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Comparative Analysis of Rumen Fermentation and Microbial Communities in Yaks of Different Ages

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ABSTRACT

Background: This study investigates the differences in rumen fermentation parameters and microbial communities in yaks aged 3 (MarG), 4 (AprG) and 5 months (MayG). As crucial livestock on the Qinghai-Tibet Plateau, optimizing yak health and productivity relies on understanding their rumen microbial structure and fermentation efficiency. These findings provide insights into their adaptation to the plateau environment and support strategies for improving livestock management.

Methods: The study analyzed rumen fermentation parameters (NH3-N, acetate, propionate, isobutyrate, butyrate, valerate) and microbial composition in three age groups of yak calves. Microbial richness and composition were assessed, with network and correlation analyses identifying key microbial phyla associated with fermentation. Functional predictions explored gene abundances related to transcription, viral infection, cancer types and the immune system across the groups.

Result: NH3-N and acetate levels were significantly higher in the MarG group compared to AprG and MayG ($p<0.05$), while MayG showed elevated propionate, isobutyrate, butyrate and valerate levels ($p<0.05$). Microbial composition varied, with Firmicutes and Actinobacteriota dominating MarG, Christensenellaceae and Oscillospiraceae in AprG and Desulfobacterota in MayG. Network analysis identified Proteobacteria as central in MarG, Firmicutes in AprG and both in MayG, forming the most complex network. UCG-005 showed strong positive correlations with all fermentation parameters. Functional predictions indicated higher cell motility in MarG and more immune-related genes in MayG.

Key words: Different ages, Microbial communities, Rumen fermentation, Yak.

INTRODUCTION

The yak is a unique livestock species native to the high-altitude pastoral regions of the Qinghai-Tibet Plateau (Huang *et al.,* 2022; Peng *et al.,* 2020). It is also the sole member of the genus Bos, capable of surviving in extreme environments characterized by high altitudes and low oxygen levels (Zhang *et al.,* 2022). Through prolonged natural selection and evolution, yaks have developed a distinctive rumen micro-ecosystem and robust fiber degradation capabilities, resulting in lower methane emissions and urine nitrogen content. Consequently, yaks are considered an important genetic resource for breeding "low-carbon, nitrogen-efficient" environmentally friendly livestock on the Qinghai-Tibet Plateau and also represent a rich "microbial resource repository (Zhang *et al.,* 2020)."

Rumen development presents a significant physiological challenge for young ruminants (Jiao *et al.,* 2015). Their rumens are underdeveloped and lack the ability to ferment and digest coarse fibers. During this period, the abomasum and small intestine are primarily relied upon for digesting and absorbing glucose and other nutrients for energy (Guo *et al.,* 2020). To quickly compensate for growth losses and effectively utilize forage-based diets, the development of the rumen and the establishment of its microbial community are crucial (Deng *et al.,* 2019). It has been found that rumen tissue morphology and metabolic functions gradually develop from 14 to 42 days, while microbial colonization occurs most rapidly from birth to 28 days (Jiao *et al.,* 2015). A developed rumen not only ensures efficient digestion and absorption of feed nutrients but also forms a vital foundation for optimal ruminant health and production (Zhang *et al.,* 2016). Recent studies using rumen metagenomics and host transcriptomics have shown that the ability of yaks to adapt to the harsh environment of the Qinghai-Tibet Plateau and long-term nutritional stress may be related to the substantial enrichment of microbial functional genes involved in volatile fatty acid (VFA) fermentation pathways

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in their rumens (Brulc *et al.,* 2009; Ishaq and Wright, 2012). Additionally, it has been reported that the presence and absorption of VFAs not only promote the metabolic functions of rumen epithelial tissues, serving as a key stimulus for their development, but also suggest that the establishment and activity of rumen-associated microbes may influence this development (Dill-McFarland *et al.,* 2017; Jami *et al.,* 2013; Rey *et al.,* 2014).

Recent awareness has underscored the importance of early microbial colonization and its impact on animal productivity and health throughout their lifetimes (Ye *et al.,* 2022). Studies have shown that the rumen bacterial community begins to form before the intake of solid food and that its composition changes with age (Dill-McFarland *et al.,* 2019; Yáñez-Ruiz *et al.,* 2015). Early intervention studies on rumen microbial communities have found that the intake of solid feed is a key turning point for microbial colonization (Rey *et al.,* 2014). Substantial evidence indicates that anaerobic microorganisms colonize the rumen at an early stage. However, few studies have compared the colonization patterns of early-stage microorganisms with factors influencing microbial community colonization, such as maternal influences, calf management practices, liquid versus solid feed and the use of additives (Huws *et al.,* 2018). Additionally, knowledge is lacking on how the rumen microbial community develops at different growth stages in yaks and when it fully matures throughout their lifetime. Therefore, 16S rRNA sequencing technology was employed to analyze the phylogenetic composition of rumen microbial communities in yaks of different ages.

Materials and Methods

Location and time of the study

The study was conducted at Meilongzhang Cooperative, Qilian County, Haibei Prefecture, Qinghai Province, from March to August 2023. Testing and experimental procedures were carried out at the Academy of Animal Science and Veterinary Medicine, Qinghai University.

Animals, sample collection and measurements

In this study involved 18 healthy male yak calves, aged 3 months (MarG), 4 months (AprG) and 5 months (MayG), sourced from Meilongzhang Cooperative, Qilian County, Haibei Prefecture, Qinghai Province, China. Each group consisted of six calves (n=6), raised under consistent feeding conditions. The experiment was approved by the Qinghai University Committee on Animal Care (approval number: QUA-2020-5572). Rumen samples (50 ml) were collected post-slaughter and pH was measured using a pH meter (LE438-2M, Mettler Toledo, Switzerland). Samples were stored at -80°C for later analysis. NH3-N content was determined via colorimetry and volatile fatty acids (VFAs) were measured using gas chromatography (GC-2014; Shimadzu, Japan).

16S rRNA gene amplification and sequencing

Microbial DNA was extracted using the CTAB method (Sigma-Aldrich, Milan, Italy) according to the manufacturer's instructions. DNA concentration and purity were verified on a 1% agarose gel and the DNA was diluted to 1 ng/μL with sterile distilled water. The V4 region of the bacterial 16S rRNA gene was amplified using universal primers F515/R806 via PCR. The reaction mixture contained 4 μL 5× Fast Pfu buffer, 2 μL 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL Fast Pfu polymerase, 10 ng template DNA and ddH2O to 20 μL. PCR cycling conditions included: 95°C for 3 min; 27 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 45 s; final extension at 72°C for 10 min. PCR products were purified using a Clean-Up Kit (YuHua, Shanghai, China), quantified via Qubit 4.0 (Thermo Fisher Scientific, USA) and sequenced on the Illumina PE300/PE250 platform (Illumina, San Diego, USA) following Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China) protocols.

Amplicon sequence processing and analysis

After demultiplexing, sequences were quality-filtered with fastp (v0.19.6) and merged using FLASH (v1.2.11). High-quality sequences were de-noised with DADA2 (Callahan *et al*., 2016) in the Qiime2 (v2020.2) pipeline, achieving single nucleotide resolution. The resulting amplicon sequence variants (ASVs) were taxonomically classified using the Naive Bayes classifier in Qiime2 with the SILVA 16S rRNA database (v138). Functional predictions were performed using PICRUSt2 (v2.2.0).

Statistics and analysis of data

Experimental data were organized in Excel 2019 and one-way ANOVA was performed in SPSS 24.0 (IBM, Armonk, NY, USA) to compare rumen fermentation parameters among the three groups ($p<0.05$ considered significant). Alpha diversity indices, including Chao 1 and Shannon, were computed using mothur, with the Wilcoxon rank-sum test assessing inter-group differences. Principal Coordinate Analysis (PCoA), based on Bray-Curtis distance, was used to evaluate microbial community similarities, while PERMANOVA was used to test the significance of group differences. LEfSe analysis (LDA > 2, $p<0.05$) identified significantly different bacterial taxa across groups. Microbial networks were constructed using Gephi (v0.9.2) to analyze correlations among dominant taxa. Pearson correlation coefficients between bacterial genera and fermentation parameters were calculated using the heatmap package in R (v4.0.2). Functional predictions were conducted with PICRUSt2, identifying differences in KEGG pathways at level 2.

Results and Discussion

Analysis of rumen fermentation parameters in yaks of different ages

The comparison of rumen fermentation parameters among yaks of different ages is shown in Table 1. The levels of NH3-N ($p=0.005$) and Acetate ($p=0.005$) in MarG group were significantly higher than those in MayG and AprG groups. In MayG group, the levels of Propionate ($p<0.001$),

Isobutyrate (p=0.039), Butyrate (p=0.002) and Valerate (p=0.013) were significantly higher than those in MarG and AprG groups.

Analysis of richness, diversity estimates and composition of rumen bacteria in yaks of different ages

From Fig 1A, it is evident that a total of 11,215 ASVs were identified in this experiment, with 2,261, 1,704 and 1,932 specific ASVs found in the MarG, AprG and MayG groups, respectively. The PCoA analysis (Fig 1B) reveals significant variations in the rumen microbiota across yaks of different ages. Additionally, alpha diversity metrics (Fig 2) show significant differences in the Chao, Shannon, sobs and ace indices among the groups.

Analysis of rumen bacterial composition in yaks of different ages

As shown in Fig 3, the phylum-level abundance for the three age groups was ranked as Firmicutes > Bacteroidetes > Proteobacteria > Spirochaetota. Firmicutes abundance was 60.40%, 66.55% and 58.61% in the MarG, AprG and MayG groups, respectively (Fig 3A). A total of 55 genera were identified, with UCG-005 (14.45%) and Rikenellaceae_RC9_gut_group (10.24%) being the most dominant, followed by norank_f_Muribaculaceae (9.40%), unclassified_f_Lachnospiraceae(8.75%), Christensenellaceae_R-7_group (7.99%) and Bacteroides (6.10%; Fig 3B). LEfSe analysis (Fig 4) revealed age-specific dominance: Actinobacteriota and its families (*e.g.*, Bacteroidaceae, Lachnospiraceae, Tannerellaceae) dominated MarG, with Bacteroides, Olsenella and Subdoligranulum. In AprG, Christensenellaceae and Oscillospiraceae were dominant, including Christensenellaceae _R-7_group and UCG-010. In MayG, Desulfobacterota and families such as Prevotellaceae and Ruminococcaceae were prominent, with genera like Mailhella and Ruminococcus_gauvreauii_group.

Network analysis of bacterial communitieses

Microbial networks were used to examine interactions among rumen bacterial communities in yaks (Fig 5). In MarG, Proteobacteria occupied the core position, showing

Table 1: Comparative analysis of rumen fermentation parameters in yaks of different months of age.

Parameters	Groups			SEM	*p*-Value
	MarG	AprG	MayG		
pH	6.23	6.15	6.11	0.78	0.074
NH3-N, mg/dL	13.57^{a}	11.68^{b}	10.9^{b}	0.43	0.005
Acetate, mmol/L	3.59^{a}	2.64^{b}	2.68^{b}	0.38	0.005
Propionate, mmol/L	0.89^{b}	0.85^{b}	1.28^{a}	0.10	<0.001
Isobutyrate, mmol/L	0.05^{b}	0.05^{b}	0.09^{a}	0.01	0.039
Butyrate, mmol/L	0.33^{c}	0.41^{b}	0.63^{a}	0.07	0.002
Valerate, mmol/L	0.05^{b}	0.04^{b}	0.09^{a}	0.01	0.011
Isovalerate, mmol/L	0.03^{b}	0.03^{b}	0.05^{a}	0.01	0.013
TVFA, mmol/L	4.94^{a}	4.02^{b}	4.82^{a}	0.43	<0.001

MarG, 5-month-old group; AprG, 4-month-old group; MayG, 3-month-old group. TVFA: Total volatile fatty acids; SEM: Standard error of the mean. a, b, c: Values with different superscript letters in the same row differ significantly (p<0.05).

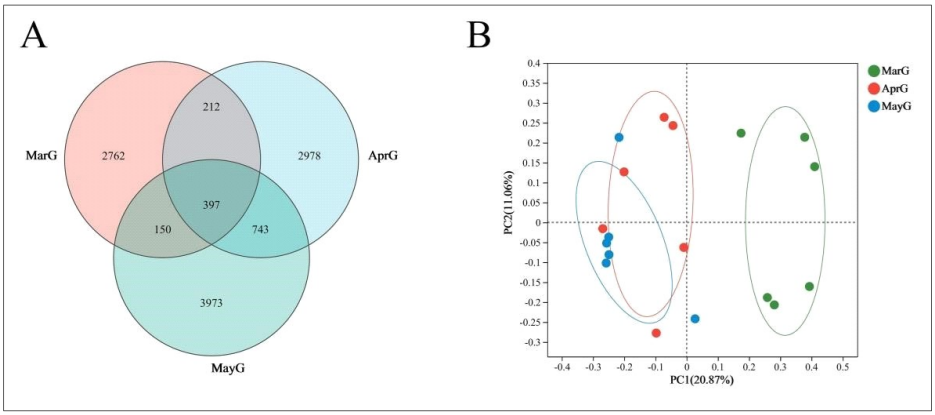


Fig 1: (A) Venn diagram showing the number of shared and unique amplicon sequence variants (ASVs) identified in yaks of different ages. (B) Principal Coordinate Analysis (PCoA) showing the differences in the rumen microbiota of yaks from different age groups.

strong positive correlations with phyla like Firmicutes and Actinobacteriota. In AprG, Firmicutes became the core, closely linked to Actinobacteriota, Proteobacteria and Bacteroidota. In MayG, both Proteobacteria and Firmicutes occupied core positions, displaying extensive positive correlations with multiple phyla. Negative correlations, though fewer, were more dispersed in MarG and AprG, indicating competition among certain phyla, while in MayG, they were more evenly distributed, suggesting widespread competition. The positive correlation network was densest in MarG, more concentrated in AprG and most complex in MayG.

Analysis of the correlations between rumen microbiota and ruminal fermentation parameters

The Spearman correlation analysis between rumen bacterial genera and fermentation parameters is shown in Fig 6. pH exhibited significant positive correlations with several taxa, including UCG-005 and Rikenellaceae_ RC9_ gut_group. NH3-N also showed notable positive correlations with various groups. Acetate was negatively correlated withun classified_f_ Lachnospiraceae and Christensenellaceae_R-7_group. Butyrate displayed both positive and negative correlations, with a positive correlation to Rikenellaceae_ RC9_gut_group. Isobutyrate and valerate showed generally weaker correlations. UCG-005 correlated positively with all fermentation parameters, while Bacteroides was negatively correlated with acetate, propionate, isobutyrate, butyrate and valerate.

PICRUSt2 function prediction

PICRUSt 2 gene function assessment was utilized to predict the function of rumen microflora in three groups of yaks according to KEGG pathway 2 (Fig 7). In the categories of Transcription, Infectious disease: viral, Cancer: specific types and Nervous system, there were significant differences in gene abundance among the three groups. In the categories of Cell motility and Immune system, it was observed that MarG had a slightly higher gene abundance in Cell motility, while MayG had a slightly higher gene abundance in the Immune system.

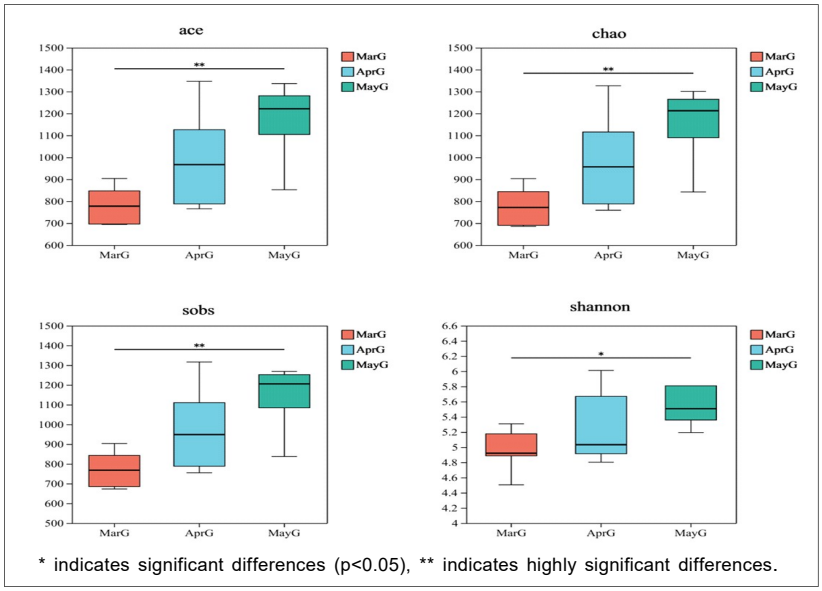


Fig 2: Diversity of rumen microbial communities in yaks of different months of age.

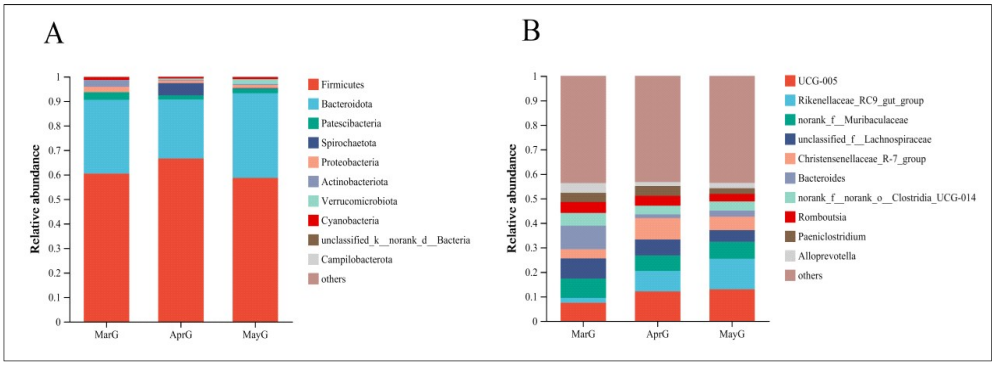


Fig 3: The relative abundances of microbial phyla (A) and genera (B) in the rumen of yaks at different ages are shown.

A stable rumen environment is crucial for ruminants. Key indicators of a stable rumen environment include rumen pH, ammonia nitrogen (NH3-N) and the molar concentration of volatile fatty acids (VFAs), which collectively reflect the state of rumen fermentation (Tomczak *et al*., 2019). Stability in rumen pH suggests that the acid-base balance remains relatively constant across different developmental stages, which helps maintain a suitable

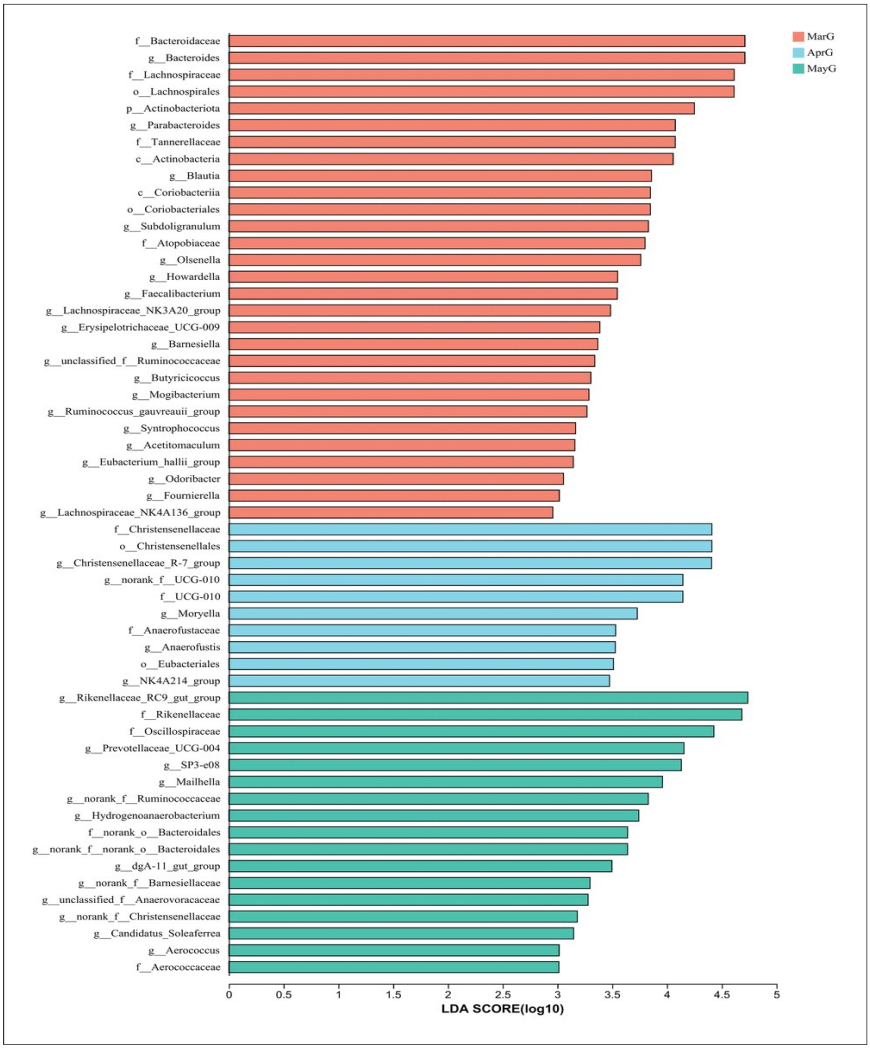


Fig 4: LEfSe analysis of rumen microflora of yaks of different ages.

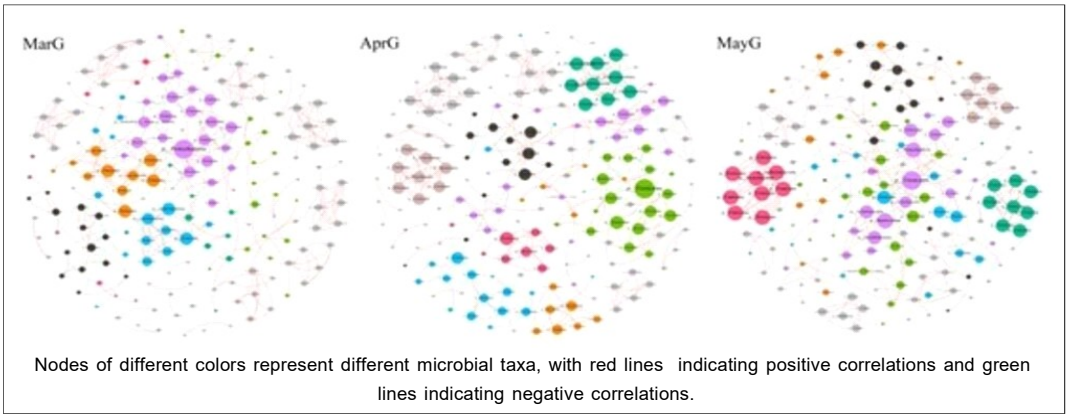
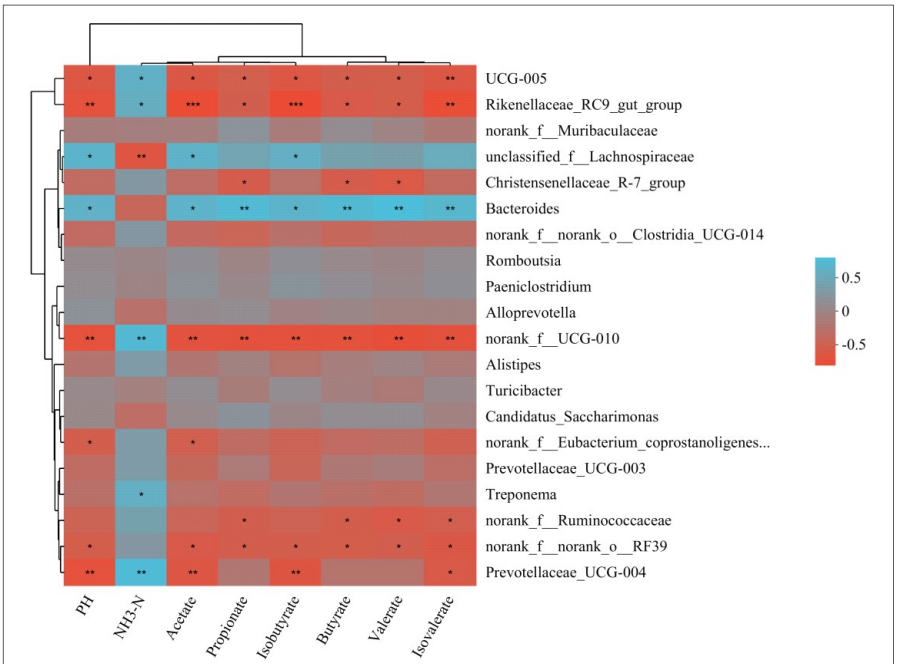


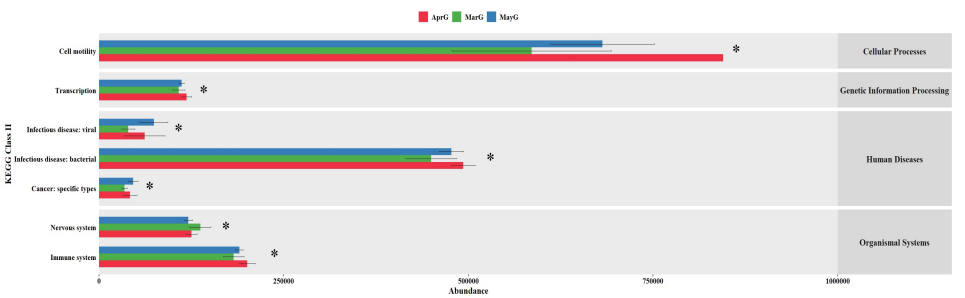
Fig 5: Microbial network analysis of rumen bacterial communities in yaks of different ages.

microbial environment and promotes efficient fermentation (Lee *et al.,* 2019). The study results show that although there was a slight decrease in rumen pH of yak calves between the MarG and MayG groups, this change was not statistically significant (p=0.074). Ammonia nitrogen (NH3-N) is produced by the decomposition of protein and non-protein nitrogen (NPN) during rumen fermentation and serves as a key indicator of nitrogen metabolism, reflecting how effectively nitrogen sources are utilized by rumen microorganisms (García-González *et al.,* 2010; Lv *et al.,* 2020). In this study, the ammonia nitrogen concentration in the MarG group was significantly higher compared to the AprG and MayG groups (p=0.005). This may indicate a lower efficiency in ammonia nitrogen utilization during the early developmental stages of the MarG group. As development progresses, ammonia nitrogen concentration decreases, likely due to the maturation of the rumen microbial community, which enhances ammonia utilization efficiency and reduces ammonia accumulation. Volatile fatty acids (VFAs), produced by rumen microorganisms during fermentation, are essential for providing energy and maintaining the stability and normal function of therumen environment (Li *et al.,* 2016). The concentration and proportions of VFAs reflect the state of rumen fermentation and microbial activity (Jiao *et al.,* 2015). In this study, acetate concentration was highest in the MarG group and significantly decreased in the AprG and MayG groups (p=0.005), likely due to dynamic shifts in the rumen microbial



Colors indicate the direction and strength of correlations, with red representing negative correlations and blue representing positive correlations. The intensity of the color corresponds to the correlation coefficient and the symbols *, **, *** denote levels of statistical significance (p<0.05, p<0.01, p<0.001), respectively.

Fig 6: Heatmap of Spearman correlation analysis between rumen bacterial genera and ruminal fermentation parameters.



The graph shows the KEGG pathways at level 2. * indicate significant differences in the abundance of certain functional pathways among the groups.

Fig 7: Functional predictions of the rumen microbiota KEGG pathways in yaks of different ages.

community and dietary adjustments. The propionate concentration in the MayG group was significantly higher than in the MarG and AprG groups ($p<0.001$), indicating increased propionate production as development progresses. Since propionate is a crucial precursor for gluconeogenesis, its increase may enhance the energy metabolism efficiency of yak calves, supporting growth. Additionally, isobutyrate and isovalerate concentrations in the MayG group were significantly higher compared to the MarG and AprG groups ($p=0.039$ and $p=0.013$, respectively).This increase in branched-chain fatty acids may reflect a greater diversity and complexity in the rumen microbial community. Butyrate, an important indicator of rumen health with anti-inflammatory properties that promotes intestinal cell proliferation and maintains intestinal barrier function (Guilloteau *et al.,* 2010), was found at the highest concentration in the MayG group ($p=0.002$), correlating with enhanced fermentation activity. The valerate concentration in the MayG group was significantly higher than in the MarG and AprG groups ($p=0.011$), suggesting an increase in the diversity of rumen fermentation metabolites as development advances.

This study found that during various developmental stages post-birth, the dominant microbial communities in the rumen of yak calves are mainly composed of Firmicutes and Bacteroidetes. This observation is consistent with findings from other studies on calf rumen microbiota (Malmuthuge *et al.,* 2014; Oikonomou *et al.,* 2013; Yeoman *et al.,* 2018). Firmicutes help break down fibrous materials in the gastrointestinal tract into short-chain fatty acids for the host, whereas Bacteroidetes are mainly involved in carbohydrate degradation and the development of the gastrointestinal immune system(Fernando *et al.,* 2010; Hu *et al.,* 2017; Nuriel-Ohayon *et al.,* 2016). Over time, the relative abundance of Firmicutes and Bacteroidetes in the rumen microbial community of yak calves initially increases and then decreases. This pattern may be linked to the increased consumption of forage by the calves, which influences the abundance of microorganisms that degrade forage cellulose. Moreover, our sampling was conducted between 3 and 5 months post-birth, a period marked by transitional weather conditions around the pasture, deteriorating climate, declining forage quality and increasing cellulose content. These factors might significantly contribute to the observed changes in microbial community structure at various developmental stages. Proteobacteria, a key marker of intestinal health in mammals, typically shows reduced abundance in healthier hosts (Shin *et al.,* 2015; Su *et al.,* 2020). It was observed that as the rumen microbial community of yak calves gradually establishes, the relative abundance of Proteobacteria decreases across developmental stages, indicating that the microbial community is approaching a stable state.

Microbial network analysis of the rumen bacterial communities in yaks reveals dynamic changes and adaptability across various months (MarG, AprG, MayG). In the MarG group, Proteobacteria is the core phylum, showing a strong positive correlation with Firmicutes and Actinobacteriota. In the AprG group, the core shifts to Firmicutes, while both Proteobacteria and Firmicutes are central in the MayG group. Although there are fewer negative correlations within each group, these correlations suggest competition between certain phyla. The dispersed negative correlations in MarG and AprG groups indicate that competition is not limited to specific interactions but spans multiple bacterial phyla, which may help maintain microbial diversity and prevent dominance by any single phylum. The even distribution of negative correlations in the MayG group suggests a more balanced competitive pattern, possibly reflecting a stable microbial community where competitive interactions are uniformly distributed, promoting ecological balance.

Gene function predictions of the yak rumen microbial communities in KEGG pathway 2 reveal significant dynamic changes across various months. These changes reflect the microbial communities' adaptability to environmental conditions, host metabolic demands and immune status. Significant differences observed in categories such as transcription, viral infections, specific cancers and neurological conditions highlight microbial activity in various functional areas, while trends in cell motility and immune system categories further indicate specific adaptive strategies of the microbial communities under changing environmental conditions (Hooper *et al.,* 2012; Tremaroli and Bäckhed, 2012). In this study, the MarG group shows slightly higher gene abundance related to cell motility, suggesting that rumen microbes in March may be more active in regulating cell motility and structural dynamics. Conversely, the MayG group exhibits slightly higher gene abundance in immune system functions, indicating that microbes in May may play a more active role in immune interactions and regulation. This increase in gene abundance might be attributed to heightened immune demands of the host, leading to a greater role for the microbial community in immune regulation and defense mechanisms.

Conclusion

The levels of NH3-N and acetate were higher in the 3-month-old yaks, while the levels of propionate, isobutyrate, butyrate and valerate were significantly higher in the 5-month-old yaks compared to the other groups. Microbial community analysis revealed significant structural differences among the groups: Proteobacteria was the central phylum in the 3-month-old yaks, Firmicutes in the4-month-old yaks and the 5-month-old yaks exhibited a complex network with both Proteobacteria and Firmicutes occupying central positions. These findings highlight significant structural and functional changes in the rumen microbial communities of yaks at different ages, providing new insights into the physiological adaptation mechanisms of yaks on the Qinghai-Tibet Plateau.

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Disclaimers

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Informed consent

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.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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