N.A.® Stool DNA Kit (D4015, Omega, Inc., USA) and then eluted with elution buffer (50 μL) following the manufacturer's instructions. The V3–V4 region of the 16S rRNA gene was amplified from extracted DNA as previously described using the universal primers of 341 (5′- CCTACGGGNGGCWGCAG-3′) and 805 (5′-GACTACHVGGGTATCTAATCC-3′) (Logue et al., 2016). The PCR products were extracted and purified by 2% agarose gel electrophoresis and AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, United States), respectively. The sequencing library was prepared using the gDNA samples applying the Illumina library quantification kit (Kapa Biosciences, Woburn, MA, United States). The amplicon libraries were evaluated and characterized for size distribution and number using an Agilent 2100 Bioanalyzer (Agilent, United States). The sample libraries were sequenced using the NovaSeq PE250 platform. Sequencing data for the 16S rRNA gene sequence of the rumen samples in the two FTMR diet groups were uploaded and stored in NCBI BioProject, and the accession number can be found under PRJNA888233.

Bioinformatics analysis

The 16S rRNA gene sequences were performed using an Illumina NovaSeq platform according to the recommended vendor's instructions (LC-Bio Technology Co. Ltd., Hangzhou,

TABLE 3 Muscle fatty acid composition of longissimus lumborum muscle (mg/100 g of fatty acid methyl esters).

Items	OH	AH	SEM	*P*-value
C14:0	4.55	5.99	0.904	0.529
C15:0	0.49	1.44	0.263	0.091
C15:1	0.25	0.13	0.079	0.557
C16:0	51.06	86.18	8.982	0.057
C16:1	3.77	5.98	0.554	0.049
C17:0	1.85	4.69	0.844	0.033
C17:1	1.95	1.94	0.010	0.547
C18:0	67.83	54.74	10.580	0.629
C18:1n9t	5.44	5.06	0.265	0.581
C18:1n9c	128.87	122.66	0.332	0.004
C18:2n6t	0.63	0.70	0.034	0.368
C18:2n6c	19.73	19.66	1.188	0.889
C20:0	0.32	0.57	0.092	0.253
C20:1	0.37	0	0.075	<0.001
C18:3n3	1.66	1.22	0.101	0.022
C21:0	0.75	1.22	0.145	0.151
C22:0	0.54	0.54	0.037	0.984
C20:3n6	0	0.55	0.113	<0.001
C22:6n3	0.47	0.38	0.018	<0.001
SFA	128.83	153.92	10.339	0.318
PUFA	22.38	22.61	0.283	0.756
MUFA	140.53	135.89	1.021	0.008

Means within the same rows with different letters are significantly different ($p < 0.05$). SEM, standard error of means; OH, high oat percentages group; AH, high alfalfa percentage group; *c*, cis-fatty acid; *t*, trans-fatty acid.

Zhejiang Province, China). Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. FLASH (v1.2.8, http://ccb.jhu.edu/software/FLASH/) was used to merge the paired-end reads. Quality filtering was performed on the raw reads under specific filtering conditions to obtain high-quality clean reads according to fqtrim (v0.94, http://ccb.jhu.edu/software/fqtrim/), and Vsearch (v2.3.4) was performed to filter the chimeric sequences. QIIME2 (v2019.7) was selected to import and process the sorted reads for bioinformatics analysis. The imported paired reads were quality filtered, denoised, and merged by plugin DADA2 (v3.11) to produce the amplicon sequence variants (ASV) feature table. The taxonomy classification was carried out using the q2-feature-classifier, a taxonomic classifier plugin for the QIIME 2 microbiome analysis platform (https://qiime2.org/) based on the scikit-learn naive Bayes classifier. After that, a SILVA database (Release 138, http://www.arb-silva.de) was used to assign taxonomy to filtered ASVs, predicting 99% identity of bacteria and representative sequences. To determine the species diversity in each sample of the two treatments, alpha diversity and beta diversity analyses were conducted using the procedure of plugin q2-diversity in QIIME2 (v2019.7). To assess bacterial communities between individuals and groups in the current research, weighted UniFrac outputs were evaluated and visualized using PCoA. The communities that showed statistical differences among the two groups were identified using PERMANOVA (vegan 2.5.4). Treatment-dependent features were identified using LEfSe. A size-effect threshold of 3.0 on the logarithmic LDA score was used to identify discriminating taxa, as proposed by Segata et al. (2011). Using the Mann–Whitney *U*-test, the differences in relative abundance were identified between groups. To illuminate the interactions between microbes and meat quality, the relationships between the top 20 genera bacterial community and meat quality were visualized by Pearson's correlation heatmap, which was performed using R Core Team (2014) after ranking and normalizing the correlation matrix. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) was used to infer the rumen microbiota functional pathways based on the information from the KEGG database (Douglas et al., 2020). Bar graphs were drawn with the software GraphPad Prism 9 (San Diego, CA, United States).

Statistical analysis

Analyses of feed nutrition, growth performance, meat quality, and muscle fatty acid composition were performed using SAS ver. 9.2 (SAS Inc, 2007 Cary, NC, USA). The statistical model of the SAS was as follows: $Y = \mu + \alpha + \varepsilon$, where Y = observation, μ = overall mean, α = diet effect, and ε = error. Differences in means among the two groups were evaluated using an independent sample *t*-test, and a *p-value of* <0.05 was considered to be statistically significant.

Results

Animal performance

The initial BW, final BW, ADG, and DMI of all lambs across the entire fattening trial are shown in Table 1. Interestingly, no statistically significant ($p > 0.05$) difference in final BW between these two different diet groups was observed. There were no significant ($p > 0.05$) differences observed in DMI or ADG between the two diet treatments. As shown in Figure 1, the dynamic changes in DMI and ADG followed the same trend and gradually increased in the two diet treatments.

Meat quality

Table 2 shows that there was no statistically significant ($p > 0.05$) difference in pH_{24} value between the two diet treatments. The dripping loss rate of the *longissimus lumborum* muscle was obviously ($p < 0.05$) increased in the AH group, whereas no significant ($p > 0.05$) qualitative difference was detected in the cooking loss rate between the two diet

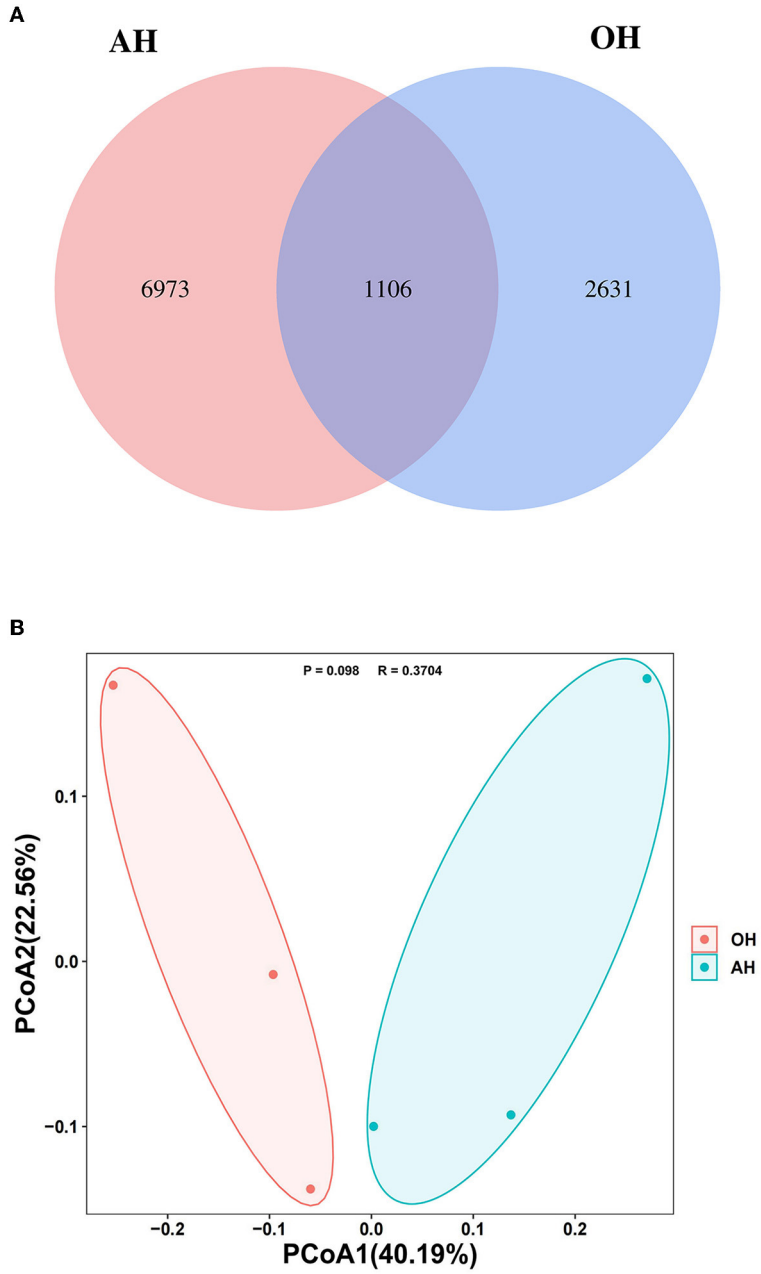

FIGURE 2
Microbial community among different treatments ($n = 3$). **(A)** Venn diagram representing the common and unique amplicon sequence variants (ASVs) found at each treatment. **(B)** Principal coordinates analysis (PCoA) of samples conducted based on weighted UniFrac distance. OH, high oat percentage group; AH, high alfalfa percentage group.

groups. No statistically significant ($p > 0.05$) differences were observed in muscle moisture or protein of lambs in the two diet treatments. Compared with the OH group, the cholesterol and intramuscular fat were significantly ($p < 0.05$) higher in the AH group, while ash was significantly ($p > 0.05$) lower.

FA components of the *longissimus lumborum* muscle of lambs allocated to each treatment group are shown in Table 3. Notably, the muscle fatty acid compositions of lambs were significantly ($p < 0.05$) influenced by the diet treatments. Differences in C14:0, C15:0, C16:0, C15:1, C16:0, C17:1, C18:0, C18:1n9t, C18:2n6t, C18:2n6c, C20:0, C21:0, and C22:0 content were not detected among the two diet treatments. Compared with the OH group, the AH group greatly ($p < 0.05$) increased the contents of C16:1, C17:0, and C20:3n6. The OH group expressed significantly higher ($p < 0.05$) C18:1n9c, C20:1, C18:3n3, and C22:6n3 content relative to the AH group. Finally, the MUFA contents were greatly ($p < 0.05$) reduced in the OH group, whereas the SFA and PUFA contents were not affected by diet treatment.

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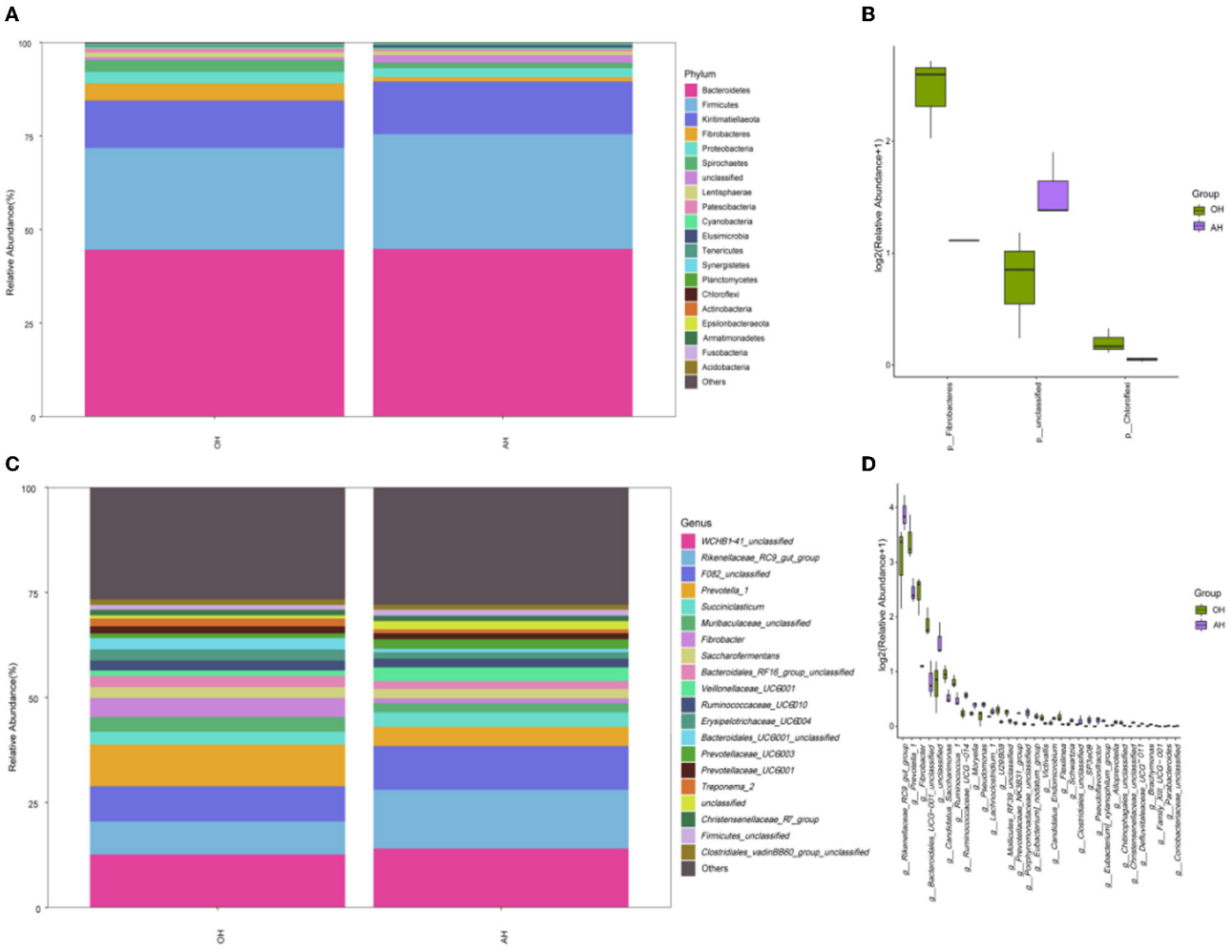

FIGURE 3
Relative abundance (%) of bacterial phyla (1% at least in one group) of ruminal microbiome of lambs fed FTMR with different alfalfa and oat ratios (n = 3). **(A)** Phylum level. **(B)** Extended error bar plot showing the bacteria at the phylum level that had significant differences among the AH and OH groups. **(C)** Genus level. **(D)** Extended error bar plot showing the bacteria at the genus level that had significant differences among the AH and OH groups. OH, high oat percentage group; AH, high alfalfa percentage group.

TABLE 4 Diversity indices of ruminal microbiota of lambs.

Items	OH	AH	SEM	*P*-value
Observed_asvs (ASVs)	1,423	2,859	368.079	0.081
Shannon	9.58	8.59	0.245	0.141
Simpson	1.00	0.99	0.001	0.184
Chao1	3,141.20	1,525.10	415.820	0.086
Goods_coverage	0.99	0.99	0.003	0.184

Means within the same rows with different letters are significantly different ($p < 0.05$). SEM, standard error of means; OH, high oat percentage group; AH, high alfalfa percentage group.

Rumen microbiota

The Venn diagram of the rumen samples from the two FTMR diet groups revealed that a total of 11,816 ASVs were identified, and the groups shared 1,106 ASVs, whereas the OH and AH groups had 6,973 and 2,631 exclusive ASVs, respectively (Figure 2A).

The percentage of Good's coverage index for all samples in the two diet treatments was >99%. In the current study, the AH treatment increased the ASVs index and reduced the Shannon and Simpson indexes, but the differences were not significant ($p > 0.05$) (Table 4).

PCoA plots with the weighted UniFrac distance metric revealed that the compositions of the bacterial community in the OH and the AH groups were basically distinguishable from each other ($R = 0.3704$, $P = 0.098$) (Figure 2B).

A list of rumen microbiota taxonomic distributions and relative abundances of the ruminal microbiota with a mean relative abundance of >1% and the top 20 at both phylum and genus levels are presented in Figure 3. Across all groups, 24 phyla and 413 genera were identified. At the phylum level, 15 bacteria phyla were detected (relative abundance > 1% at least in one group). The relative abundances in OH and AH groups revealed that Bacteroidetes (44.63 and 44.83%) was the most abundant

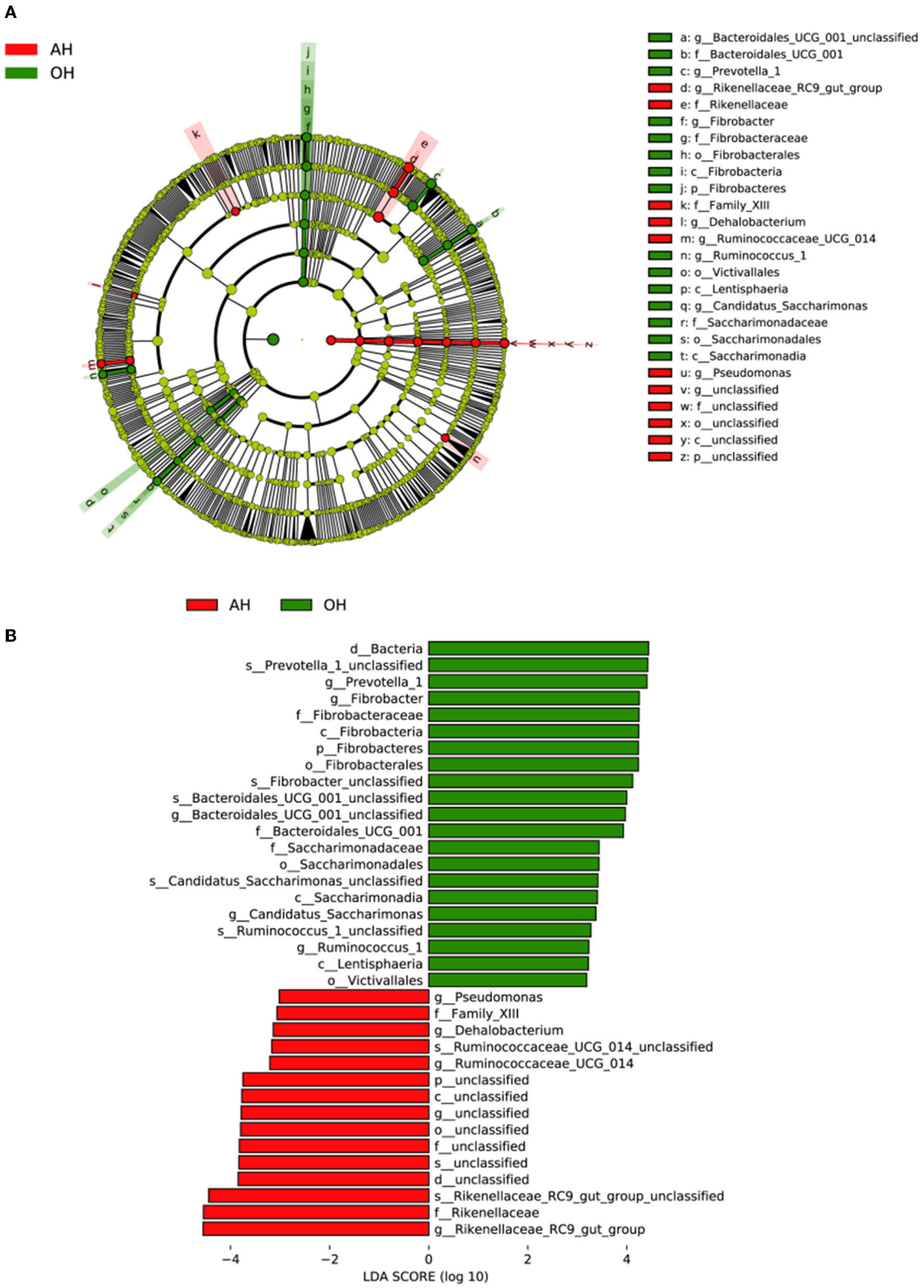

FIGURE 4
Linear discrimination analysis (LDA) coupled with effect size (LEfSe) analysis of the rumen microbial community of lamb in the AH and OH groups (n = 3). **(A)** Cladogram showing microbial species with significant differences among the two treatments. Red and green represent different groups. Species classification at the phylum, class, order, family, and genus level is displayed from inner to outer layers. The red and green nodes represent microbial species in the phylogenetic tree that play important roles in the AH and OH groups, respectively. Yellow nodes represent no significant difference between species. **(B)** Significantly different species with an LDA score greater than the estimated value (default score = 3). The length of the histogram represents the LDA score of different species in the two groups. OH, high oat percentage group; AH, high alfalfa percentage group.

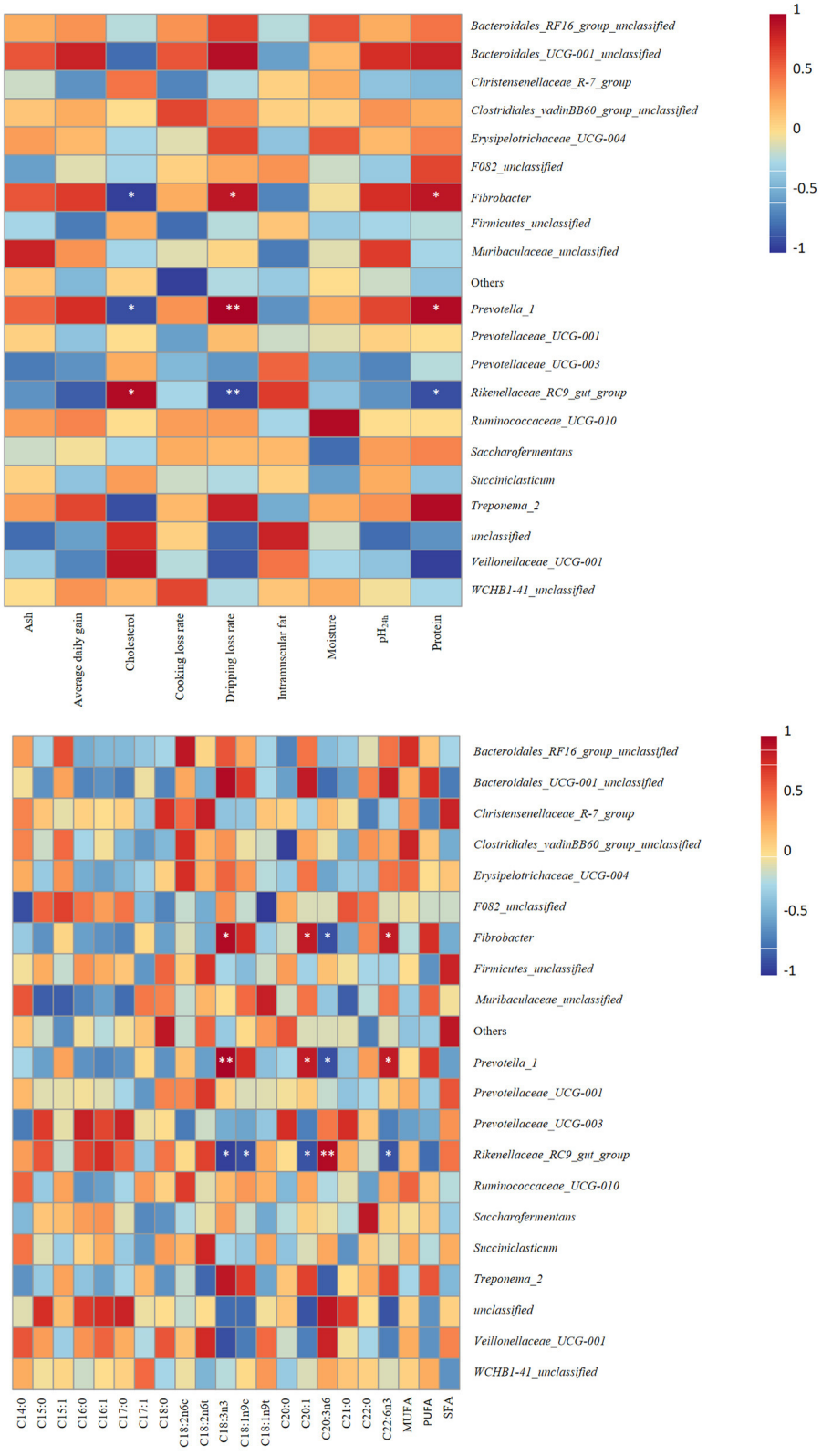

FIGURE 5
Heatmaps of Pearson's correlations between dominant genera and meat quality, and fatty acid profile. Red represents a positive correlation, while blue represents a negative correlation. Levels of significance are shown as follows: *$P < 0.05$; **$P < 0.01$.

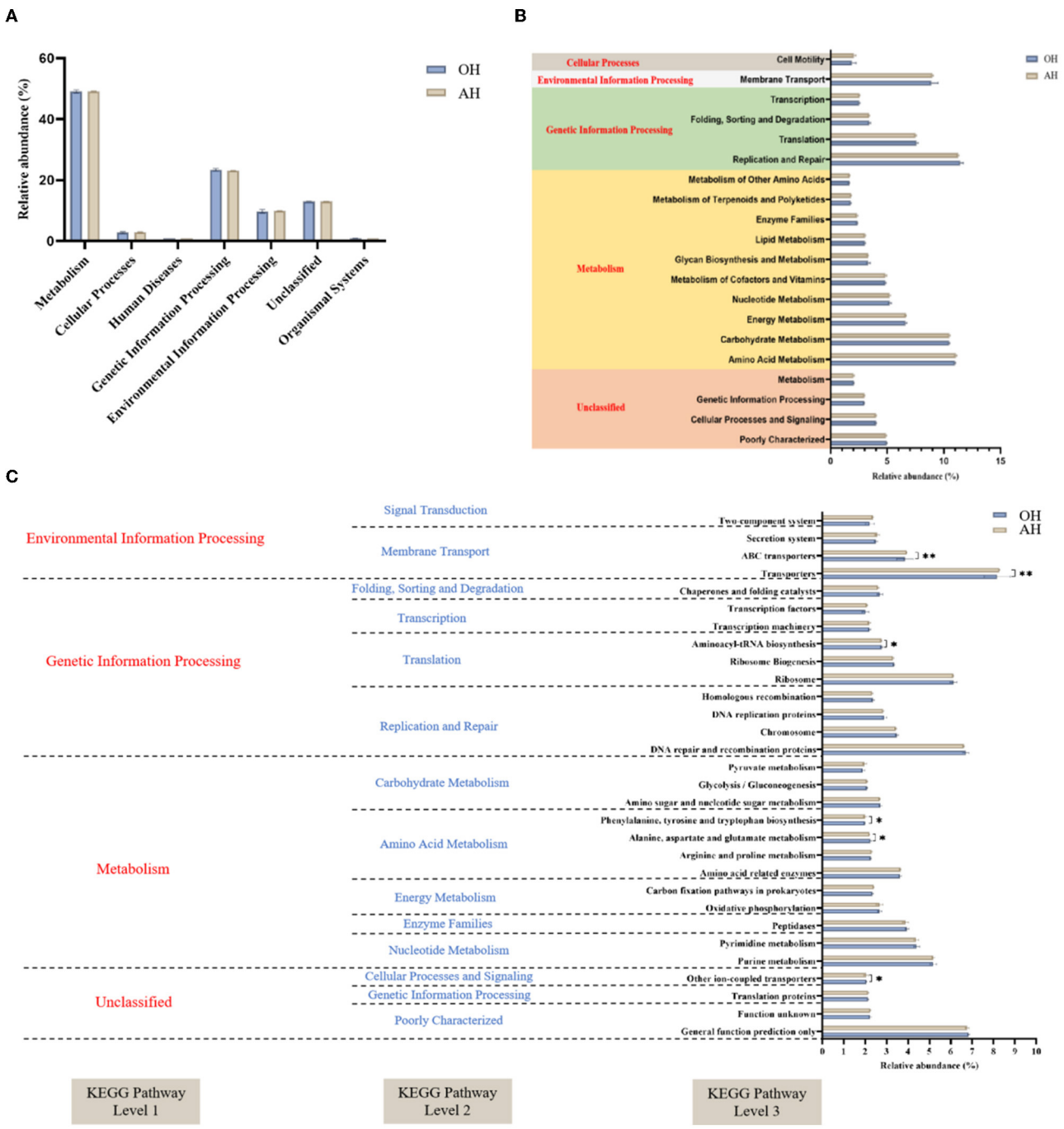

FIGURE 6
Dynamics of rumen bacterial predicted functional profiles fed with different diets analyzed by PICRUSt2 ($n = 3$). **(A)** Level 1 metabolic pathways. **(B)** Level 2 Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog functional predictions of the relative abundances of the top 20 metabolic functions. **(C)** Level 3 KEGG ortholog functional predictions of the relative abundances of the top 30 metabolic functions. OH, high oat percentage group; AH, high alfalfa percentage group. $^{*}P < 0.05$; $^{**}P < 0.01$.

bacterial phylum, followed by Firmicutes (27.18 and 30.66%), Kiritimatiellaeota (12.66% and 14.06%), Fibrobacteres (4.57 and 1.16%), Proteobacteria (3.08 and 2.40%), Spirochetes (3.12 and 1.48%), Lentisphaerae (1.36 and 1.01%) and Patescibacteria (1.04 and 0.62%), Cyanobacteria (0.48 and 0.39%), Elusimicrobia (0.11 and 0.63%), Tenericutes (0.34 and 0.25%), Synergistetes (0.27 and 0.24%), Planctomycetes (0.11 and 0.11%), and Chloroflexi (0.15 and 0.04%), respectively (Figure 3A). Abundances of Fibrobacteres and Chloroflexi were significantly ($p < 0.05$) different between the OH and AH groups (Figure 3B). At the genus level, 94 bacteria genera were thought to be the identifiable genera (relative abundance > 1% at least in one group). *WCHB1-41_unclassified* accounted for 12.66 and 14.06%, *Rikenellaceae_RC9_gut_group* accounted for 7.84 and 13.97%, *F082_unclassified* accounted for

8.40 and 10.43%, *Prevotella_1* accounted for 9.86 and 4.55%, and *Succiniclasticum* accounted for 3.09 and 3.44%, in the OH group and the AH group, respectively, and these were the dominant genera (Figure 3C). The OH and AH treatments resulted in significant ($p < 0.05$) differences in *WCHB1-41 unclassified*, *Rikenellaceae_RC9_gut_group* and F082_unclassified (Figure 3D).

Figure 4A shows a cladogram representative of the predominant bacteria and their structure, indicating that the most differentially abundant taxa enriched in the two groups. The results indicated that 15 clades were enriched in the OH group, and 11 clades were enriched in the AH group. The abundance differences among OH and AH were measured, and the results are shown in Figure 4B. The bacterial genus of *Prevotella_1*, *Fibrobacter*, *Bacteroidales_UCG_001_unclassified*, *Candidatus_Saccharimonas*, and *Ruminococcus_1* was the most enriched in the OH group, and the *Pseudomonas*, *Dehalobacterium*, *Ruminococcaceae_UCG_014*, and *Rikenellaceae_RC9_gut_group* were most enriched in the AH.

Correlation analysis

Correlation analysis provided new insights into the confirmation of several bacterial genera potentially implicated in host development and meat quality improvement (Figure 5). Notably, the results revealed that the genera *Fibrobacter* and *Prevotella_1* were strongly correlated with dripping loss rate ($r = 0.8986$, $p = 0.0149$; $r = 0.9856$, $p = 0.0003$, respectively) and protein ($r = 0.8857$, $p = 0.0333$; $r = 0.9429$, $p = 0.0167$, respectively) but negatively correlated with cholesterol ($r = -0.9429$, $p = 0.0167$; $r = -0.8857$, $p = 0.0333$, respectively). The relative abundance of the *Rikenellaceae_RC9_gut_group* was positively correlated with cholesterol ($r = 0.9429$, $p = 0.0167$) but inversely correlated with dripping loss rate ($r = -0.9276$, $p = 0.0167$). The study findings support the idea that the genera *Fibrobacter* and *Prevotella_1* were positively related to C18:3n3 ($r = 0.9429$, $p = 0.0167$; $r = 1.0000$, $p = 0.0028$, respectively), C20:1 ($r = 0.8783$, $p = 0.0213$; $r = 0.8783$, $p = 0.0213$, respectively), and C22:6n3 ($r = 0.8783$, $p = 0.0213$; $r = 0.8783$, $p = 0.0213$, respectively) content but inversely correlated with C20:3n6 ($r = -0.8804$, $p = 0.0206$; $r = -0.8804$, $p = 0.0206$, respectively) content. The genera *Rikenellaceae_RC9_gut_group* was positively related to C20:3n6 ($r = 0.9411$, $p = 0.0051$) content but inversely correlated with C18:3n3 ($r = -0.9429$, $p = 0.0167$), C18:1n9c ($r = -0.8804$, $p = 0.0206$), C20:1 ($r = -0.8783$, $p = 0.0213$), and C22:6n3 ($r = -0.8783$, $p = 0.0213$) content. No significant correlation was found between the fat performance indexes and other changed genera whose abundance differed significantly between groups ($p > 0.05$).

Predicted functional profiles

To enable a better understanding of the functions of rumen microbiota, metabolic pathways of the microbiota involved were predicted according to the PICRUSt analysis using the KEGG pathway database (Figure 6). The primary predicted functional genes at level 1 in all groups were predominantly categorized into metabolism (48.60–49.66%), environmental information processing (23.04–23.87%), and genetic information processing (9.05–10.36%), respectively (Figure 6A). Among the enriched pathways, the relative abundance of replication and repair (11.35%), amino acid metabolism (11.01%), and carbohydrate metabolism (10.49%) accounted for more than 10% among the two groups. At the three levels, differences among the two groups in the microbial gene-predicted functions of bacteria are shown in Figure 6C. Notably, the genus associated with phenylalanine, tyrosine, and tryptophan biosynthesis, ABC transporters, and transporters were mainly accumulated in the AH group, while the genus correlated with alanine, aspartate, and glutamate metabolism and aminoacyl-tRNA biosynthesis were mainly expressed in the OH group.

Discussion

Roughage serves as the major source of nutrients for ruminants and is potentially crucial for ruminant nutrient metabolism. An appropriate composition of roughage not only improves rumen microbiota but also promotes the rapid growth and development of ruminants (Cui et al., 2022). In the current research, the effect of FTMR diets with different forage proportions on fatty acid profile and rumen microbiota in lambs was investigated. The results provide a reference for promoting the formation of meat quality in lambs by regulating ruminal microbiota with roughage composition.

The results of this investigation indicated that forage proportion in FTMR had no adverse effects on the DMI of lambs, which is in agreement with Dung et al. (2020), who found the forage sources in FTMRs could directly reduce fiber characteristic effects on DM intake. Additionally, animals can adjust their feed intake behavior based on the energy level in the diet within a certain range (Wang et al., 2020). Thus, no significant difference was detected in DMI among the two groups due to their similar energy intake (Du et al., 2022). Furthermore, no remarkable difference was detected in ADG among AH and OH groups, which might be due to the similar DMI and energy intakes among the two groups (Mushi et al., 2009; Galvani et al., 2014).

The pH_{24} was kept within the normal range from 5.5 to 5.8 as suggested by Malva et al. (2016), which indicates that the lambs in this experiment did not have pre-slaughter animal stress and had a sufficient content of muscle glycogen. Intramuscular fat (IMF) is an important constituent of meat and affects its edibility (Brand et al., 2018). It has been demonstrated that the forage proportion in the diet affects the IMF content (Kotupan and Sommart, 2021). In the present investigation, intramuscular fat in lambs supplemented with AH was greater than that of the OH group. This result may be associated with the greater lactic acid bacteria amount in the diet of the AH group, which can produce the lactate to limit chylomicron secretion from enterocytes and lead to lipid synthesis and storage, a mechanism involving the conversion of lactate to malonyl-CoA, which subsequently inhibits β-oxidation and ultimately leads to IMF deposition after a series of reactions (Araujo et al., 2020). In addition, the increased lactic acid content in the AH group could be secondarily fermented in the rumen by lactate-utilizing bacteria

to produce propionic acid (Li et al., 2021), resulting in increased fat deposition (Zhou et al., 2014). The significant difference between the two groups observed in cooking loss rates, dripping loss rates, and cholesterol could be explained by the difference in IMF content, and it has been shown that fat content affects meat palatability, including flavor and tenderness (Costa et al., 2012).

The component of fatty acids (FAs) in meat is crucial for the human health effects of meat consumption (Zhu et al., 2022). Meat from lamb is characterized by an abundance of saturated fatty acids (SFAs), monounsaturated and trans-fatty acids, and a low amount of polyunsaturated fatty acids (PUFA) (Behan et al., 2021). A previous study reported that unsaturated fatty acids can have positive effects on human health and the prevention of cardiovascular disease, and the excessive intake of saturated fatty acids can cause diseases such as arteriosclerosis (Shahidi and Ambigaipalan, 2018). The FA component of meat can be easily shaped by animal diets, as indicated by goats fed *Mitragyna speciosa* Korth leaves having more monounsaturated fatty acid than those fed Pangola grass hay (Chanjula et al., 2022). The intramuscular fat content of lambs fed OH had a higher C18:3n3 concentration than the AH group ($p < 0.05$). These differences may be interpreted as the difference in the FA compositions of AH and OH treatments because there is evidence that the content of C18:3n3 in oat hay is higher than that in alfalfa hay (Abidi et al., 2009; Whitney and Smith, 2015; Zhu et al., 2022). C18:3n3 is an important precursor of n-3 PUFA, especially the C22:6n3, which can promote the development of the nervous system and brain as an important raw material for the formation of biofilms (Youdim et al., 2000; Xu et al., 2019). In the current experiment, the results suggested that oat supplementation in FTMR diets improved the C22:6n3 content of lambs in terms of fatty acid nutrition, which was generally similar to the finding of Sun et al. (2021), who showed that C18:3n3 in muscle is a precursor substance for the synthesis of C22:6n3. C18:0 was the predominant SFA of lambs in the present study. The concentration of C18:0 was decreased in the AH diet, which might be because flavonoids in alfalfa played a certain role, as described by Su et al. (2022), who reported that the flavonoids of dietary alfalfa powder could decrease C18:0 content by regulating the activity of fatty acid metabolism-related enzymes. C18:1n9c was the main MUFA, as previously reported for lamb meat (Hajji et al., 2016). Significantly increased C18:1n9c content with the supplement of oat was observed in this study, which could be explained by the higher C18:0 content of the AH group that could form the C18:1n9c by the enzyme stearoyl Co-A desaturase (Wood et al., 2008). In addition, a higher concentration of MUFA was also found in the AH group, which may be due to the conversion of SFAs into MUFAs, especially the conversation of C18:0 into C18:1n9c (Sprecher et al., 1995).

Diet is a major factor in the formation of bacterial communities in the rumen (Huang et al., 2021). In this study, we evaluated the forage proportion in FTMR using information about ruminal bacteria based on high-throughput sequencing of the 16S rRNA. Differences in alpha diversity of ruminal bacteria were not detected among the diet treatments. This is consistent with some studies on ruminants (McGovern et al., 2018; McLoughlin et al., 2020). Similarly, Yang et al. (2018) also found that the diversity of ruminal bacteria was not affected by the forage type in Hu lambs. The current study showed that most of the two core phyla were Bacteroidetes and Firmicutes, which is consistent with previous studies (Derakhshani et al., 2017), in which Bacteroidetes and Firmicutes were found predominantly in rumen fluid. A previous study indicated that these two phyla were closely correlated with the decomposition of cellulose, hemicellulose, and polysaccharides (Liang et al., 2021). However, the phylum composition of Fibrobacteres was higher in the OH group relative to the AH group. Fibrobacteres play a primal function in the decomposition of fiber and cellulose to provide nutrients for ruminants (Chen et al., 2021). The current results may be interpreted as the higher fiber content of the OH group as described by An et al. (2020), who confirmed that the number of Fibrobacteres in the rumen tended to increase with an increase in coarse feed.

At the genus level, *WCHB1-41_unclassified*, *Rikenellaceae_RC9_gut_group*, *F082_unclassified*, and *Prevotella_1* were the dominant bacteria in the two diet groups. No differences were observed in *WCHB1-41_unclassified* and *F082_unclassified* bacteria among the two groups, but the OH group had a significant influence on the content of *Rikenellaceae_RC9_gut_group*, *Prevotella_1*, and *Fibrobacter* in rumen fluid. Although we do not know with certainty the function of *Rikenellaceae_RC9_gut_group*, a previous study has suggested that *Rikenellaceae_RC9_gut_group* is closely correlated with acetate and propionate (Clarke et al., 2013; Holman and Gzyl, 2019). Taken together, an increase in *Rikenellaceae_RC9_gut_group* in the AH group is suggestive of its major role in utilizing the carbohydrate and nitrogen in ruminants. This explains, partially at least, the improvement in IMF (Table 2). *Prevotella_1* is associated with carbohydrate metabolism and propionate production, which play a significant role in the synthesis and decomposition of plant non-cellulosic polysaccharides, starch, and protein (Liu et al., 2019; Zhang X. et al., 2021). The increase in the abundance of this genus in the AH group suggested that the higher percentage of oat in FTMR could promote the degradation both in fibrous and non-fibrous carbohydrates, and these differences may be due to responses to dietary nutrition changes, such as increased dietary starch (Liu et al., 2019), as has also been shown in previous research (Cui et al., 2022). *Fibrobacter* are cellulolytic bacteria, which specialize in cellulose and hemicellulose fermentation (Zhou et al., 2019; Wei et al., 2021). In this study, the AH group had a higher abundance of *Fibrobacter*, which may indicate its higher fiber content (Supplementary Table 1). This suggestion agrees with the finding of An et al. (2020), who reported that supplementing with oat hay in the diet promotes the decomposition of carbohydrates and dietary cellulose in the rumen, and then increased the content of *Fibrobacter*.

Correlation analysis revealed relationships between meat quality, fatty acid profile, and rumen microbial abundances. In this study, the AH treatment resulted in a higher abundance of *Rikenellaceae_RC9_gut_group* and higher cholesterol content, which is mainly due to the *Rikenellaceae_RC9_gut_group* belonging to the butyrate-producing bacteria, which could increase AMPK activity and further regulate lipid deposition traits by regulating the production of VFAs (Zhang Y. K. et al., 2021; Cheng et al., 2022). Furthermore, the increase in *Rikenellaceae_RC9_gut_group* abundance may affect C18:1n9c content (Figure 6), which can

regulate cholesterol (Li et al., 2022). Ruminal microorganisms establish the key link between dietary nutrition and the fatty acids in ruminant products (Abuelfatah et al., 2016). Precursors for *de novo* fatty acid synthesis are mainly produced by rumen microbial fermentation (Shingfield et al., 2013). Therefore, ruminal rumen microbial metabolism is crucial in determining the ideal muscle fatty acid composition of ruminant products (Wang et al., 2022). A previous study has reported that reasonable muscle fatty acid compositions could be facilitated via the regulation of the bacterial community (Bi et al., 2018). The results of this study revealed that most parameters related to muscle FA deposition were correlated with the genera *Fibrobacter*, *Prevotella_1*, and *Rikenellaceae_RC9_gut_group*. C18:3n3 and C22:6n3 are the most bioactive components of ω-3 polyunsaturated fatty acids (PUFAs) and play an important role in reducing hepatic triglyceride content (Pirillo and Catapano, 2013). In this experiment, *Fibrobacter* and *Prevotella_1* were correlated positively with the C18:3n3 and C22:6n3 content. This result is mainly due to *Prevotella_1* and *Fibrobacter* being relevant for fiber digestion, which plays a crucial role in energy harvesting in the rumen ecosystem and provides precursors for UFA synthesis (Abuelfatah et al., 2016; Wang et al., 2019). Similar findings have been reported in fattening yaks (Hu et al., 2020). *Rikenellaceae_RC9_gut_group* was negatively correlated with the concentrations of C18:3n3 and C22:6n3. This result may be explained by the *Rikenellaceae RC9 gut group* having important metabolic functions in lipid metabolism (Ahmad et al., 2020). Research has shown that the *Rikenellaceae_RC9_gut_group* is negatively correlated with propionic acid (Cheng et al., 2022), which is produced by the biohydrogenation of PUFA (Beam et al., 2000). Furthermore, a previous study has reported that n-3 PUFA deficiency increased *Rikenellaceae* (Leyrolle et al., 2021).

Microorganisms are important in modulating the host's adaptive immunity and regulating the degradation, distribution, and absorption of nutrients (Fan et al., 2020).

In the current research, the predicted function from the level 1 gene was generally consistent among the two diet groups. However, the observations indicated that the diets directly impacted the levels 2 and 3 estimated gene functional profiles of lamb rumen microbiomes. Compared to the AH group, the metabolism of amino acids, including phenylalanine, tyrosine, and tryptophan biosynthesis, was decreased in the OH group. These results may be interpreted as due to the significant enrichment of *Prevotella_1* and *Fibrobacter* regulating the rumen protein metabolism and cellulose and hemicellulose metabolism and favoring the preferential use of carbohydrates, which would then weaken amino acids fermentation (Zhang et al., 2022). Hence, the pathways of "phenylalanine, tyrosine, and tryptophan biosynthesis" identified in this experiment may be attributed to the higher fiber content in the FTMR of lambs fed OH diets. However, our study findings do not represent the actual function of rumen bacteria. Further detailed analyses are needed to investigate the potential mechanisms and reveal the gene functions of microbiota under diets with different forage sources in FTMR fed to lambs.

Conclusion

This preliminary investigation explored the effect of forage proportions in FTMR on fatting performance and the rumen bacterial community of lambs. The fatting performance analysis showed that partial replacement of alfalfa with oat in FTMR promotes a beneficial lipid pattern in the *Longissimus lumborum* muscles for lambs. In addition, the correlation analysis revealed that there was a strong correlation between specific rumen bacteria and fatty acids in the *Longissimus lumborum* muscles. Of course, the lack of rumen fermentation parameters and quantification of probiotics as well as the small sample size are potential limitations of this study. Regardless, the results of the current research suggested that oat-based FTMR was more conducive to the production of beneficial fatty acids in the *Longissimus lumborum* muscles for lambs. These results can also provide some references for the application of FTMR in animal production, and information on interactions of the fatting performance of lamb and microbiota of the rumen, which may help make decisions regarding feeding.

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/, PRJNA888233.

Ethics statement

The study protocol selected for this experimental was based on the Institutional Guidelines for Animal Experiments and the Regulations for the Administration of Affairs Concerning Experimental Animals of the College of Grassland, Resources and Environment, Inner Mongolia Agricultural University, Hohhot, China. All the experimental protocols carried out in this study were approved by the Animal Care Committee of Inner Mongolia Agricultural University. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

ML: conceptualization, methodology, data curation, writing—original draft preparation, and writing—reviewing and editing. ZW: investigation and resources. YW: methodology. LS: software. JL: validation and formal analysis. GG: writing—reviewing and editing. YJ: project administration and funding acquisition. SD: writing—original draft preparation and writing—reviewing and editing. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

JL was employed by Inner Mongolia Yili Industrial Group Co., Ltd.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1197059/full#supplementary-material

References

Abidi, S., Salem, H. B., Vasta, V., and Priolo, A. (2009). Supplementation with barley or spineless cactus (*Opuntia ficus* indica f. inermis) cladodes on digestion, growth and intramuscular fatty acid composition in sheep and goats receiving oaten hay. *Small Rumin. Res.* 87, 9–16. doi: 10.1016/j.smallrumres.2009.09.004

Abuelfatah, K., Zuki, A. B., Goh, Y. M., Sazili, A. Q., and Abubakr, A. (2016). Effects of feeding whole linseed on ruminal fatty acid composition and microbial population in goats. *Anim. Nutr.* 2, 323–328. doi: 10.1016/j.aninu.2016.10.004

Ahmad, M. I., Ijaz, M. U., Hussain, M., Haq, I. U., Zhao, D., and Li, C. B. (2020). High-fat proteins drive dynamic changes in gut microbiota, hepatic metabolome, and endotoxemia-tlr-4-nfκb-mediated inflammation in mice. *J. Agric. Food Chem.* 68, 11710–11725. doi: 10.1021/acs.jafc.0c02570

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

AOAC (2005). *Official Methods of Analysis. 18th Edn.* Gaithersburg, MD: AOAC International.

Araujo, J. R., Tazi, A., Defranoux, O. B., Guerre, S. V., Nigro, G., Licandro, H., et al. (2020). Fermentation products of commensal bacteria alter enterocyte lipid metabolism. *Cell Host Microbe* 27, 358–375. doi: 10.1016/j.chom.2020.01.028

Beam, T. M., Jenkins, T. C., Moate, P. J., Kohn, R. A., and Palmquist, D. L. (2000). Effects of amount and source of fat on the rates of lipolysis and biohydrogenation of fatty acids in ruminal contents. *J. Dairy Sci.* 83, 2564–2573. doi: 10.3168/jds.S0022-0302(00)75149-6

Behan, A. A., Akhtar, M. T., Loh, T. C., Fakurazi, S., Kaka, U., Muhamad, A., et al. (2021). Meat quality, fatty acid content and NMR metabolic profile of dorper sheep supplemented with bypass fats. *Foods* 10, 1133. doi: 10.3390/foods10051133

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

Brand, T. S., Merwe, D. A. V., Hoffman, L. C., and Geldenhuys, G. (2018). The effect of dietary energy content on quality characteristics of Boer goat meat. *Meat Sci.* 139, 74-81. doi: 10.1016/j.meatsci.2018.01.018

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

Carballo, O. C., McCoard, S. A., Cookson, A. L., Laven, R. A., Ganesh, S., Lewis, S. J., et al. (2021). Effect of divergent feeding regimes during early life on the rumen microbiota in calves. *Front. Microbiol.* 12, 711040. doi: 10.3389/fmicb.2021.711040

Carrasco, J. M. D., Cabral, C., Redondo, L. M., Viso, N. D. P., Colombatto, D., Farber, M. D., et al. (2017). Impact of chestnut and quebracho tannins on rumen microbiota of bovines. *Biomed Res. Int.* 2017, 9610810. doi: 10.1155/2017/9610810

Chanjula, P., Wungsintaweekul, J., Chiarawipa, R., Rugkong, A., Khonkhaeng, B., Suntara, C., et al. (2022). Effect of feed supplement containing dried kratom leaves on apparent digestibility, rumen fermentation, serum antioxidants, hematology, and nitrogen balance in goats. *Fermentation* 8, 131. doi: 10.3390/fermentation8030131

Chávez, C. C. M., Ramírez, P. N., Hoyos, L. R., Durán, M. G. R., Madrigal, J. F., Sánchez, M. C. C., et al. (2022). Retos y perspectivas del sector acuí-cola rumbo al 2050. *Cienc. Nicolait.* 83. doi: 10.35830/cn.vi83.572

Chen, H., Guo, B. B., Yang, M. R., Luo, J. R., Hu, Y. Q., Qu, M. R., et al. (2021). 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.* 8, 681389. doi: 10.3389/fvets.2021.681389

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

Clarke, S. F., Murphy, E. F., O'sullivan, O, Ross, R. P., O'toole, PW, Shanahan, F., et al. (2013). Targeting the microbiota to address diet-induced obesity: a time dependent challenge. *PLoS ONE* 8, e65790. doi: 10.1371/journal.pone.0065790

Costa, P., Lemos, J. P., Lopes, P. A., Alfaia, C. M., Costa, A. S. H., Bessa, R. J. B., et al. (2012). Effect of low- and high-forage diets on meat quality and fatty acid composition of Alentejana and Barrosã beef breeds. *Animal* 6, 1187–1197. doi: 10.1017/S1751731111002722

Cui, Y. L., Liu, H., Gao, Z. M., Xu, J. Y., Liu, B. S., Guo, M., et al. (2022). Whole-plant corn silage improves rumen fermentation and growth performance of beef cattle by altering rumen microbiota. *Appl. Microbiol. Biotechnol.* 106, 4187–4198. doi: 10.1007/s00253-022-11956-5

Derakhshani, H., Tun, H. M., Cardoso, F. C., Plaizier, J. C., Khafipour, E., and Loor, J. J. (2017). Linking peripartal dynamics of ruminal microbiota to dietary changes and production parameters. *Front. Microbiol.* 7, 2143. doi: 10.3389/fmicb.2016.02143

Douglas, G. M., Maffei, V. J., Zaneveld, J., Yurgel, S. N., Brown, J. R., Taylor, C. M., et al. (2020). PICRUSt2: an improved and extensible approach for metagenome inference. *BioRxiv. [Preprint].* doi: 10.1101/672295

Du, S., You, S. H., Sun, L., Wang, X. L., Jia, Y. S., and Zhou, Y. L. (2022). Effects of replacing alfalfa hay with native grass hay in pelleted total mixed ration on physicochemical parameters, fatty acid profile, and rumen microbiota in lamb. *Front. Microbiol.* 13, 861025. doi: 10.3389/fmicb.2022.861025

Dung, D. V., Phung, L. D., Ngoan, L. D., Ba, N. X., Van, N. H., and Thao, L. D. (2020). Effects of different forages in fermented total mixed rations on nutrient utilization and ruminating behaviours of growing yellow cattle in vietnam. *Adv. Anim. Vet. Sci.* 8, 245-251. doi: 10.17582/journal.aavs/2020/8.3.245.251

Ellison, M. J., Conant, G. C., Lamberson, W. R., Cockrum, R. R., Austin, K. J., Rule, D. C., et al. (2017). Diet and feed efficiency status affect rumen microbial profiles of sheep. *Small Rumin. Res.* 156, 12–19. doi: 10.1016/j.smallrumres.2017.08.009

Enjalbert, F., Combes, S., Zened, A., and Meynadier, A. (2017). Rumen microbiota and dietary fat: a mutual shaping. *J. Appl. Microbiol.* 123, 782–97. doi: 10.1111/jam.13501

Fan, Q. S., Wanapat, M., Yan, T. H., and Hou, F. J. (2020). Altitude influences microbial diversity and herbage fermentation in the rumen of yaks. *BMC Microbiol.* 20, 370. doi: 10.1186/s12866-020-02054-5

Freer, M., Dove, H., Nolan, J. V. (2007). *Nutrient Requirements of Domesticated Ruminants*. Melbourne, VIC: CSIRO Publishing.

Galvani, D. B., Pires, A. V., Susin, I., Gouvêa, V. N., Berndt, A., Chagas, L. J., et al. (2014). Energy efficiency of growing ram lambs fed concentrate-based diets with different roughage sources. *J. Anim. Sci.* 92, 250–263. doi: 10.2527/jas.2012-6017

Hajji, H., Joy, M., Ripoll, G., Smeti, S., Mekki, I., Gahete, F. M., et al. (2016). Meat physicochemical properties, fatty acid profile, lipid oxidation and sensory

characteristics from three North African lamb breeds, as influenced by concentrate. *J. Food Compos. Anal.* 48, 102–110. doi: 10.1016/j.jfca.2016.02.011

Harper, M. T., Oh, J., Giallongo, F., Lopes, J. C., Roth, G. W., and Hristov, A. N. (2017). Using brown midrib 6 dwarf forage sorghum silage and fall-grown oat silage in lactating dairy cow rations. *J. Dairy Sci.* 100, 5250–5265. doi: 10.3168/jds.2017-12552

Holman, D. B., and Gzyl, K. E. (2019). A meta-analysis of the bovine gastrointestinal tract microbiota. *FEMS Microbiol. Ecol.* 6, fiz072. doi: 10.1093/femsec/fiz072

Honikel, K. O. (1998). Reference methods for the assessment of physical characteristics of meat. *Meat Sci.* 49, 447–457. doi: 10.1016/S0309-1740(98)00034-5

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

Huang, G. X., Guo, L. Y., Chang, X. F., Liu, K. Z., Tang, W. H., Zheng, N., et al. (2021). Effect of whole or ground flaxseed supplementation on fatty acid profile, fermentation, and bacterial composition in rumen of dairy cows. *Front. Microbiol.* 12, 760528. doi: 10.3389/fmicb.2021.760528

Jia, T. T., Yun, Y., and Yu, Z. (2021). Propionic acid and sodium benzoate affected biogenic amine formation, microbial community, and quality of oat silage. *Front. Microbiol.* 12, 750920. doi: 10.3389/fmicb.2021.750920

Kotupan, S., and Sommart, K. (2021). Broken rice in a fermented total mixed ration improves carcass and marbling quality in fattened beef cattle. *Anim. Biosci.* 34, 1331–1341. doi: 10.5713/ajas.20.0288

Krause, T. R., Lourenco, J. M., Welch, C. B., Rothrock, M. J., Callaway, T. R., and Pringle, T. D. (2020). The relationship between the rumen microbiome and carcass merit in Angus steers. *J. Anim. Sci.* 98, skaa287. doi: 10.1093/jas/skaa287

Kumagai, H., Hishinuma, M., and Takahashi, J. (1983). Whole crop silage diets for beef production in Japan. *J. Jpn. Soc. Zootech. Sci.* 54, 37-43.

Leyrolle, Q., Decoeur, F., Briere, G., Amadieu, C., Quadros, ARAA, Voytyuk, I., Lacabanne, C., et al. (2021). Maternal dietary omega-3 deficiency worsens the deleterious effects of prenatal inflammation on the gut-brain axis in the offspring across lifetime. *Neuropsychopharmacology* 46, 579–602. doi: 10.1038/s41386-020-00793-7

Li, F., Zhang, Z. A., Li, X. Y., Zhu, B. Z., Guo, L., Li, F. D., et al. (2020). Effect of duration of linseed diet supplementation before slaughter on the performances, meat fatty acid composition and rumen bacterial community of fattening lambs. *Anim. Feed Sci. Technol.* 263, 114457. doi: 10.1016/j.anifeedsci.2020.114457

Li, L., Sun, X. H., Luo, J. Y., Chen, T., Xi, Q. Y., Zhang, Y. L., et al. (2022). Effects of herbal tea residue on growth performance, meat quality, muscle metabolome, and rumen microbiota characteristics in finishing steers. *Front. Microbiol.* 12, 821293. doi: 10.3389/fmicb.2021.821293

Li, Y., Lv, J. Y., Wang, J. H., Zhou, S., Zhang, G. N., Wei, B. D., et al. (2021). Changes in carbohydrate composition in fermented total mixed ration and its effects on *in vitro* methane production and microbiome. *Front. Microbiol.* 12, 738334. doi: 10.3389/fmicb.2021.738334

Liang, J. S., Zheng, W. G., Zhang, H. B., Zhang, P. Y., Cai, Y. J., Wang, Q. Y., et al. (2021). Transformation of bacterial community structure in rumen liquid anerobic digestion of rice straw. *Environ. Pollut.* 269, 116130. doi: 10.1016/j.envpol.2020.116130

Liu, H. J., Xu, T. W., Xu, S. X., Ma, L., Han, X. P., Wang, X. G., et al. (2019). Effect of dietary concentrate to forage ratio on growth performance, rumen fermentation and bacterial diversity of Tibetan sheep under barn feeding on the Qinghai-Tibetan plateau. *PeerJ* 7, e7462. doi: 10.7717/peerj.7462

Liu, Y., Wu, H., Chen, W. B., Liu, C., Meng, Q. X., and Zhou, Z. M. (2022). Rumen microbiome and metabolome of high and low residual feed intake angus heifers. *Front. Vet. Sci.* 9, 812861. doi: 10.3389/fvets.2022.812861

Logue, J. B., Stedmon, C. A., Kellerman, A. M., Nielsen, N. J., Andersson, A. F., Laudon, H., et al. (2016). Experimental insights into the importance of aquatic bacterial community composition to the degradation of dissolved organic matter. *ISME J.* 10, 533–545. doi: 10.1038/ismej.2015.131

Malva, A. D., Albenzio, M., Annicchiarico, G., Caroprese, M., Muscio, A., Santillo, A., et al. (2016). Relationship between slaughtering age, nutritional and organoleptic properties of Altamurana lamb meat. *Small Rumin. Res.* 135, 39–45. doi: 10.1016/j.smallrumres.2015.12.020

McGovern, E., Kenny, D. A., Mccabe, M. S., Fitzsimons, C., Mcgee, M., Kelly, A. K., et al. (2018). 16S rRNA sequencing reveals relationship between potent cellulolytic genera and feed efficiency in the rumen of bulls. *Front. Microbiol.* 9, 1842. doi: 10.3389/fmicb.2018.01842

McLoughlin, S., Spillane, C., Claffey, N., Smith, P. E., Rourke, T. O., Diskin, M., et al. (2020). Rumen microbiome composition is altered in sheep divergent in feed efficiency. *Front. Microbiol.* 11, 1981. doi: 10.3389/fmicb.2020.01981

Ministry of Agriculture (2019). *Operating Procedures of Livestock and Poultry Slaughtering- Sheep and Goat (NY/T 3469-2019)*. Beijing: China Agricultural Press.

Mushi, D. E., Safari, J. G., Mtenga, L. A., Kifaro, G. C., and Eik, L. O. (2009). Effects of concentrate levels on fattening performance, carcass and meat quality attributes of Small East African × Norwegian crossbred goats fed low quality grass hay. *Livest. Sci.* 124, 148–155. doi: 10.1016/j.livsci.2009.01.012

Nasehi, M., Torbatinejad, N. M., Rezaie, M., and Ghoorchi, T. (2018). Effects of partial substitution of alfalfa hay with green tea waste on growth performance and in vitro methane emission of fat-tailed lambs. *Small Rumin. Res.* 168, 52–59. doi: 10.1016/j.smallrumres.2018.09.006

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

Pirillo, A., and Catapano, A. L. G. (2013). Omega-3 polyunsaturated fatty acids in the treatment of hypertriglyceridaemia. *Int. J. Cardiol.* 170, S16–S20. doi: 10.1016/j.ijcard.2013.06.040

R Core Team (2014). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

SAS Inc (2007). *SAS OnlineDoc®*. Cary, NC: 9.1.3 SAS Inc.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12, R60. doi: 10.1186/gb-2011-12-6-r60

Seman, D. L., Boler, D. D., Carr, C. C., Dikeman, M. E., Owens, C. M., Keeton, J. T., et al. (2017). "Meat science lexicon," in *Meat Science and Muscle Biology*. p. 1–15. doi: 10.22175/mmb2017.12.0059

Shahidi, F., and Ambigaipalan, P. (2018). Omega-3 polyunsaturated fatty acids and their health benefits. *Annu. Rev. Food Sci. Technol.* 9, 345–381. doi: 10.1146/annurev-food-111317-095850

Shingfield, K. J., Bonnet, M., and Scollan, N. D. (2013). Recent developments in altering the fatty acid composition of ruminant-derived foods. *Animal* 7, 132–162. doi: 10.1017/S1751731112001681

Sprecher, H., Luthria, D. L., Mohammed, B. S., and Baykousheva, S. P. (1995). Reevaluation of the pathways for the biosynthesis of polyunsaturated fatty acids. *J. Lipid Res.* 36, 2471–2477. doi: 10.1016/S0022-2275(20)41084-3

Su, Y. Y., Sun, X., Zhao, S. M., Hu, M. L., Li, D. F., Qi, S. L., et al. (2022). Dietary alfalfa powder supplementation improves growth and development, body health, and meat quality of Tibetan sheep. *Food Chem.* 396, 133709. doi: 10.1016/j.foodchem.2022.133709

Sun, C. H., Lee, J. S., Nejad, J. G., Kim, W. S., and Lee, H. G. (2021). Effect of a rumen-protected microencapsulated supplement from linseed oil on the growth performance, meat quality, and fatty acid composition in korean native steers. *Animals* 11, 1253. doi: 10.3390/ani11051253

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

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

Wang, J., Yang, B. Y., Zhang, S. J., Amar, A., Chaudhry, A. S., Cheng, L., et al. (2021). Using mixed silages of sweet sorghum and alfalfa in total mixed rations to improve growth performance, nutrient digestibility, carcass traits and meat quality of sheep. *Animal* 15, 100246. doi: 10.1016/j.animal.2021.100246

Wang, Q. Y., Wang, Y. C., Hussain, T., Dai, C. P., Li, J. Z., Huang, P. F., et al. (2020). Effects of dietary energy level on growth performance, blood parameters and meat quality in fattening male Hu lambs. *J. Anim. Physiol. Anim. Nutr.* 104, 418–430. doi: 10.1111/jpn.13278

Wang, Y. Y., Li, T. F., Chen, X. Y., Liu, C. Y., Jin, X. M., Tan, H., et al. (2022). Preliminary investigation of mixed orchard hays on the meat quality, fatty acid profile, and gastrointestinal microbiota in goat kids. *Animals* 12, 780. doi: 10.3390/ani12060780

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

Welkie, D. G., Stevenson, D. M., and Weimer, P. J. (2010). ARISA analysis of ruminal bacterial community dynamics in lactating dairy cows during the feeding cycle. *Anaerobe* 16, 94–100. doi: 10.1016/j.anaerobe.2009.07.002

Whitney, T. R., and Smith, S. B. (2015). Substituting redberry juniper for oat hay in lamb feedlot diets: carcass characteristics, adipose tissue fatty acid composition, and sensory panel traits. *Meat Sci.* 104, 1–7. doi: 10.1016/j.meatsci.2015.01.010

Wood, J. D., Enser, M., Fisher, A. V., Nute, G. R., Sheard, P. R., Richardson, R. I., et al. (2008). Fat deposition, fatty acid composition and meat quality: a review. *Meat Sci.* 78, 343–358. doi: 10.1016/j.meatsci.2007.07.019

Xu, X. J., Chen, X. L., Chen, D. W., Yu, B., Yin, J. D., and Huang, Z. Q. (2019). Effects of dietary apple polyphenol supplementation on carcass traits, meat quality, muscle amino acid and fatty acid composition in finishing pigs. *Food Funct.* 10, 7426–7434. doi: 10.1039/C9FO01304K

Yang, B., Le, J. Q., Wu, P., Liu, J. X., Guan, L. L., and Wang, J. K. (2018). Alfalfa intervention alters rumen microbial community development in hu lambs during early life. *Front. Microbiol.* 9, 574. doi: 10.3389/fmicb.2018.00574

You, S. H., Du, S., Ge, G. T., Wan, T., and Jia, Y. S. (2021). Selection of lactic acid bacteria from native grass silage and its effects as inoculant on silage fermentation. *Agron. J.* 113, 3169–3177. doi: 10.1002/agj2.20720

Youdim, K. A., Martin, A., and Joseph, J. A. (2000). Essential fatty acids and the brain: possible health implications. *Int. J. Diabetes Dev. Ctries.* 18, 383–399. doi: 10.1016/S0736-5748(00)00013-7

Zhang, G. N., Li, Y., Fang, X., Cai, Y. M., and Zhang, Y. G. (2020). Lactation performance, nitrogen utilization, and profitability in dairy cows fed fermented total mixed ration containing wet corn gluten feed and corn stover in combination replacing a portion of alfalfa hay. *Anim. Feed Sci. Technol.* 269, 114687. doi: 10.1016/j.anifeedsci.2020.114687

Zhang, R. Y., Liu, J. H., Jiang, L. S., Wang, X. F., and Mao, S. Y. (2022). The remodeling effects of high-concentrate diets on microbial composition and function in the hindgut of dairy cows. *Front. Nutr.* 8, 809406. doi: 10.3389/fnut.2021.809406

Zhang, X., Wang, H. C., and Guo, X. S. (2021). Comparative analysis of rumen fermentation parameters and bacterial profiles during adaption to different fattening stages in beef cattle fed TMR with various forage silage. *Anim. Feed Sci. Technol.* 278, 115006. doi: 10.1016/j.anifeedsci.2021.115006

Zhang, Y. K., Zhang, X. X., Li, F. D., Li, C., Zhang, D. Y., Li, X. L., et al. (2021). Exploring the ruminal microbial community associated with fat deposition in lambs. *Animals* 11, 3584. doi: 10.3390/ani11123584

Zhao, J., Dong, Z. H., Chen, L., Wang, S. R., and Shao, T. (2020). The replacement of whole-plant corn with bamboo shoot shell on the fermentation quality, chemical composition, aerobic stability and in vitro digestibility of total mixed ration silage. *Anim. Feed Sci. Technol.* 259, 114348. doi: 10.1016/j.anifeedsci.2019.114348

Zhao, S. S., Yang, F. Y., Wang, Y., Fan, X. M., Feng, C. S., and Wang, Y. P. (2021). Dynamics of fermentation parameters and bacterial community in high-moisture alfalfa silage with or without lactic acid bacteria. *Microorganisms* 9, 1225. doi: 10.3390/microorganisms9061225

Zhou, K., Bao, Y., and Zhao, G. Y. (2019). Effects of dietary crude protein and tannic acid on rumen fermentation, rumen microbiota and nutrient digestion in beef cattle. *Arch. Anim. Nutr.* 73, 30–43. doi: 10.1080/1745039X.2018.1545502

Zhou, Z. M., Zhou, B., Ren, L. P., and Meng, Q. X. (2014). Effect of ensiled mulberry leaves and sun-dried mulberry fruit pomace on finishing steer growth performance, blood biochemical parameters, and carcass characteristics. *PLoS ONE* 9, e85406. doi: 10.1371/journal.pone.0085406

Zhu, X. Y., Liu, B. S., Xiao, J. N., Guo, M., Zhao, S. M., Hu, M. L., et al. (2022). Effects of different roughage diets on fattening performance, meat quality, fatty acid composition, and rumen microbe in steers. *Front. Nutr.* 9, 885069. doi: 10.3389/fnut.2022.885069

Zou, Y., Zou, X. P., Li, X. Z., Guo, G., Ji, P., Wang, Y., et al. (2018). Substituting oat hay or maize silage for portion of alfalfa hay affects growth performance, ruminal fermentation, and nutrient digestibility of weaned calves. *Asian Aust. J. Anim. Sci.* 31, 369–378. doi: 10.5713/ajas.17.0210

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