Original Article

Survey and analysis of rumen microbiota in yaks at different growth stages

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Abstract

This study investigated changes in the richness and composition of the rumen microbiome in yaks at various developmental stages. Eighteen healthy male yaks at the ages of 5 months (M5), 12 months (M12), and 24 months (M24) had samples of their rumen digesta taken for 16S rRNA gene sequencing. The 11087 amplicon sequence variations (ASVs) in total were found; the M5, M12, and M24 groups had 3585, 1146 and 5545 distinct ASVs, respectively. Alpha diversity indices Chao, Shannon, Sobs, and ace indicated significant differences among the groups. The principal coordinate analysis (PCoA) indicated significant changes in microbial community composition across age stages. Firmicutes and Bacteroidetes were the two most prevalent phyla across all groups at the phylum level; Firmicutes had the highest relative abundance in the M5 group, which declined significantly with age. In contrast, Spirochaetota and Proteobacteria were relatively more abundant in the M24 group. At the genus level, there was a notable increase in Prevotella in the M12 and M24 groups, whereas UCG-005 continued to dominate in the M5 group. Microbial network analysis indicated increasing complexity of microbial interactions from M5 to M12, while the network became more dispersed in the M24 group. LEfSe analysis identified 65 microbial taxa with significant differences. This work provides new insights into the successional changes in yak rumen microbiota and their potential impact on digestive efficiency across developmental stages.

Keywords: microbial communities, rumen, yak

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Introduction

Yaks, indigenous to high-altitude regions such as the Himalayas, are highly adapted to harsh environments and possess unique genetic characteristics (Long *et al*., 2008). China has roughly 16 million yaks, accounting for more than 95% of the total global population (Huang *et al*., 2022). These yaks are mostly spread in Tibet, Qinghai, Gansu, and Sichuan, where they play an important role in the productivity and subsistence of local herders (Guo *et al*., 2015). Similar to other ruminants, yaks have developed a highly efficient foregut fermentation system. However, unlike other ruminants, yaks inhabit more extreme environments with limited and nutritionally poor feed sources. Despite these challenges, environmental stress has driven the evolution of more efficient feed utilization and a more advanced rumen fermentation function in yaks, providing essential physiological support for their survival (Zhao *et al*., 2022; Liu *et al*., 2019).

Rumen serves as the primary nutritional source in the digestive tract of ruminants, fulfilling multiple roles in protection, immunity, development, and nutrient provision (Jiao *et al*., 2015; Guo *et al*., 2020). As an efficient and orderly "fermentation tank," the rumen converts low-quality fibrous plants into high-quality meat and milk, primarily through the action of its complex and diverse microbiota (Guo, Zhou *et al*., 2020). These microorganisms produce enzymes necessary for feed fermentation and, through synergistic interactions, convert plant-based feed into volatile fatty acids (VFAs), microbial cell protein (MCP), amino acids, and vitamins (Jami *et al*., 2013). These fermentation products provide the host with essential energy and nutrients to support healthy growth. As a result, the formation and stability of rumen microecology are critical to maintaining ruminant growth and reproduction. An imbalance in the rumen microecology can lead to decreased growth and reproductive performance and may even trigger a series of nutritional metabolic diseases, resulting in significant losses in ruminant production. The ruminant stomach is divided into four compartments according to the sequence of feed passage: the rumen, reticulum, omasum, and abomasum. A variety of microorganisms, including bacteria, fungi, archaea, and protozoa, are contained in the rumen. These microorganisms utilize fibrous plant materials and serve as the primary energy source for ruminants (Liang *et al*., 2024). Among these microorganisms, various relationships, such as predation, antagonism, symbiosis, and competition, exist, forming a symbiotic system with the host that maintains the balance and stability of the rumen environment. Ruminants provide a suitable habitat for the growth and reproduction of these microorganisms, while the microorganisms, in turn, supply the host with energy, proteins, fatty acids, and vitamins, creating a dynamic symbiotic balance (Pan *et al*., 2021; Baker *et al*., 2022).

There is currently little research on the succession patterns of rumen bacteria in grazing yaks of varying ages exposed to severe natural environments and nutritional stress throughout time. During natural selection and evolution, yaks have developed mechanisms to regulate their adaptation to the cold stress of the Qinghai-Tibet Plateau through the succession and functional changes in their rumen and intestinal microbiota (Fan *et al*., 2020). In-depth research into the sources, colonization, succession, and driving factors of rumen microbial communities in yaks at various developmental stages within the same environment can reveal how yaks adapt to cold stress through dynamic changes in the structure and function of their unique rumen and intestinal microbiota (Li *et al*., 2022). This study enhances the understanding of yak rumen microbiota while providing valuable insights into breeding herbivorous livestock varieties with high feed conversion rates and low methane emissions by investigating their effects on digestive efficiency at various developmental stages. Additionally, it offers theoretical insights into nutritional regulation during the early life stages of yaks.

Materials and Methods

All animal procedures for experiments were approved by the Committee of Experimental Animal Care and Handling Techniques were approved (QUA-2020-5572) by the Qinghai University of Animal Care Committee.

Animal and Sample Collection: In this study, 18 healthy male yaks aged 5 months (M5), 12 months (M12), and 24 months (M24) (n=6) were selected from Meilongzhang Farm in Haibei Tibetan Autonomous Prefecture, Qinghai Province, China. This area is located in the western part of Qilian County, with an altitude of 3500 meters. The average annual temperature here is -2.8 °C, and the average annual precipitation is 280.5 millimeters. This region is mainly characterized by alpine meadows and grasslands. It has abundant water sources and lush pastures. The yaks on this farm are weaned at the age of 3 months. The experimental animals of the three groups were separately grouped and grazed freely with the large herd, allowing them to feed ad libitum. In the morning before grazing, rumen fluid was collected using a sampling device that was inserted into the rumen through the mouth and esophagus. Approximately 15 mL of rumen fluid was collected from each experimental yak, filtered through four layers of gauze, and kept in a refrigerator at -80°C for future examination.

16S rRNA Gene Amplification and Sequencing: After the rumen fluid was thawed at room temperature, the total microbial genomic DNA was extracted from the rumen fluid using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.). The quality and concentration of DNA were determined by 1.0% agarose gel electrophoresis and a NanoDrop2000 spectrophotometer (Thermo Scientific, United States) and kept at -80°C prior to further use. The hypervariable region V3-V4 of the bacterial 16S rRNA gene was amplified with primer pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R(5'-GGACTACHVGGGTWTCTAAT-3') by T100 Thermal Cycler PCR thermocycler (BIO-RAD, USA). The PCR

reaction mixture including 12.5 µL Phusion Hot start flex 2X Master Mix, 0.8 µL each primer (5 µM), 50 ng of template DNA, and ddH2O to a final volume of 25 µL. PCR amplification cycling conditions were as follows: initial denaturation at 98°C for 3 min, followed by 35 cycles of denaturing at 98°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 45 s, and single extension at 72°C for 10 min, and end at 4°C. The PCR product was extracted from 2% agarose gel and purified using the PCR Clean-Up Kit (YuHua, Shanghai, China) according to the manufacturer's instructions and quantified using Qubit 4.0 (Thermo Fisher Scientific, USA).

Statistics and analysis of data: After demultiplexing, the sequences underwent quality filtering using fastp (v0.19.6) and were then merged with FLASH (v1.2.11). The high-quality sequences were denoised through the DADA2 plugin in Qiime2 (version 2020.2) with default settings, achieving single-nucleotide resolution based on sample error profiles. The resulting denoised sequences, termed amplicon sequence variants (ASVs), were classified taxonomically using the Naive Bayes classifier in Qiime2, referencing the SILVA 16S rRNA database (v138). Alpha diversity indices, such as Chao1 and Shannon, were computed via Mothur software, and inter-group differences in alpha diversity were assessed using the Wilcoxon rank-sum test. Principal Coordinate Analysis (PCoA) based on Bray-Curtis distance was applied to explore similarities in microbial community structure across samples. Additionally, the significance of microbial community structure differences among groups was evaluated using PERMANOVA (Permutational Multivariate Analysis of Variance). LEfSe (Linear Discriminant Analysis Effect Size) analysis (http://huttenhower.sph.harvard.edu/LEfSe) (LDA > 3.5, $p < 0.05$) was used to identify bacterial taxa with significantly different abundances across taxonomic levels, from phylum to genus. To analyze correlations among dominant taxa, microbial networks were constructed using Gephi software (version 0.9.1)

Results

Richness, diversity estimates, and rumen bacteria composition: Figure 1A indicates that a total of 11,087 ASVs were identified in this experiment, with 3,585, 1,146, and 5,545 distinct ASVs discovered in the M5, M12, and M24 groups, respectively. Principal component analysis (PCoA) was performed on the bacteria in each group, with a 95% confidence interval, revealing significant compositional differences in the rumen microbiota of yaks at different ages (Figure 1B). Furthermore, alpha diversity metrics (Figure 2) indicate significant variation in the Chao, Shannon, Sobs, and ace indices across the groups.

Bacterial composition among groups: As depicted in Figure 3, the phylum-level abundance rankings across the three age groups were as follows: Firmicutes > Bacteroidetes > Spirochaetota > Proteobacteria > Patescibacteria. The relative abundances of Firmicutes were 58.13%, 55.73%, and 48.80% in the M5, M12, and M24 groups, respectively (Figure 3A). The genera level abundance ranking for the three age groups was Rikenellaceae_RC9_gut_group > Prevotella > norank_f__F082 > Christensenellaceae_R-7_group > UCG-005 Rikenellaceae_RC9_gut_group was 12.74%, 13.80%, and 12.44% in the M5, M12, and M24 groups, respectively (Figure 3B). Notably, the M5 group exhibited a significantly higher relative abundance of Firmicutes compared to the M12 and M24 groups. Conversely, the M24 group displayed a markedly higher relative abundance of Spirochaetota and Proteobacteria compared to the M5 and M12 groups (Figure 3C). In the M5 group, the dominant bacterial taxa were UCG-005 (12.86%) and Rikenellaceae_RC9_gut_group (12.74%), with norank_f__Muribaculaceae (7.15%) and Christensenellaceae_R-7_group (5.45%) also representing a significant proportion. In the M12 group, the primary genera were Prevotella (14.02%) and Rikenellaceae_RC9_gut_group (13.80%), with Succiniclasticum (8.47%), Christensenellaceae_R-7_group (5.82%), and norank_f__F082 (5.66%) also showing substantial relative abundance. In the M24 group, Rikenellaceae_RC9_gut_group (12.44%) and Prevotella (10.27%) were the most abundant genera, with norank_f__F082 accounting for 9.79%, and Christensenellaceae_R-7_group and Succiniclasticum contributing 4.43% and 3.40%, respectively. The relative abundance of bacterial genera varied significantly among different age groups (Figure 3D). Prevotella was significantly more abundant in the M12 and M24 groups compared to the M5 group, while UCG-005 showed significantly higher abundance in the M5 group than in the M12 and M24 groups. Moreover, norank_f__F082 was significantly more abundant in the M24 group compared to the M5 and M12 groups, and Succiniclasticum was significantly more abundant in the M12 group. To further investigate the bacterial taxa with differential abundances, LEfSe software was used to identify the distinct bacterial groups across the three age groups. As shown in Figure 4, a total of 65 differential microorganisms were identified at the phylum to genus levels ($p < 0.05$, LDA > 3.5).

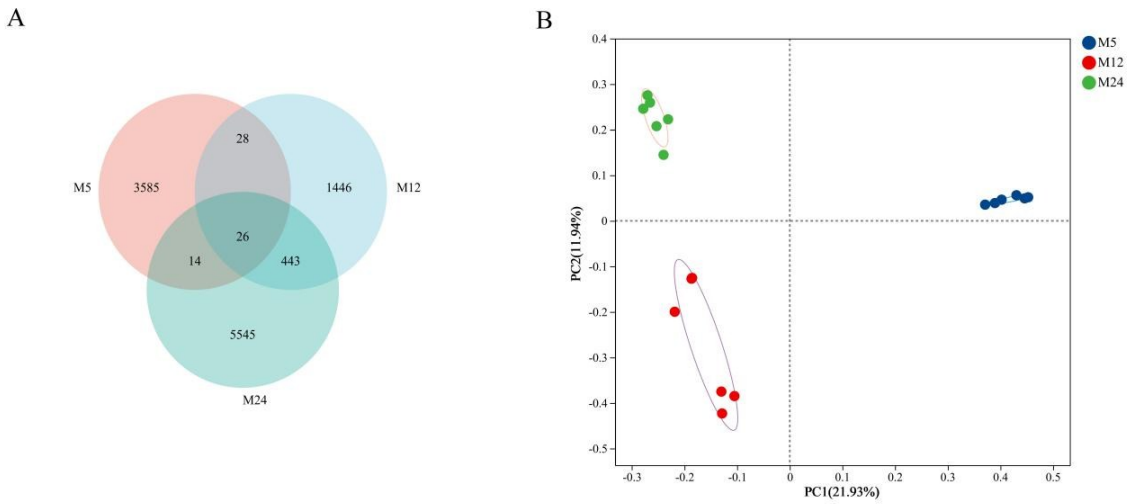

Figure 1 (A) Venn diagram of shared and unique ASVs in yaks of different age groups; (B) PCoA analysis of rumen microbiota differences in yaks from different age groups.

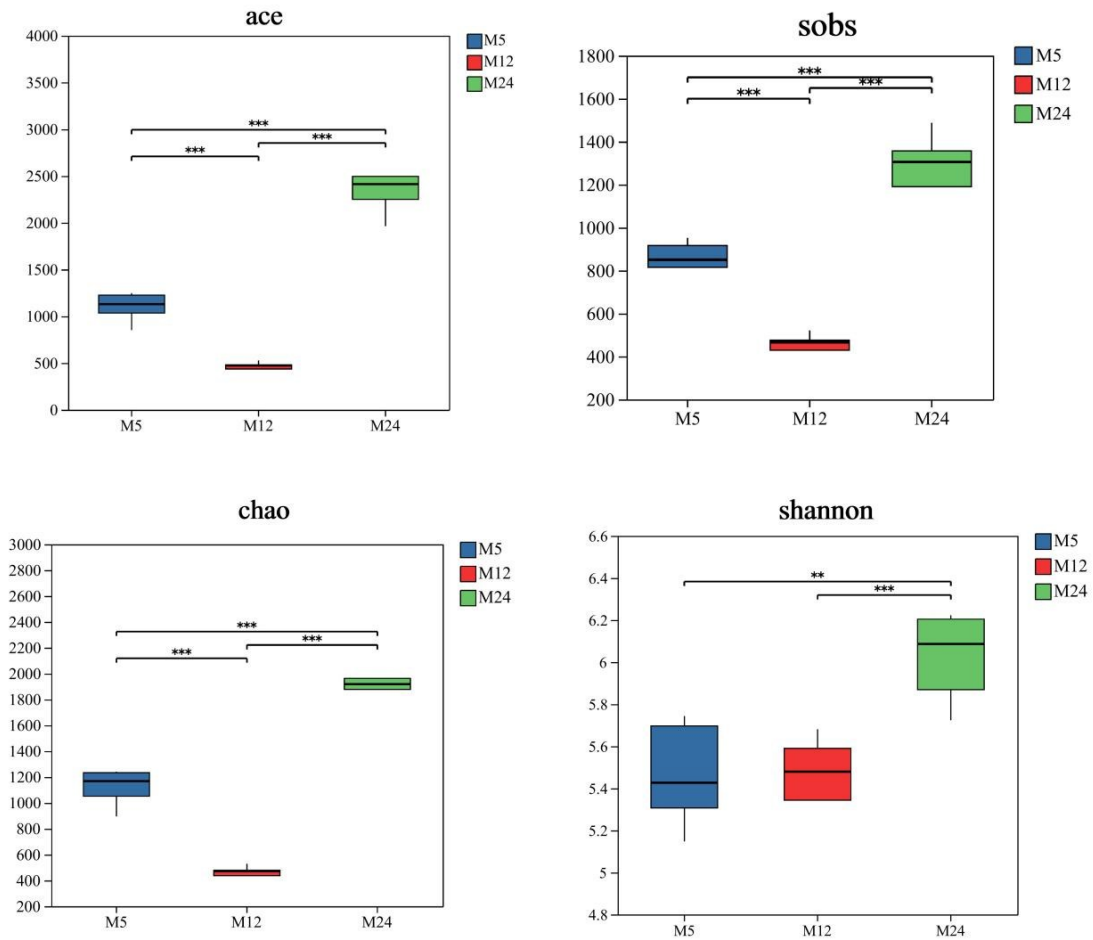

Figure 2 Diversity of rumen microbiota in yaks from different age groups. * indicates significant differences ($p < 0.05$), ** indicates highly significant differences ($p < 0.01$), and *** indicates extremely significant differences ($p < 0.001$).

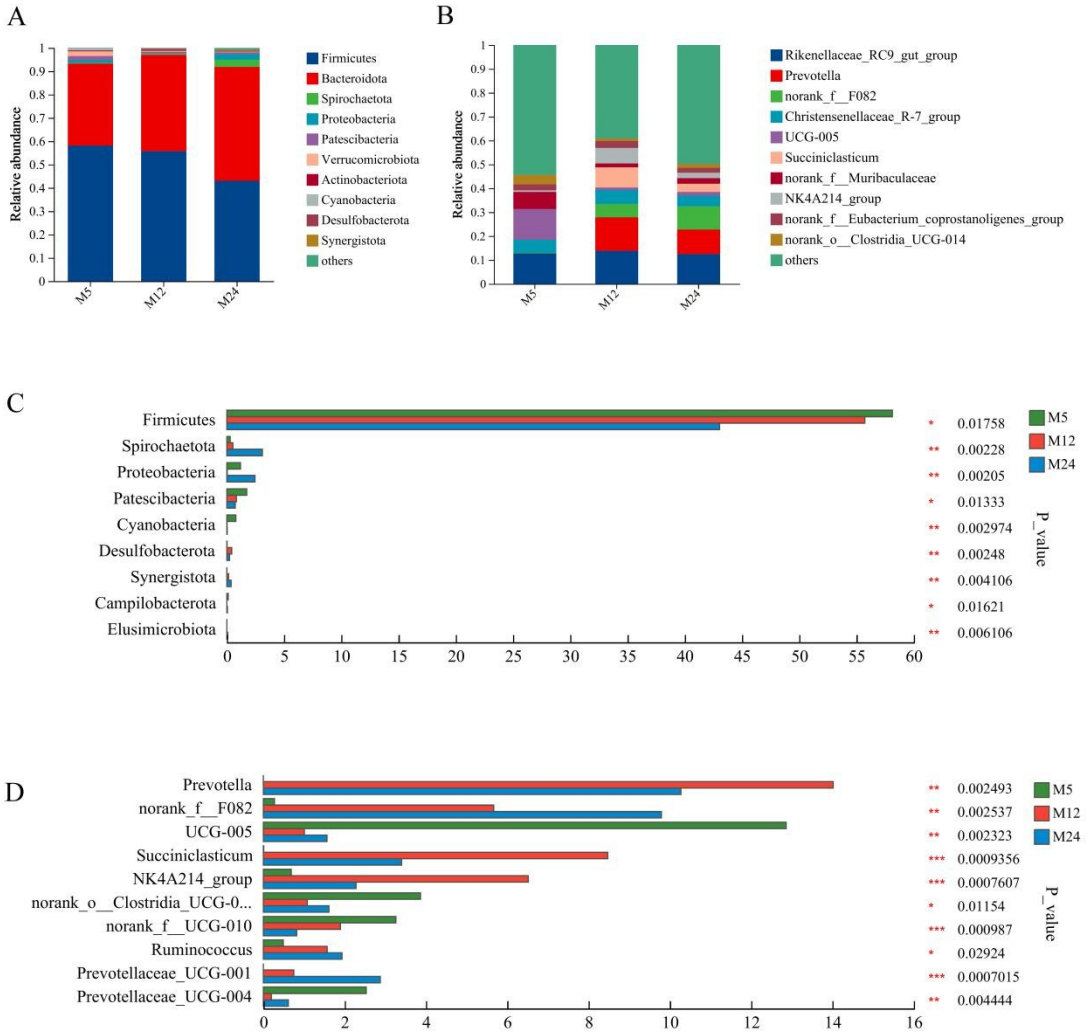

Figure 3 Composition of rumen bacterial communities in yaks of different age groups. (A) Phylum-level classification. (B) Genus-level classification. (C) Differences in bacterial composition between groups at the phylum level. (D) Differences in bacterial composition between groups at the genus level.

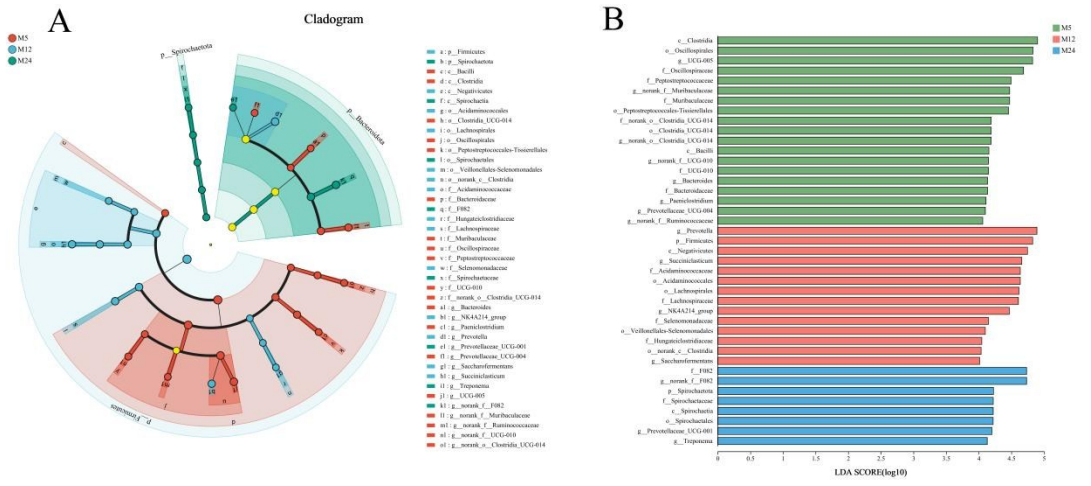

Figure 4 LEfSe analysis of rumen microbiota in yaks of different age groups. (A) Histogram of linear discriminant analysis (LDA) scores based on categorical information. (B) Linear discriminant analysis effect size (LEfSe) classification plot based on categorical information.

Rumen bacteria correlation analysis: Microbial networks were employed to analyze the interactions among bacterial communities in the yak rumen (Figure 5). In the M5 group, Firmicutes and Bacteroidota dominate, displaying a relatively complex microbial community network with extensive associations with several other phyla, such as Proteobacteria and Actinobacteriota. Firmicutes and Bacteroidota

continue to dominate the microbial community structure in the M24 group, but network interactions with other groups are sparse, and the community network structure is more dispersed than in the M5 group. Firmicute remains the main microbial group in the M12 group, with a stronger association with Patescibacteria and Proteobacteria.

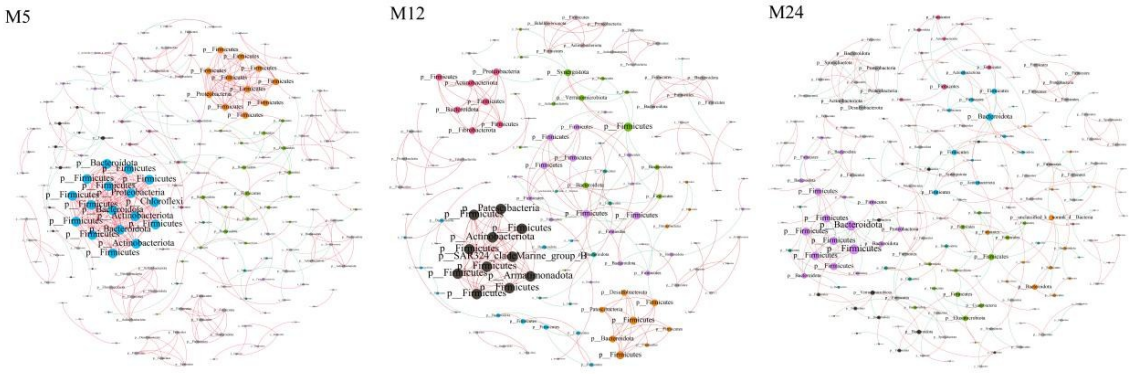

Figure 5 Interaction network of the yak rumen microbiota across different age groups. The size of the nodes is proportional to the abundance of the taxa, with red lines indicating positive correlations and green lines representing negative correlations.

Discussion

A diverse microbial community inhabits the rumen of ruminants, playing a critical role in fermenting and digesting nutrients, enhancing the host's resistance to external stress, and promoting growth and development (Ahmad *et al*., 2020). Bacterial populations are the most significant microbial group in the rumen, and they usually maintain a condition of dynamic equilibrium. However, this equilibrium may be regulated and influenced by various factors, including dietary composition, the physiological age of the animal, and the use of feed additives (Ahmad *et al*., 2022; Stefańska *et al*., 2021; Jayanegara *et al*., 2020). Significant changes in the Chao, Shannon, Sobs, and ace indices were seen between groups in this experiment, suggesting that microbial diversity evolves in a complicated manner with increasing age. The M12 group, in particular, exhibited a marked increase in microbial diversity, likely due to the dietary shift from milk to more fibrous and complex feed at this developmental stage. This shift enriched the microbial environment, fostering greater diversity. The pronounced separation between groups in the PCoA plot further highlights substantial variations in microbial community composition across age groups. As yaks matured from juveniles to adults, their rumen microbial communities transitioned from relatively simple to more complex ecosystems. This progression reflects not only the adaptation of microbial communities to diverse feed sources but also the gradual functional maturation of the rumen across developmental stages.

This investigation elucidated the colonization process of rumen bacteria in yaks, as well as the maturation phase of several rumen microbiomes. Firmicutes and Bacteroidetes are the major components of the rumen microbiota in all age groups, consistent with previous study findings (Ahmad *et al*., 2022; Furman *et al*., 2020; Sha *et al*., 2020). Firmicutes had a considerably greater relative abundance in the M5 group (58.13%) compared to the M12 and M24 groups. Bacteria within the phylum Firmicutes typically possess the ability to break down starch and simple carbohydrates (Sun *et al*., 2023). The increased abundance of Firmicutes in the M5 group might be attributed to the milk-based diet that yaks consume throughout their early lives. According to studies, the phylum Spirochaetes is strongly connected with the fermentation of complex carbohydrates (Fernando *et al*., 2010). Proteobacteria play an important part in rumen metabolism, including the breakdown of complex organic molecules and glucose fermentation. Some bacteria within this phylum are involved in the degradation of proteins, lipids, and other nutrients, contributing to the rumen's ability to adapt to different diets (Cendron *et al*., 2020, Mtshali *et al*., 2022). In the M12 and M24 groups, there were significant differences in the abundance of Spirochaetes and Proteobacteria, indicating that as yaks gradually consumed more fibrous feed, the rumen microbiota adapted to more complex carbon sources.

At the genus level, UCG-005 and Rikenellaceae_RC9_gut_group dominated the bacterial community in the M5 group, accounting for 12.86% and 12.74% of relative abundance, respectively. In M12 group, the relative abundance of Prevotella increased to 14.02%, becoming one of the predominant genera. Concurrently, the relative abundance of Succiniclasticum also significantly rose. Previous research has indicated that Prevotella can effectively degrade carbohydrates and proteins (Shah *et al*., 2015). Succiniclasticum predominantly converts succinate to propionate, a crucial stage in ruminant energy metabolism (van Gylswyk, 1995). The increased prevalence of these taxa indicates that the yak's diet has a larger proportion of complex polysaccharides and proteins at this stage, which corresponds to the progressive introduction of fibrous feed into the yak diet. This indicates the onset of maturation of the rumen metabolic function in yaks. In the M24 group, the relative abundance of the genus norank_f__F082 with in the phylum Bacteroidetes reached 9.79%. Research conducted by (Ma *et al*., 2024). revealed that norank_f__F082 is an unclassified genus with in the Bacteroidetes phylum, which is widely distributed in

the rumen and primarily involved in carbohydrate degradation. These findings suggest that yaks at 24 months of age exhibit enhanced carbohydrate digestion capabilities.

Studies have shown that rumen microorganisms in ruminants synergistically convert indigestible plant fibers into nutrients that the host can absorb (Keum *et al*., 2024). The rumen microbial network analysis might identify positive and negative interactions between species (Pang *et al*., 2022). Negative contacts can exacerbate competitive connections, whilst good interactions can improve cooperation and competition among organisms. In this experiment, the microbial network in the M5 group was relatively complex, with Firmicutes and Bacteroidetes dominating and showing extensive interactions with other phyla, such as Proteobacteria and Actinobacteria. This may be due to the limited nutritional sources for young yaks, requiring close cooperation among microbial communities to maximize nutrient utilization. As the yaks aged, the microbial community network in the M12 group became more complex, indicating tighter interactions between different phyla. The enhanced interaction between Firmicutes and Bacteroidetes suggests that the microbial community at this stage had adapted to a more complex diet. In the M24 group, although Firmicutes and Bacteroidetes still dominated, the microbial network became more dispersed, with overall connectivity decreasing. We hypothesize that this shift in community structure reflects the functional maturity of the bacterial community in older yak rumens, where particular bacterial populations dominate ecological niches, reducing reliance on other communities. A more dispersed structure may help optimize energy utilization and improve fiber digestion efficiency.

The microbial community evolved from a simple structure in early stages to a more complex and diversified system. Firmicutes were first dominant, but Prevotella, Spirochaetes, and Proteobacteria became more common as time passed. Microbial network analysis showed that the interaction network became more dispersed and functionally specialized as yaks matured, which contributed to enhanced digestive efficiency and nutrient utilization at different life stages. This study provides a scientific basis for optimizing feeding strategies tailored to the growth stages of yaks.

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Conflicts of interest: There were no conflicts of interest that may have biased the work reported in this study.

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