

COMBINED ANALYSIS OF THE EFFECTS OF CHANGES IN FEEDING SYSTEMS ON RUMEN MICROBIOTA IN GOATS

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ABSTRACT

Different feeding methods are associated with differences in the health status and rumen microbial community of goats, but the underlying mechanism remains unclear. In this experiment, Nanjiang Yellow sheep (NYG) were randomly divided into the barn feeding group (BF) and the grazing group (GF) (16 sheep in each group; 8 replicates, 2 sheep in each replicate). After the experiment, 6 sheep were randomly selected from each group for subsequent sample collection ($n = 6$). Serum biochemical indicators, rumen enzyme activities and rumen microbial flora composition were comprehensively analyzed by ELISA, colorimetric method and 16S rRNA sequencing respectively to evaluate the effects of pen feeding and grazing on NYG. The results showed that these two feeding systems, which differ in diet composition, nutrient consistency and forage physical structure, were associated with differences in the composition and abundance of rumen microbiota. Compared to GF, the relative abundances of Armatimonadota, Patescibacteria, Desulfobacterota, and Planctomycetota in the rumen were significantly lower in the BF group ($P \leq 0.05$), while Elusimicrobiota, unclassified Synergistota and Cyanobacteria were significantly higher ($P \leq 0.05$). Functional prediction analysis using PICRUSt2 revealed that amino acid metabolism, cofactor and vitamin metabolism were the most abundant metabolic pathways in the rumen. These findings suggest that alterations in management systems are associated with changes in the abundance of specific rumen microbial phyla, including Armatimonadota, Desulfobacterota, Elusimicrobiota, and Cyanobacteria. Additionally, these systems are associated with differences in amino acid, cofactor, and vitamin metabolic pathways. These results enhance our understanding of diet- and management system-microbiota in goats and provide a basis for optimizing feeding strategies under different management systems.

Keywords: Goat; Feeding systems; Serum biochemical indexes; Rumen enzyme activity; Rumen microbiota

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INTRODUCTION

Nanjiang Yellow goats (NYG) are a high quality goat breed indigenous to Nanjiang County, in Sichuan Province. This breed demonstrates multiple advantages, including tolerance to coarse feed, rapid growth, excellent meat quality, high feed conversion rate, strong reproductive capacity and strong adaptability (Li et al., 2016; Zhao et al., 2018). The NYG are mainly raised by grazing. Studies have shown that during this process, goats may consume a diverse microbial community, which has a positive impact on their health (Shah et al., 2019). However, the NYG are reared in a mountainous region where the species and quantity of pasture grasses significantly decline in winter, accompanied by a reduction in their nutrient content. Combined with the harsh winter climate, these factors considerably reduce GF efficiency (Stephan et al., 2020). Therefore, a comprehensive analysis of the effects of housing with formulated TMR versus natural pasture grazing on rumen microbiota can provide valuable theoretical insights for goat feeding management, while recognizing that the observed differences may reflect the combined effects of diet composition, nutritional consistency, forage physical structure, and management practices.

Rumen microbes play a critical role in host growth, reproduction, immune function, and product quality (Huang et al., 2021; Zhu et al., 2022). Additionally, Various factors, including age, sex, altitude and diet composition particularly feeding systems affect rumen bacterial composition (Guo et al., 2014; Zhang et al., 2021). For example, weaned lambs fed high-concentrate diets showed higher rumen bacterial counts and 3.5 fold more protozoa than grazing lambs (Ji et al., 2016). Similarly, BF Aberdeen Angus cattle exhibited greater microbial diversity and a higher Firmicutes abundance

than GF cattle (Zhang et al., 2021). These findings indicate that feeding strategies and dietary compositions significantly influence gastrointestinal microbial diversity and composition.

In livestock production systems, barn feeding with TMR and natural pasture grazing represent two fundamentally different management paradigms that differ simultaneously in diet composition, nutrient consistency, forage physical structure, and environmental conditions (Huang et al., 2021). While this integrated comparison precludes attribution of observed effects to any single factor, it reflects the practical reality faced by producers who must choose between these complete management systems rather than between isolated variables (Huang et al., 2021). We hypothesized that BF with a formulated TMR would alter rumen microbiota composition and diversity in NYG goats compared to GF on natural pasture, potentially enriching beneficial taxa, though we recognize that this comparison confounds feeding system with differences in dietary composition, nutrient consistency, and forage physical form. This study compared the effects of BF and GF on rumen microbiota, serum biochemistry, and enzyme activity in NYG goats. The findings aim to provide a theoretical basis for optimizing winter feeding strategies and promoting sustainable development of the NYG industry.

MATERIALS AND METHODS

Ethics statement: The sample collection plan has been approved by the Biotechnology and Livestock Breeding Committee of Southwest University of Science and Technology (SWUST, China). To ensure the highest standards of animal welfare and rights, all animal breeding experiments and related animal sample collection are strictly carried out in accordance with relevant regulations and requirements (Approval No. : L2022026-2022-03).

Animal feeding management: This experiment was conducted in December 2022 at the Nanjiang Yellow Sheep Farm in Nanjiang County, Bazhong City, Sichuan Province. Thirty-two 3-month-old weaned Nanjiang yellow sheep were selected and randomly divided into two treatment groups: the barn feeding group (BF group) and the grazing group (GF group), with 16 sheep in each group. Each group has 8 repetitions, with 2 sheep in each repetition. At the conclusion of the experiment, following a 12 hour fasting period, 6 replicates were randomly selected from each group, and 1 goat was randomly chosen from each selected replicate for sample collection (n=6). The experimental period spanned 67 days, which included a 7 day pretest phase and a 60 day main test period. GF goats were released to pasture at 7:00 a.m. and herded back to the pen at 6:00 p.m. BF goats were fed twice daily at 8:00 a.m. and 5:00 p.m. The total mixed ration (TMR) formulation was based on Lee et al. (Lee et al., 2021). The nutrient composition of the TMR was as follows: the dry matter (DM) content was 91.60%, and on a DM basis, it contained 87.30% organic matter, 8.15% crude ash, 12.00% crude protein (CP), 3.10% ether extract (EE), 46.85% neutral detergent fiber (NDF), 26.35% acid detergent fiber (ADF), and 34.30% non-fiber carbohydrate (NFC). The TMR formulation was adapted from Lee et al (Lee et al., 2021). and was designed to meet the nutritional requirements of growing goats (NRC, 2007). During the GF period, the goats in the GF group were permitted to freely graze on ryegrass, oleander leaves, elm leaves, rice straw, and other withered grasses. The GF system represented traditional GF practice, where goats freely grazed on open, unfenced mountain pastures. Owing to the extensive and heterogeneous topography of the GF area, quantitative assessment of pasture biomass availability and dynamic forage quality was not feasible within the scope of this study. However, throughout the entire experiment, visual monitoring was conducted on the botanical composition of the forage grass, with the main species being ryegrass, oleander leaves, elm leaves, straw and other withered weeds. To control the potential confounding effect of differences in forage species on the composition of rumen microbiota, we designed the roughage portion of the total mixed diet (BF TMR) to contain the same botanical species as the forage in the grazing group (GF). Namely, ryegrass (*Lolium* sp.), oleander leaves (*Nerium oleander*), elm leaves (*Ulmus* sp.), straw (*Oryza sativa*) and other withered weeds. These forage grasses account for 50% of the total dry matter (DM) of the BF TMR roughage (the remaining 50% is composed of alfalfa hay and corn silage). It should be pointed out, however, that in the BF group, roughage was mechanically chopped to 3-5 cm and fed in the form of prepared TMR. In contrast, the GF group freely consumed intact forage plants with natural stem and leaf structures, and their nutritional content fluctuated with the change of seasons. Therefore, the composition and physical structure of the diet are systematically mixed with the management model, resulting in fundamental differences between the two feeding models in terms of the physical form of roughage, diet uniformity and nutritional stability.

Sample collection: On the last day of the experiment, blood and rumen fluid samples were collected from the animals after they had fasted for 12 hours. Blood samples (5 mL) were collected from the jugular vein using a 20-gauge needle without anesthesia. The serum was then separated by centrifugation at 3500 rpm for 15 minutes at 4 ° C for subsequent analysis of serum indicators. Rumen fluid was collected under artificial preservation and without sedation through a flexible gastric tube (16 mm in diameter and 55 cm in length) inserted into the rumen through the oral cavity. Discard the first 10 mL of rumen fluid to minimize contamination by saliva and oral microorganisms. Then, use a vacuum pump to

extract approximately 10 mL of representative rumen fluid under mild negative pressure. The extracted rumen fluid was immediately filtered through four layers of sterile gauze and transferred to cryotubes for rumen enzyme activity analysis and rumen microbial DNA extraction. All samples were temporarily stored in liquid nitrogen and then kept at -80°C .

Determination of serum biochemical indices and rumen enzyme activities: The collected serum was thawed at 4°C . The levels of total protein (TP), blood urea nitrogen (BUN), glucose (GLU), total cholesterol (TC), and triglycerides (TG) were detected by enzyme-linked immunosorbent assay (ELISA) in accordance with the recommended operating procedures of the Nanjing Jiancheng Kit. Take a portion of the rumen fluid from each goat, thaw it at 4°C , and then centrifuge it in a low-temperature centrifuge at 3500 rpm for 10 minutes. Collect the supernatant. Subsequently, the cellulase activity was analyzed by the 3, 5-dinitrosalicylic acid (DNS) colorimetric method; The activity of α -amylase was determined by the iodine-starch colorimetric method (Bernfeld method). The activity of pepsin was determined by the flinphenol colorimetric method. The lipase activity was determined by the copper soap colorimetric method. All the above operations were carried out strictly in accordance with the recommended procedures of the corresponding kits (Nanjing Jiancheng Institute of Biotechnology, Nanjing, China).

Microbiome sample processing and sequencing: The Tian Gen Fecal Genomic DNA Extraction Kit is specifically designed to efficiently extract genomic DNA from rumen fluid samples using the CTAB method. The extracted DNA is evaluated on a 1% agarose gel to ensure satisfactory quality and concentration. Based on the assessed DNA concentration, an appropriate volume of DNA is precisely extracted into a centrifuge tube and diluted to a concentration of 1 ng/ μL in sterile water for subsequent PCR amplification. The amplification of the 16S rRNA gene is performed using the 16S V3-V4 primers, where the forward primer sequence is CCTACGGGNGGCWGCAG, and the reverse primer sequence is GACTACHVGGGTATCTAATCC (Hugerth *et al.*, 2014). The target fragment length is 464 bp. The PCR reaction system comprises 15 μL of Phusion Master Mix (2 $\times$), 0.2 μL each of upstream and downstream primers, and 10 μL of the diluted 1 ng/ μL DNA sample, with 4.6 μL of ddH₂O added to achieve a total volume of 30 μL . The PCR amplification program was as follows: pre denaturation at 98°C for 1 min; 30 cycles including (denaturation at 98°C for 10 sec; annealing at 50°C for 30 s ; extension at 72°C for 30 s); and finally extension at 72°C for 5 min. PCR products were mixed in equal amounts with 1 $\times$ buffer containing fluorescence and purified by agarose gel electrophoresis at a concentration of 2%, followed by recovery of the products using the Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries are created using the TruSeq® DNA PCR-Free Sample Preparation Kit, with the constructed libraries evaluated for quality using Qubit and Agilent Bioanalyzer 2100, and quantified by System/Q-PCR. Once the libraries are confirmed to be of adequate quality, sequencing is conducted using the Illumina NovaSeq 6000.

Bioinformatics and statistical analysis of bacterial metagenomics: Raw data were filtered, spliced and compared to obtain valid data using Fastp (v0.22.0), FLASH (v1.2.11) and Vsearch (v2.22.1). Primer sequences were trimmed and chimeric sequences were removed using Cutadapt (v1.9.1) and UCHIME (v4.2) software. Utilizing Quantitative Insights into Microbial Ecology (QIIME) software (Upase v7.0.1001), all sequences and microbial compositions were classified and clustered into operational taxonomic units (OTUs) with 97% similarity to the sample sequences to calculate alpha diversity. Alpha diversity was analyzed in depth using the R program (v3.6.0) to explore species abundance and evenness, verifying that the current sequencing depth is sufficient to cover most species information. Beta diversity was evaluated by principal coordinate analysis (PCoA) based on Bray-Curtis distance matrices. The statistical significance of clustering patterns between groups was assessed using PERMANOVA (Adonis) with 999 permutations using the vegan package in R (v3.6.0). The shared file was generated using the 'make. shared' command in Mothur, and the relative abundance of each taxonomic unit was calculated using the 'summary'. shared' command. Differential abundance analysis of rumen microbiota at the phylum level was performed using the Wilcoxon rank-sum test (Mann-Whitney U test) with Benjamini-Hochberg false discovery rate (FDR) correction. Taxa with $q \leq 0.05$ and $|\log_2\text{FC}| > 1$ were considered significantly different. Using PICRUST2 (version 2.2.0b), the predict metagenomes function is employed to predict the functional profiles of each sample. Subsequently, statistical analyses and visualization tools were utilized to compare the functional differences among different sample groups, thereby revealing the functional characteristics of the rumen microbiota.

Statistical analysis of data: Experimental data were statistically analyzed using SPSS 26.0 software and t-test. The results are expressed as mean and standard error of the mean (SEM). $P \leq 0.05$ indicates significant difference, and $P \leq 0.01$ indicates extremely significant difference. Graphs were generated using GraphPad Prism version 9.

RESULTS

Effects of different feeding system on serum biochemical indexes of goats: The results of the serum biochemical indices showed that the contents of TP and BUN in the serum of BF were significantly higher than those of GF ($P \leq 0.05$), while the content of TC was significantly lower than that of the GF goats ($P \leq 0.05$). No significant differences were observed in the contents of GLU and TG in the serum between the BF and GF groups (Table1).

Effects of different feeding system on rumen enzyme activities of goats: In rumen enzyme activity, the relative content of α -amylase, lipase and pepsin in the rumen of the GF group was higher than that of the BF group, with no significant differences observed ($P > 0.05$); however, the cellulase activity in the GF group was significantly higher than that of the BF group ($P \leq 0.05$; Table1).

Table 1 Comparison of serum biochemistry and rumen enzyme activity in GF vs BF goats

Items	GF (Grazing)	BF(Barn feeding)	P-value
TP (mg/ml)	60.02±3.04	66.56±5.10	0.030
BUN (mmol/L)	2.47±0.90	5.30±1.70	0.002
GLU (mmol/L)	5.82±0.54	5.50±0.27	0.154
TC (mmol/L)	2.29±0.26	1.66±0.13	0.047
TG (mmol/L)	0.80±0.06	1.00±0.15	0.087
α -amylase (U/dl)	575.63±8.04	532.26±15.10	0.125
Lipase (U/dl)	57.10±3.31	44.93±2.12	0.340
Pepsin (U/ml)	86.98±21.61	55.44±9.96	0.590
Cellulase (U/ml)	475.63±8.04	448.93±15.63	0.023

16S rRNA Diversity analysis: To understand the changes in rumen microbiota, alpha and beta diversity analyses of the rumen microbiota were conducted (Fig. 1). The results of the alpha diversity analysis indicated that the Chao1 index of the BF group was significantly higher than that of the GF group, and the number of operational taxonomic units OTUs was also significantly greater in the BF group compared to the GF group ($p \leq 0.05$). Although the Shannon index of the BF group was slightly lower than that of the GF group, this difference was not statistically significant (Fig. 1A). The PCoA results showed that PC1 explained 33.15% of the variance, while PC2 accounted for only 1.83%, indicating that the majority of the between group variation was captured primarily along the first principal coordinate. Despite the low variance explained by PC2, the samples from the two groups formed distinctly separated clusters along PC1, with the BF group exhibiting tighter clustering (Fig. 1B). PERMANOVA (Adonis) analysis based on Bray-Curtis distance matrices further confirmed that the observed separation between the BF and GF groups was statistically significant ($R^2 = 0.270$, $P = 0.007$). To further investigate the differences between the two groups, Venn diagram analysis revealed that 258 OTUs were shared between the BF and GF groups, whereas 129 OTUs were unique to the GF group and 63 OTUs were unique to the BF group (Fig. 1C). Notably, despite the GF group exhibiting more unique OTUs, its Chao1 index was significantly lower than that of the BF group, suggesting that the GF-unique OTUs were unevenly distributed across individuals as transient, low-abundance taxa acquired from the heterogeneous grazing environment, whereas the BF group maintained a more uniform and richer resident microbial community within each sample. These results suggest that BF system was associated with higher species richness and structural stability of the rumen microbial community, whereas GF system contribute to greater inter-individual variability in microbiota composition, possibly due to fluctuations in the foraging environment.

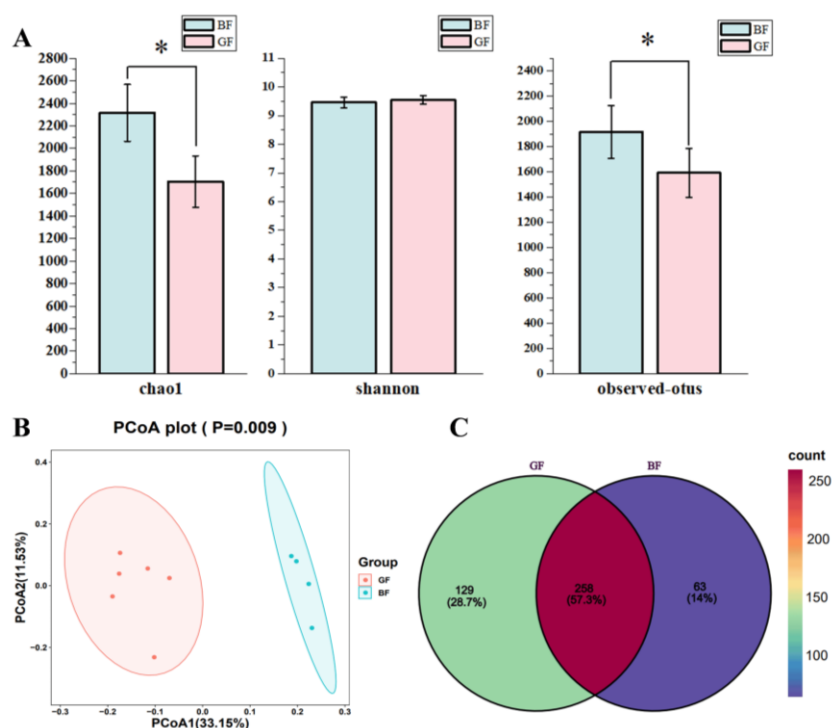
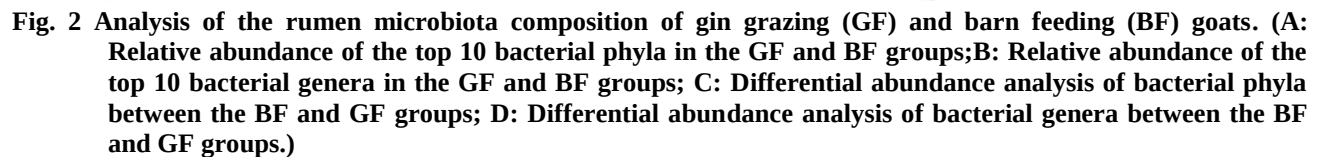


Fig. 1 Diversity analysis of the rumen microbial of in grazing (GF) and barn feeding (BF) goats (A: Chao1 index, Shannon index, and Observed index; Data are presented as mean $\pm$ SEM, Error bars represent the standard error of the mean (SEM). * $P \leq 0.05$, ** $P \leq 0.01$. B: PCoA analysis. C: Venn diagram).

Difference analysis of different feeding systems on rumen microbial composition of goats: At the phylum level, Firmicutes, Bacteroidota and Patescibacteria represented the highest bacterial percentages in the GF group, comprising 54.45%, 30.19% and 3.97%, respectively. In contrast, the highest phylum level abundances in the BF group were Firmicutes, Bacteroidota and Verrucomicrobiota, accounting for 37.89%, 41.49% and 7.08%, respectively. In the BF at the phylum level, the abundance of Bacteroidota and Verrucomicrobiota increased while Firmicutes and Patescibacteria were lower (Fig. 2A). Differential analysis of the abundance of the microbiota revealed significant differences in the abundance of a total of 11 species between the BF and GF at the phylum level, as shown in Fig. 2C, Compared to the BF group, Armatimonadota, Patescibacteria, Desulfobacterota, Planctomycetota, SAR32 clade (Marine group B), and Chloroflexi were significantly more abundant in the GF group ($P \leq 0.05$), whereas Fibrobacterota, Elusimicrobiota, unclassified Synergistota, and Cyanobacteria were significantly less abundant in the GF group ($P \leq 0.05$).

At the genus level, the groups with higher abundance in the GF group included the *Rikenellaceae RC9 gut group*, *Prevotella*, *Christensenellaceae R7 group*, and *Firmicutes*, which accounted for 7.39%, 5.17%, 5.18% and 4.95% respectively. In contrast, the relatively high abundance groups in the BF group were *Prevotella*, *F082 unclassified*, *Rikenellaceae RC9 gut group*, and *WCHB141 unclassified*, accounting for 9.98%, 9.93%, 7.66%, and 6.70%, respectively. Notably, *Prevotella*, *F082 unclassified*, *WCHB141 unclassified*, and *Rikenellaceae RC9 gut group* all had higher relative abundances in the BF than in the GF, although *Christensenellaceae R7 group* had considerably lower abundance (Fig. 2B). Genus level differential abundance analysis showed that, compared to the BF group, the GF group had significantly reduced abundance of *Bacteroides*, *Family XIII UCG001*, *Olsenella*, *Clostridiales unclassified*, and *Candidatus Saccharimonas*, while Bifidobacteriaceae, *Fretibacterium*, *Bacteroidota unclassified*, and *V9D2013 group et al.* abundance significantly increased ($P \leq 0.05$, Fig. 2D).



Effects of different feeding system on rumen microbial metabolic pathways and functions in goats: Utilizing the PICRUSt2 tool to analyze the functional profiles of the rumen microbial community, it was found at the KEGG2 functional classification level (Fig. 3A) that the GF group exhibited significantly higher proportions in the categories of amino acid metabolism and metabolism of cofactors and vitamins compared to the BF group ($P \leq 0.05$). In contrast, the BF group demonstrated a higher proportion of functions related to cell motility and metabolic diseases ($P \leq 0.05$). Further analysis at the KEGG3 functional classification level revealed that the GF group exhibited significantly higher average proportions in several metabolic pathways, including transcription factors, bacterial secretion systems, and carbon fixation pathways in prokaryotes, compared to the BF group ($P \leq 0.05$; Fig. 3B). These results suggest associations between management system and distinct biological functional differences, which may reflect differences in available dietary substrates and management conditions.

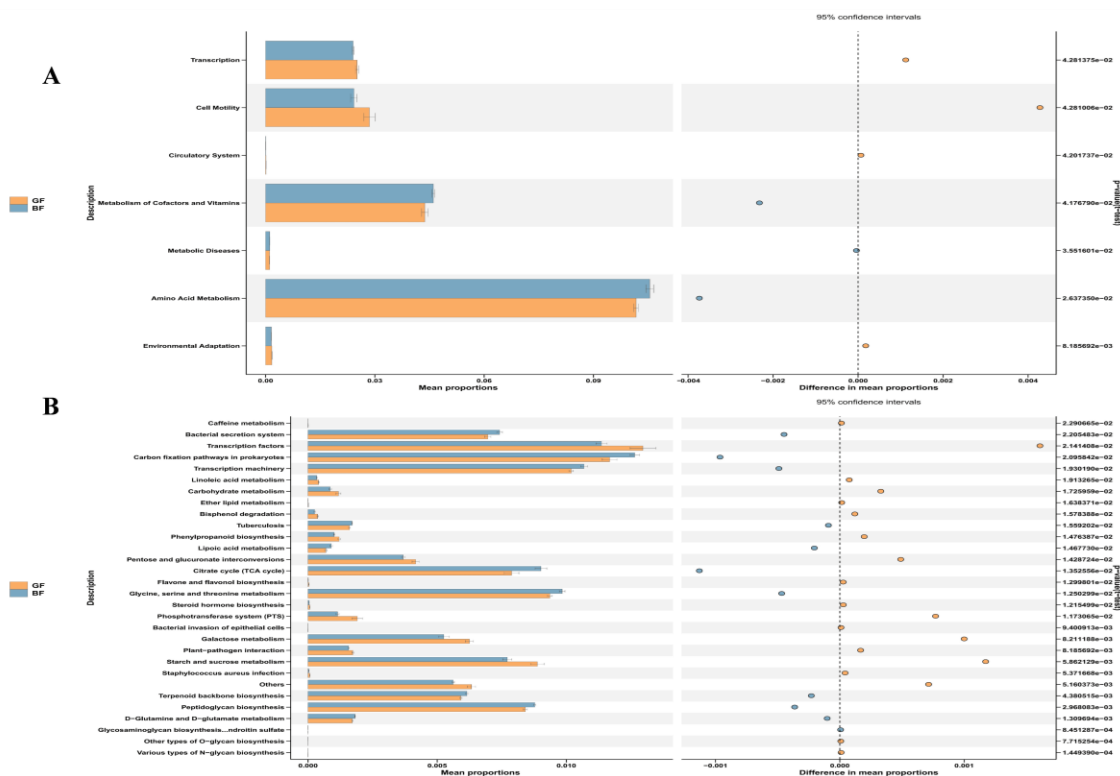


Fig. 3 KEGG metabolic pathway analysis of rumen microbiota in grazing (GF) and barn feeding (BF) goats (A: KEGG2 level; B: KEGG3 level)

DISCUSSION

The main feeding systems for goats are grazing on GF and BF. These two strategies differ in the composition of the diet, the physical structure of roughage and management methods, and thus may have a significant impact on the rumen microbiota of ruminants. The rumen is home to a variety of microorganisms, including bacteria, protozoa and fungi, which play a crucial role in the digestive physiology of ruminants (Qu et al., 2023; Weimer, 2015). Studies have shown that changes in feeding management and its related dietary composition have a more significant impact on the rumen microbiota than changes in feeding locations (Dande et al., 2015). Furthermore, studies have reported that restricted GF combined with supplementary feeding is linked to changes in gut microbiota composition and nutrient utilization patterns in sheep (Bolte et al., 2021; Jin et al., 2022). Consequently, this experiment employed high throughput sequencing of the 16S rRNA gene, alongside serum biochemical indicators and rumen enzyme activity, to conduct a comprehensive analysis of the rumen microbiota under two distinct feeding systems.

Serum biochemical indicators reflect the health status of animals and are influenced by factors such as environment, age, and feeding management (Li et al., 2022; Molefe et al., 2019; Vicari et al., 2008; Wu et al., 2022). In ruminants, urea is secreted via saliva or blood into the gastrointestinal tract, where it produce ammonia. Appropriate BUN decomposition coupled with increased urease activity can enhance protein utilization efficiency (Patra & Aschenbach, 2018). Previous studies reported higher serum TP and ALB in Tibetan sheep under GF than BF, with TP positively correlated with growth (Ji et al., 2024), but the direction depends on grazing environment and BF diet. In this study, BF group showed significantly higher TP and BUN than GF, contradicting previous findings. This discrepancy likely reflects combined effects of diet and feeding system: TMR provides synchronous nitrogen and energy, increasing total protein, while high protein load raises nitrogen metabolic burden, elevating BUN (Piao et al., 2012; Zurak et al., 2023). However, since GF consumed natural winter pasture with variable CP, while BF received formulated TMR with consistent nutrient density, these metabolic differences cannot be solely attributed to management. Furthermore, winter pasture CP decline limits ruminal ammonia and microbial activity in GF, resulting in lower TP and BUN (Dias et al., 2025). Elevated BUN in BF represents adaptive response to high-protein TMR, mediated by rumen microbes regulating nitrogen utilization efficiency (Zhi et al., 2025; Zhou et al., 2025a). TMR improves nitrogen efficiency and reduces ammonia accumulation, promoting microbial protein synthesis and increasing total protein, yet also raises BUN due to

high protein load (Piao et al., 2012; Zurak et al., 2023). In GF systems, ammonia nitrogen variability is greater; high-quality pasture reduces nitrogen efficiency, while winter low-quality pasture limits microbial protein synthesis, forcing reliance on endogenous urea recycling constrained by energy supply (Mahboobi et al., 2023; Nichols et al., 2022). The increase in blood urea nitrogen in the BF reflects a dual effect: the consistent availability of high-protein feed both promote liver protein synthesis and increases nitrogen metabolic burden, suggesting that dietary crude protein needs to be optimized to balance synthetic benefits and metabolic costs (Chen et al., 2024; Zeleke et al., 2025). TC can reflect the physiological and growth levels of animals, with serum concentration increases with the rise in dietary energy level, which can affect the host's liver function and protein metabolism (Wang et al., 2020). In a research report on Huanghuai sheep, BF and GF had no significant effect on serum GLB, TC, and TG (Zhao et al., 2023a). In this study, no significant differences were observed in GLU and TG levels between the two groups, but the serum total TC content of GF goats was significantly higher than that of the captive group. This may be related to the intake of phytosterols and polyunsaturated fatty acids in the pasture, which can exert regulatory effects by competitively inhibiting cholesterol absorption or promoting its metabolic pathways (Lv et al., 2023). The above results indicate that these distinct feeding systems, differing in nutritional profile, nutrient consistency, and forage sources, have selective effects on basal energy metabolism parameters.

In ruminant nutrition research, rumen enzyme activity serves as a critical indicator for evaluating feed utilization efficiency and microbial metabolic function. Its dynamic changes are significant for understanding the mechanisms of action of various feed additives (Jia et al., 2020). Wu et al (Wu et al., 2022). reported that feeding high concentration diets reduced the activities of digestive enzymes such as lipase, α -amylase, pepsin and cellulase in ruminants, leading to rumen acidosis. Recent research reports indicate that BF with TMR can shift the rumen metabolic pattern of Gangba sheep: characterized by increased activities of lipase and α -amylase, and decreased activity of cellulase, suggesting a shift in digestive focus from fiber breakdown to starch and lipid utilization, thereby enhancing energy acquisition efficiency (Xie et al., 2026). However, in this study, cellulase activity was significantly higher in the GF group than in the BF group, while α -amylase, lipase, and pepsin also showed an increasing trend, though without significant differences, consistent with the higher intact fiber content and physical structure of natural pasture in the GF system (Xie et al., 2026). These patterns likely reflect the combined effects of forage physical form and nutrient availability rather than management practice alone. This phenomenon may stem from microbial adaptive changes associated with increased roughage intake and intact fiber structure under GF system (Mizrahi et al., 2021; Zhao et al., 2023b). Specifically, the enhancement of cellulase activity was closely associated with the enrichment of rumen fiber degrading microbiota. For example, bacterial genera with cellulolytic functions, such as *Prevotella* 9 and *Faecalibacterium*, exhibited higher relative abundances in the GF (Wu et al., 2023), which is consistent with previous findings that high fiber diets promote the proliferation of fiber degrading microorganisms (Stewart et al., 2019; Wu et al., 2023). Notably, the enhancement of cellulase activity not only reflects differences in microbial metabolic capacity, but also suggests that the combination of intact forage structure and diverse pasture substrates in the GF system may have stimulated strategic adjustments in rumen microbial secretion of extracellular enzymes by altering the physical structure of forage (Mizrahi et al., 2021). Under GF conditions, goats consume intact forage with longer fiber length, rougher surface texture, and higher lignification, which could provide greater attachment surface area and ecological niches for fiber-degrading microorganisms such as *Prevotella* and *Rikenellaceae*, potentially enhancing microbial colonization stability and promoting substrate-induced cellulase complex secretion (Huang et al., 2021; Mizrahi et al., 2021). By contrast, Under BF system, while chopping forage can increase the substrate surface area, the shortened fiber length may compromise the colonization and stability of microbial communities, potentially inhibiting the sustained synthesis and secretion of cellulases (He et al., 2024; Huws et al., 2014). However, since this study primarily investigates the effects of the BF and GF feeding systems on NYG, further validation of this mechanism requires measurements of forage physical characteristics, microbial attachment dynamics, or enzyme secretion kinetics. No significant differences were observed in the activities of α -amylase, lipase, and pepsin, primarily attributable to similar macro-ingredients between the two groups, resulting in no substantial differences in substrate availability (Hassan et al., 2020; Xie et al., 2026). However, the physical form and nutrient consistency still differed. Additionally, after 12-h of fasting, soluble substrates were nearly depleted, causing the activities of α -amylase, lipase, and pepsin to decline to comparable baseline levels (Moharrery & Das, 2001; Wang & Mcallister, 2002). In summary, cellulase activity may serve as a useful indicator distinguishing these two feeding systems. However, this distinction likely reflects the combined effects of forage physical structure, nutrient availability, and microbial colonization stability, rather than management practice per se.

The stability of rumen microbiota is crucial for the health of ruminants and is influenced by diet and feeding system. Shah et al (Shah et al., 2022) found that GF yaks exhibit higher gut microbiota diversity compared to those fed in BF. Under BF conditions for Tibetan sheep, the Firmicutes/Bacteroidota ratio can be increased, while α -diversity decreases (Xie et al., 2026). In this study, the Firmicutes/Bacteroidota (F/B) ratio in the BF group significantly decreased from 1.80 to 0.91, potentially reflecting a shift in the gut microbiota structure toward a protein-metabolism-dominant

phenotype (Zhou et al., 2025b). This shift may be related to the higher and more stable protein supply in the TMR diet, although in the design of this study, the relative contributions of diet composition and management measures are difficult to completely distinguish. Bacteroidota, as a core functional bacteria phylum in the gastrointestinal tract of ruminants, plays a leading role in protein hydrolysis and peptide degradation. An increase in its relative abundance can enhance protein degradation and microbial protein synthesis (Fan et al., 2021; Zhou et al., 2025b). The transformation of this microbial community was consistent with the elevated levels of serum total protein (TP) and blood urea nitrogen (BUN) observed in the BF group, indicating that under BF conditions, the host nitrogen utilization efficiency was improved and the protein metabolic turnover rate was increased (He et al., 2024; Ren et al., 2025). In this study, the Chao1 index and the number of OTUs in the BF group were significantly higher than those in the GF group, suggesting that stable nutrient supply promoted an increase in microbial species richness (Li et al., 2025a; Sun et al., 2025). The β -diversity PCoA analysis further confirmed the significant separation of community structures between the two groups. The tighter clustering of BF group samples reflects the niche convergence effect under standardized management, while the dispersed distribution of GF group samples demonstrates the shaping role of GF environmental heterogeneity on the microbiome (Song et al., 2020). Firmicutes, *Bacteroidetes* and *Proteobacteria* are the main bacterial groups in ruminants, and their abundance is influenced by management environment, feeding system and feed composition. Under GF conditions, the relative abundance of Firmicutes is higher, and high energy feed promotes their growth and reproduction (Guo et al., 2008). In a study on Tibetan sheep, it was reported that the abundances of fiber-degrading bacteria such as *Prevotella*, *Rikenellaceae RC9 gut group*, *Butyrivibrio* and *Lachnospiraceae* significantly increased under GF conditions (Ji et al., 2024). In this study, both groups had Firmicutes and Bacteroidetes as the dominant phyla, with higher rumen bacterial diversity in the BF group, along with increased abundance of *Verrucomicrobia* and decreased abundance of *Patescibacteria*. These changes in the microbiota are associated with an adaptive reprogramming of host energy metabolism, though whether this is driven by diet composition, nutrient consistency or management practice per se requires further investigation. In other host systems, *Verrucomicrobiota* has been reported to stimulate intestinal mucus turnover and potentially increase the thickness of the intestinal mucus layer, which could contribute to the physical barrier against pathogen invasion and may be associated with reduced systemic inflammation (Montenegro et al., 2023). Likewise, acetic acid, propionic acid and butyric acid reportedly produced by *Verrucomicrobiota* fermentation have been suggested to regulate host energy metabolism by activating free fatty acid receptors and promoting fatty acid oxidation through activation of the AMPK signaling pathway (Bar-Shalom et al., 2023; Miao et al., 2023). In summary, the abundance of *Verrucomicrobiota* in the BF group significantly increased from 2.45% to 7.08%. This enrichment of the bacterial group may be associated with the improvement of intestinal barrier function, enhancement of mucosal integrity, and regulation of host energy metabolism (Bar-Shalom et al., 2023; Miao et al., 2023). However, the increased abundance of *Verrucomicrobiota* in the BF group is merely a compositional finding, and its functional significance has not been verified, requiring further multi-omics validation. The *Patescibacteria* group lacks complete biosynthetic pathways for amino acids, nucleotides, and lipids, and depends on obtaining essential nutrients from host bacteria or the environment, exhibiting obligate symbiotic or parasitic lifestyles (Brown et al., 2015; Castelle & Banfield, 2018). Under nutrient-limited conditions, such as those encountered in the GF system during winter, *Patescibacteria* may thrive by exploiting metabolic byproducts from other bacteria or by attaching to host cells to scavenge essential nutrients (Brown et al., 2015; Castelle & Banfield, 2018). In contrast, under the nutrient-rich and stable conditions of BF, Bacteroidota and Firmicutes can directly utilize abundant substrates and outcompete *Patescibacteria* for ecological niches (Huang et al., 2023). In this study, the abundance of *Patescibacteria* in the BF group decreased from 3.97% to 1.89%, indirectly reflecting an increase in nutrient availability in the BF group and a reduction in the host's dependence on metabolic intermediates. However, the functional consequences of these compositional changes on host metabolism and rumen health require further investigation. In the present experiment, compared with the GF, the BF group exhibited higher abundances of *Rikenellaceae RC9 gut group* and *Prevotella*, whereas the abundance of *Christensenellaceae R7 group* was lower. The enrichment of *Rikenellaceae RC9 gut group* in the BF group may modulate host lipid and glucose metabolism, thereby reduce visceral fat deposition and supporting healthy host metabolism (Tavella et al., 2021). Studies have demonstrated that *Rikenellaceae RC9 gut group* is negatively correlated with glucose metabolism parameters; its enrichment contributes to the maintenance of glucose homeostasis and may exert adiposity-modulating effects via the production of short-chain fatty acids, including acetate and propionate (Tavella et al., 2021; Teng et al., 2023). *Prevotella* is a dominant bacterial group in the rumen, renowned for its capacity to degrade proteins, peptides, and complex polysaccharides, and plays a pivotal role in pectin and protein metabolism (Precup & Vodnar, 2019; Shah et al., 2022). Through the secretion of diverse glycoside hydrolases and proteases, *Prevotella* efficiently utilizes both fibrous and non-fibrous polysaccharides in the diet, thereby enhancing feed digestibility (Precup & Vodnar, 2019; Shah et al., 2022). In this study, the abundances of microbiota such as *Prevotella* and *Rikenellaceae RC9 gut group* were increased in the BF group, and synergistically and significantly elevated serum TP and BUN levels. It can be inferred that under the BF feeding system, host nitrogen assimilation and protein metabolic turnover were enhanced via the

microbe-metabolite axis. While the *Christensenellaceae* R7 group has been associated with host metabolic regulation and intestinal homeostasis (Gong et al., 2020). *Christensenellaceae* R7 group participates in host amino acid, peptide, and lipid metabolism, maintains intestinal barrier function through the production of short-chain fatty acids such as acetate and butyrate, and is positively correlated with skeletal muscle mass index while negatively correlated with visceral adipose tissue content, exerting a protective role in promoting lean body mass and reducing abdominal fat accumulation (Jiang et al., 2023; Pan et al., 2023). In this study, since BF outperformed GF in terms of nutrient supply, substrate availability, and environmental stability, this may be an important reason for promoting the growth of beneficial bacteria in BF. However, given the confounding factors present in the forage species, physical form, nutritional consistency, and management practices in this study, these patterns cannot be attributed to any single factor. Their specific functional significance for host metabolism remains speculative and needs to be validated through metagenomic or metabolomic analyses.

PICRUSt2-predicted functional profiles suggest putative changes in rumen microbial pathways may reflect functional shifts under specific conditions, and these inferred differences between these two feeding systems could alter rumen microbial KEGG pathways (Ge et al., 2023). Such predicted differences likely reflect the distinct dietary substrates available in natural winter pasture versus TMR, rather than management practice alone. PICRUSt2-predicted KEGG pathway analysis indicated a tendency toward higher predicted proportions of amino acid metabolism and cofactor/vitamin metabolism pathways in the GF group compared to the BF group. This pattern is potentially associated with the diversified plant resources ingested by animals under the GF system. Nevertheless, these functional predictions should be interpreted cautiously, as they may primarily reflect dietary substrate differences rather than management effects per se. For example, GF animals might activate the phenylpropanoid metabolic pathway by consuming natural vegetation rich in polyphenolic compounds, which could promote the synthesis of amino acid derivatives (Bešlo et al., 2023; Li et al., 2025b; Ninkuu et al., 2025). These predicted functional differences are provisional and need to be verified through metagenomic or metabolomics methods. Furthermore, the prediction results suggested that the GF group exhibited a putatively higher degree of enrichment in pathways related to cell motility and metabolic diseases. These predictions may reflect the adaptive adjustments made to the variable foraging environment and diverse plant resources during GF. However, their direct physiological consequences remain to be verified experimentally (Douglas et al., 2020; Sen & Orešič, 2019). It should be noted, however, that the above findings are based on PICRUSt2 predictions of 16S rRNA data rather than directly determined metabolic activities, and their reliability is limited by the coverage of the database and the classification resolution. Consequently, these results should be regarded as functional hypotheses to be experimentally verified. Future research should integrate multi-omics technologies and controlled dietary interventions to further clarify the molecular mechanisms of host-microbial interactions under different feeding systems, thereby promoting the sustainable development of precision animal husbandry.

Conclusions: In summary, different feeding regimes can significantly influence the health status, rumen enzyme activity, and rumen microbial community of goats. The results indicate that under BF conditions, the structural diversity, stability, and metabolic pathways of the rumen microbiota were improved, with significant increases in the abundance of beneficial genera such as *Prevotella* and *Rikenellaceae* RC9 gut group. Additionally, serum total protein and blood urea nitrogen levels were significantly elevated in the BF group, suggesting enhanced nitrogen utilization efficiency and increased protein metabolic turnover. These differences may arise from the combined effects of diet composition, nutritional consistency, physical structure of roughage, and management practices, rather than the feeding regime itself. These findings indicate that BF may confer advantages in terms of rumen microbial health, particularly during the winter period when pasture quality and quantity decline.

The present study has several limitations. First, individual feed intake was not quantified in the GF group due to free GF, and the BF group received TMR with potentially different nutrient profiles; thus, the observed differences likely reflect combined effects of feeding system and diet composition rather than feeding system alone. Second, KEGG functional predictions by PICRUSt2 are based on phylogenetic inference rather than metagenomic or transcriptomics sequencing; therefore, the observed pathway differences should be interpreted as putative functional potentials rather than definitive evidence of differential metabolic activity. Future studies should integrate marker based methods for quantifying individual DMI under GF conditions with shotgun metagenomic and transcriptomics analyses to validate the predicted functions and elucidate the actual transcriptional responses of rumen microbiota to different feeding strategies.

Data Availability Statement: All data included in this study are available upon request by contact with the corresponding author. The datasets generated for this study can be found in the NCBI Sequence Read Archive under BioProject PRJNA1019472. The data are publicly accessible at <https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1019472>

Author contribution: HL and CP were responsible for writing and revising the first draft and were involved in sample collection and processing. YZ and HL were responsible for Conceptualization and design of experiments. YZ and HL were responsible for project data processing and thesis revision. WZ, TS and YZ are responsible for project management and fund preparation. All authors have read and agreed to the final manuscript.

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Ethics approval: Animal care and experiments were carried out in accordance with the criteria set by the Chinese Guidelines for the Care and Use of Laboratory Animals, and all methodologies were supervised and authorized by the Southwest University of Science and Technology's Institutional Review Board (Approval No. L2022026-2022-03).

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