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# Dynamic changes in the yak rumen eukaryotic community and metabolome characteristics in response to feed type

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With diversification of yak breeding, it is important to understand the effects of feed type on the rumen, especially microbiota and metabolites. Due to the unique characteristics of yak, research on rumen microbes and metabolites is limited. In this study, the effects of two diet types on rumen eukaryotic microflora and metabolites were evaluated using the Illumina MiSeq platform and liquid chromatography-mass spectrometry (LC-MS). All identified protozoa belonged to *Trichostomatia*. At the genus level, the relative abundance of *Metadinium* and *Eudiplodinium* were significantly ( $p < 0.05$ ) higher in the roughage group than that of concentrate group, while the concentrate group harbored more *Isotricha*. *Ascomycota*, *Basidiomycota*, and *Neocallimastigomycota* were the main fungal phyla, and the *Wallemia*, *Chordomyces*, *Chrysosporium*, *Cladosporium*, *Scopulariopsis*, and *Acremonium* genera were significantly ( $p < 0.05$ ) more abundant in the roughage group than the concentrate group, while the concentrate group harbored more *Aspergillus*, *Neocallimastix*, *Thermoascus*, and *Cystofilobasidium* ( $p < 0.05$ ). Metabolomics analysis showed that feed type significantly affected the metabolites of rumen protein digestion and absorption (L-proline, L-phenylalanine, L-tyrosine, L-leucine, L-tryptophan, and  $\beta$ -alanine), purine metabolism (hypoxanthine, xanthine, guanine, guanosine, adenosine, and adenine), and other metabolic pathway. Correlation analysis revealed extensive associations between differential microorganisms and important metabolites. The results provide a basis for comprehensively understanding the effects of feed types on rumen microorganisms and metabolites of yaks. The findings also provide a reference and new directions for future research.

## KEYWORDS

yak, rumen, microbiota, fungi, protozoa, metabolomics, feed type

## 1. Introduction

Ruminant and rumen microbes are closely dependent on each other. These bacteria, make up 90% of the microbial population, and eukaryotes (fungi and protozoa), also make an important though poorly understood contribution (1–3). Fungi play pivotal role in the degradation of dietary fiber (4, 5), and protozoa function in carbohydrate degradation and maintaining rumen pH (6). Changes in the host environment affect rumen microbes, among which diet is the main factor affecting the microbial community (7). Many previous studies analyzed microbial and metabolite changes to comprehensively explore the effects of diet type on the rumen and ruminants. The findings suggest that dietary changes can alter the rumen microbiota, modifying rumen metabolites. Metabolites can reflect microbial interactions with diet, and this can impact the health and performance of ruminants (8–10).

Yak is an important ruminant animal in the plateau area of China, accounting for >90% of worldwide yak production (11, 12). When a traditional grazing pattern is employed, yak weight varies seasonally, which affects animal productivity and the income of herdsman (13, 14). Nowadays, two modes are common; two-stage feeding (grazing in the warm season, shelter feeding in the cold season) and shelter fattening (12, 15). Supplementary feeding with total mixed rations of available roughage and grains is not entirely dependent on grazing (16). However, yak has evolved a unique rumen microflora and metabolic mechanism adapted to the harsh environment (17–20). Little information is available on the effects of different feed type on yaks to make appropriate supplementary feeding strategy.

Therefore, we explored the effects of feed type on the rumen bacterial community and metabolism in yak in our previous work (21). However, our knowledge of eukaryotic communities and metabolites in yak remains limited. Only through investigating the microflora and metabolites will we be able to better explain the effects of feed type on yak rumen. Herein, we combined microbial sequencing and metabolomics techniques to explore the effects of two different feed types on rumen eukaryotes microorganisms and metabolites, and the possible relationships between ruminal microbiota and metabolites.

## 2. Materials and methods

### 2.1. Animals, treatments, and feeding regimes

The study was conducted at Haibei Tibetan Autonomous Prefecture, Qinghai Province of China. This area is over 3,000 m above sea level and the annual average temperature is 1.5°C. Sixteen male Datong yaks aged 48 months and initial

BW ( $195 \pm 50$  kg) resided at Haibei Tibetan Autonomous Prefecture Plateau Ecological Animal Husbandry science and technology Demonstration park. These animals were used as experimental animals and randomly divided into four groups. Four  $4 \times 4$  Latin square experiments were conducted, with four diets per square, making 16 different diets. Each experiment lasted 21 days, and the adaptation period lasted 15 days. According to the fiber content, feed with crude fiber content below 18% was placed in the concentrate feed group (CFG), and feed with crude fiber content of 25–45% was placed in the roughage feed group (RFG). The concentrate feed group included soybean meal, broad bean, rapeseed meal, sesame meal, oat, hulled barley, corn, barley, wheat, and wheat bran. The roughage feed group consisted of wheat straw, pea stem, broad bean stem, rape straw, oat straw, and alfalfa. Feed in the roughage feed group was fed separately, and feed in the concentrate feed group was processed into pellets using a diameter of 6 mm with 30% oat straw. Diets were supplemented with 2% buffering additives ( $\text{NaHCO}_3$  and MgO with 2:1 ratio), 0.5% limestone, 0.5% NaCl, and 30 mg/kg rumensin to maintain normal rumen fermentation. Yaks were fed 1.9% bodyweight (BW) on a dry matter basis at 0800 and 1,600. Experiments were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (The State Science and Technology Commission of P. R. China, 1988). The procedures for the care and use of animals in this study were approved and conducted according to standards established by the College of Animal Science and Technology, CAU, Beijing, P. R. China (permit number DK1402006).

### 2.2. Rumen sampling and measurements

Rumen fluid was collected from yaks before feeding on the morning of day 22. A 50 mL syringe was used to extract rumen fluid from the oral cannula. The collecting device was thoroughly washed with clean warm water and the first rumen fluid sample collected was discarded. About 50 mL of rumen fluid was then collected from each animal and transferred to a 50 mL cryopreservation tube and frozen in liquid nitrogen. A total of 64 rumen fluid samples were collected from yaks, but some samples were damaged due to improper storage, and 40 samples were used for follow-up analysis.

### 2.3. DNA extraction, PCR amplification, and high-throughput sequencing

Microbial community genomic DNA was extracted from rumen samples using an E.Z.N.A. SOIL DNA Kit (Omega Bio-Tek, Norcross, GA, U.S.) according to the manufacturer's instructions. Extract DNA was checked on a 1% agarose gel,

and the concentration and purity were determined using a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific). Protozoa primers reg1320R (5'-AATTGCAAAGATCTATCCC-3') and RP841F (5'-GACTAGGGATTGGARTGG-3') (7) and fungal primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2R (5'-GCTGCGTTCTTCATCGATGC-3') (22) were used for PCR amplification of variable regions. PCR mixtures contained 4  $\mu$ L of 5 $\times$  TransStart FastPfu buffer, 2  $\mu$ L of 2.5 mM dNTPs, 0.8  $\mu$ L of forward primer (5  $\mu$ M), 0.8  $\mu$ L of reverse primer (5  $\mu$ M), 0.4  $\mu$ L of TransStart FastPfu DNA Polymerase, 10 ng of template DNA, and double-distilled water (ddH<sub>2</sub>O) up to 20  $\mu$ L. Amplifications were performed in triplicate. PCR products were extracted from 2% agarose gels and purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions, and quantified using a Quantus<sup>TM</sup> Fluorimeter (Promega, USA). Purified amplicons were pooled in equimolar amounts and paired-end (PE) sequenced (2  $\times$  300) on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) according to standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

## 2.4. Data analysis

Raw sequencing reads were demultiplexed and quality-filtered by Fastp (version 0.19.6, <https://github.com/OpenGene/fastp>) and merged by FLASH (version 1.2.11, <https://ccb.jhu.edu/software/FLASH/index.shtml>) with the following criteria: (i) 300 bp reads were truncated at any site receiving an average quality score of <20 over a 50 bp sliding window, truncated reads shorter than 50 bp were discarded, and reads containing ambiguous bases were also discarded; (ii) according to overlap relation between PE reads, pairs were merged into sequences with a minimum length overlap of 10 bp; (iii) the maximum mismatch ratio of overlap regions was 0.2. Reads that could not be assembled were discarded. Operational taxonomic units (OTUs) with a 97% similarity cutoff were clustered using UPARSE (version 11, <http://drive5.com/uparse/>), and chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analyzed by RDP Classifier (version 2.13, <https://sourceforge.net/projects/rdp-classifier/>) against the 18S rRNA database (Protist\_PR2\_v4.5) and the ITS database (unite 8.0) using a confidence threshold of 0.7.

The analysis was conducted using the free online Majorbio I-Sanger Cloud Platform (<http://www.i-sanger.com>). The alpha-diversity index was calculated using MOTHUR (version v.1.30.2, [https://www.mothur.org/wiki/Download\\_mothur](https://www.mothur.org/wiki/Download_mothur)). The R language tool was used to generate dilution curves and plot bar charts related to results. Beta-diversity was estimated by calculating the unweighted UniFrac distance and visualized using principal coordinate analysis (PCoA), and the results

were plotted using GUniFrac and ape packages in R (23, 24). The Wilcoxon rank-sum test within STAMP (version v.2.1.3) was used to identify phyla and genera that showed significant differences in abundance between groups (confidence interval method) (25). Spearman's correlation analysis was used to evaluate correlations between different metabolites and microbial communities using the pheatmap software package in R (version 61, <http://CRAN.R-project.org/package=pheatmap>), with  $p < 0.05$  indicating statistical significance.

## 3. Results

### 3.1. Diversity of the rumen microbiota of yaks receiving different feed types

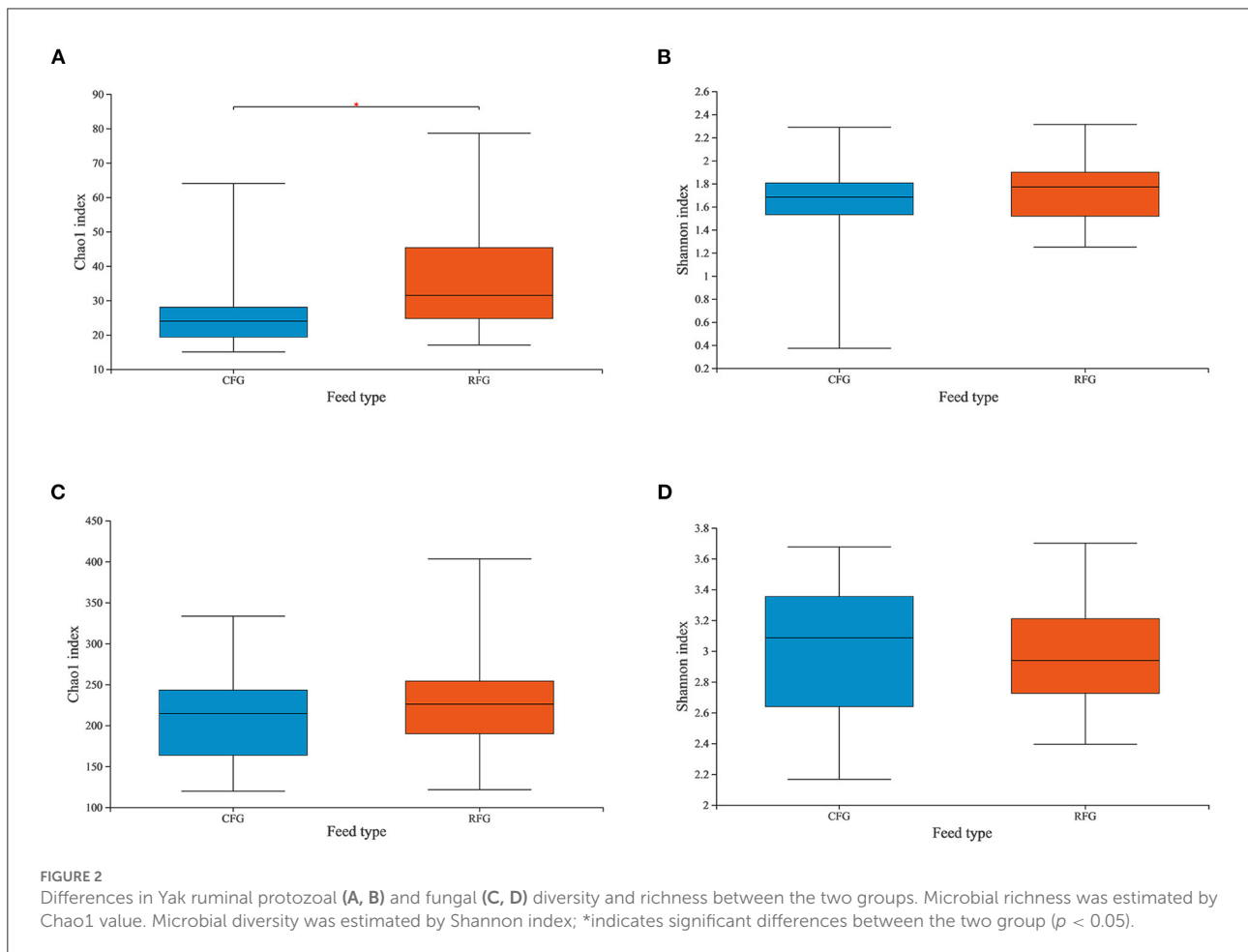
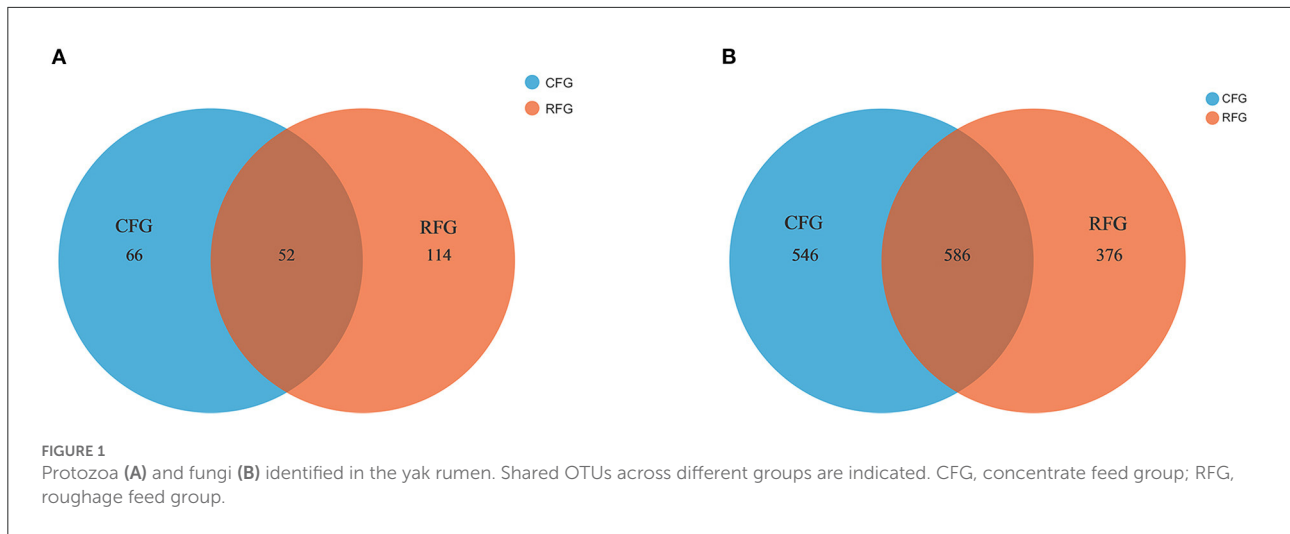
In this study, high-quality 1,771,085 protozoal sequences and 2,245,482 fungal sequences were obtained. Each sample sequence was processed according to a minimum number of samples. Venn diagrams revealed the number of core and differing microbes (Figure 1). For protozoa, the total number of OTUs in CFG and RFG was 118 and 166, respectively, and 52 shared OTUs. For fungi, a total of 546 and 376 OTUs were unique to the CFG and RFG, and 586 shared OTUs. The protozoal community richness (Chao 1) for RFG was greater than that for CFG ( $p = 0.049$ ; Figure 2). Community diversity (Shannon) of fungi and protozoa showed no significant differences between the two groups.

### 3.2. Microbial communities of the yak rumen microbiota following different feed types

In Figure 3, PCoA plot clearly showed that fungal samples were not completely separated by feed type, but protozoal samples revealed aggregation. This results indicated that there were some differences in fungal community structure between CFG and RFG, while the protozoal community structure was similar.

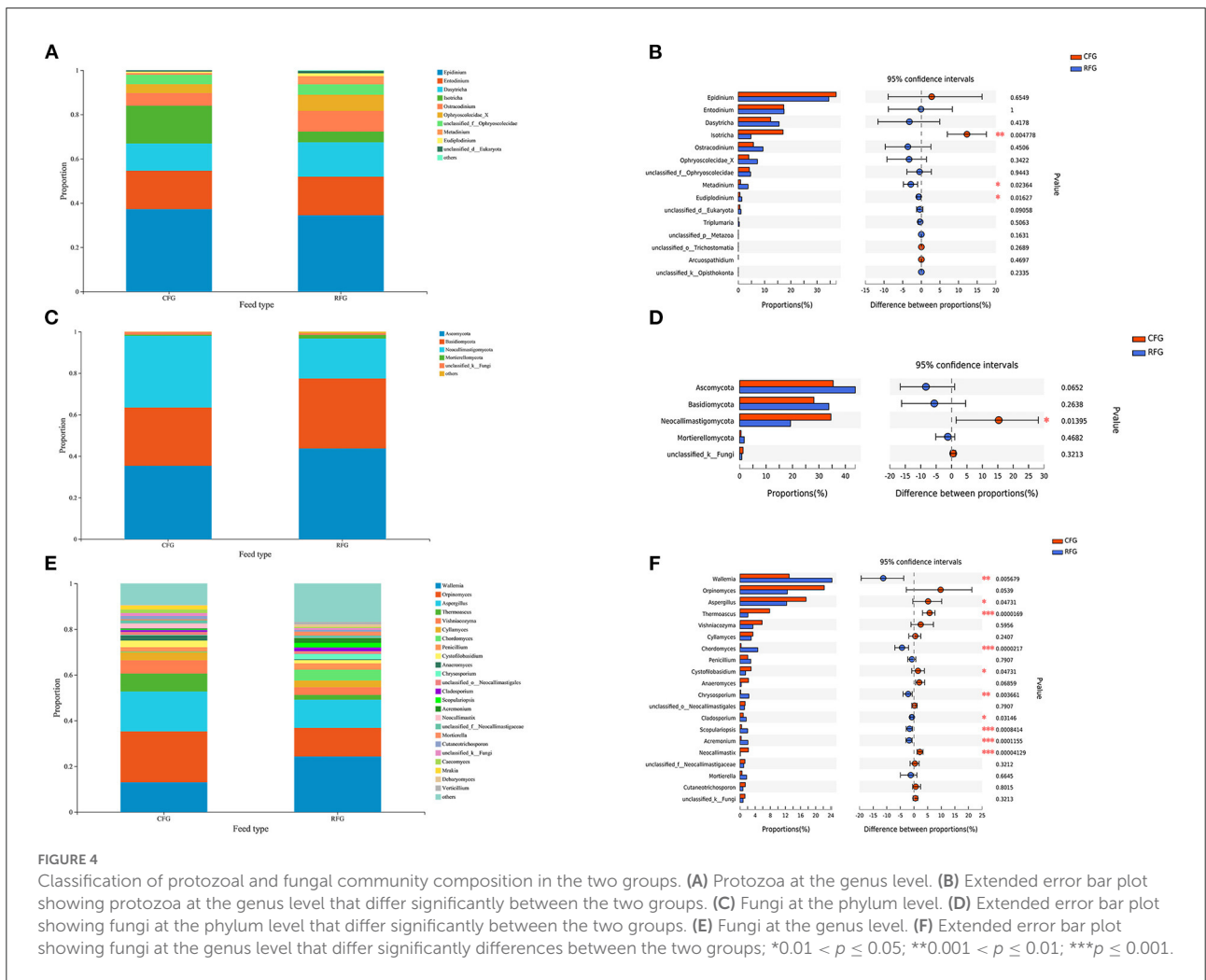
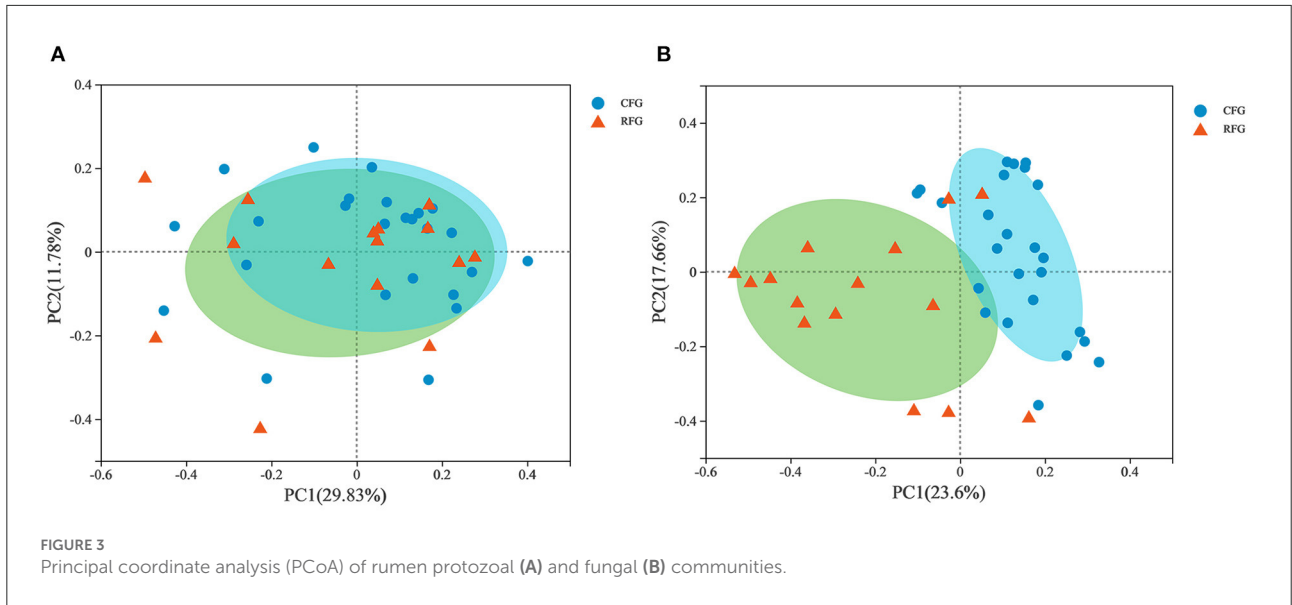
As shown in Figure 4, all protozoa identified in this study belonged to *Trichostomatia*, and they could be divided into *Ophryoscolecidae* and *Isotrichidae* at the family level. The fungal phyla *Ascomycota* (43.68%), *Basidiomycota* (33.71%), and *Neocallimastigomycota* (19.25%) were the most abundant. The relative abundance of *Neocallimastigomycota* was significantly higher CFG than in RFG ( $p = 0.014$ ).

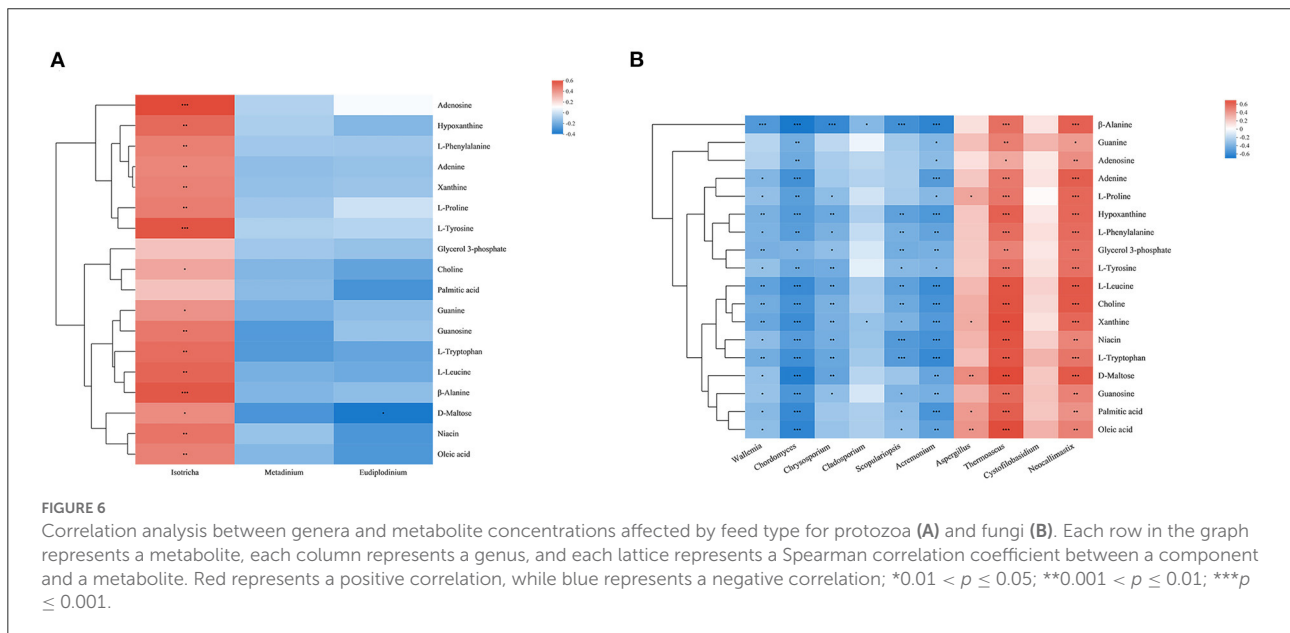
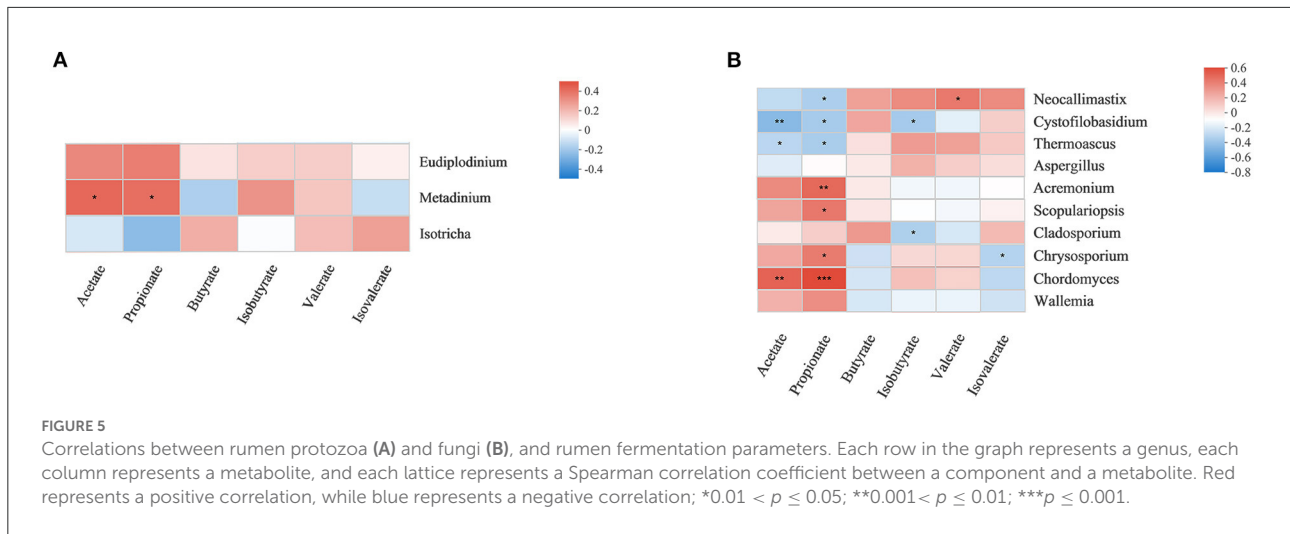
For protozoa, *Epidinium* was the most abundant genus, accounting for  $\sim$ 35%, far surpassing the second and third most abundant protozoa. The relative abundance of *Isotricha* in CFG was significantly higher than in RFG ( $p = 0.005$ ). By contrast, the relative abundance of *Metadinium* and *Eudiplodinium* in RFG was significantly higher than in CFG ( $p < 0.05$ ). At the



genus level, *Orpinomyces*, *Wallemia*, and *Aspergillus* were the dominant genera for fungi, collectively accounting for ~50% of fungal. The relative abundances of *Wallemia*, *Chordomyces*, *Chryso sporium*, *Cladosporium*, *Scopulariopsis*, and *Acremonium*

were higher in RFG than CFG ( $p < 0.05$ ). However, the relative abundances of *Aspergillus*, *Thermoascus*, *Cystofilobasidium*, and *Neocallimastix* were significantly higher in CFG than RFG ( $p < 0.05$ ).





### 3.3. Correlations between the rumen microbiome and the metabolome

Fermentation parameters and metabolomics results have been reported in previous laboratory studies (21). Herein, correlations between the relative abundances of rumen fungi and protozoa and the concentrations of metabolites were investigated by correlation analysis (Figure 5). *Metadinium* showed a significant positive correlation with acetate and propionate concentrations, and there was a significant correlation between rumen fungi and VFAs. *Chordomyces* was significantly positively correlated with acetate concentration, while *Cystofilobasidium* and *Thermoascus* were significantly negatively correlated with acetate concentration. *Chordomyces*,

*Scopulariopsis*, *Acremonium*, and *Chrysosporium* were positively correlated with propionate concentration. *Neocallimastix*, *Cystofilobasidium*, and *Thermoascus* were significantly negatively correlated with propionate concentration. In addition, *Cystofilobasidium* and *Cladosporium* were positively correlated with isobutyrate concentration. *Neocallimastix* was significantly positively correlated with valerate concentration, while *Chrysosporium* was significantly negatively correlated with isovalerate concentration.

Combined with the results of previous studies, we identified a set of metabolites that are significantly enriched in several important pathways. Metabolites were mainly divided into amino acids (L-proline, L-phenylalanine, L-tyrosine, L-leucine, L-tryptophan, and β-alanine), nucleosides, purines and purine



derivatives (hypoxanthine, xanthine, guanine, guanosine, adenosine, and adenine), fatty acids (palmitic acid, oleic acid), and other metabolites (choline, niacin, D-maltose, glycerol-3-phosphate). In order to explore the composition and functions of microbial communities, Spearman correlation analysis was performed on significantly different species of eukaryotic microbes at the genus level and metabolites (Figure 6). Except glycerol 3-phosphate and palmitic acid, *Isotricha* of protozoa showed a significant positive correlation with other metabolites, while fungi showed great correlations with metabolites, among which *Chordomyces*, *Acremonium*, *Thermoascus*, and *Neocallimastix* showed significant negative or positive correlation with all metabolites. *Walleimia* showed significant negative correlations with metabolites except for guanine and adenosine. *Chrysosporium* showed significant negative correlations with amino acids and some nucleosides and purine metabolites (guanosine, hypoxanthine, xanthine). *Cladosporium* showed significant negative correlations with  $\beta$ -alanine and xanthine. *Scopulariopsis* showed significant negative correlations with metabolites except guanine, adenosine, adenine, L-proline and D-maltose. *Aspergillus* showed significant positive correlations with L-proline, xanthine, D-maltose and fatty acids.

## 4. Discussion

Ruminants and microorganisms are in a constant dynamic balance of mutual dependence and restriction (12). The external environment can vary dramatically, and microbes change as a result, helping hosts to adapt (26). In the present work, protozoa richness changed due to dietary fiber content, but overall diversity did not change. Many studies showed that diet can affect rumen protozoa, and low concentrate diets were more conducive to the establishment of protozoa populations (27, 28). In addition, the results showed that fiber content did not change the community structure of rumen protozoa in yak, or the dominant genera. It was possible that rumen protozoa following a roughage diet were present at low density but richness is high. For fungi, changing the feed type had no effect on alpha-diversity. Consistently, previous studies also showed that dietary changes had less impact on fungal diversity (9, 10). Nevertheless, the composition of fungi varied with the type of diet. Each fungus may perform a distinct physiological function and degrade more than one substrate, which made them able to adapt to a changing diet, building resilience and resistance to change (1).

The community composition of yak protozoa was relatively simple. The rumen of most herbivorous mammals was dominated by ciliates of the subclass *Trichostomatia* (29). In this study, *Epidinium* was the dominant genus, but *Entodinium* was the most prevalent in most previous studies (9, 10, 26). *Epidinium* accounted for a large proportion of microbes in the

rumen of grazing animals (27), and members of this genus secreted cellulase and hemicellulose enzymes to destroy plant cell walls and absorb chloroplasts from plant cells during fiber fermentation (30–32). It was possible that yaks evolved to preferentially host protozoa that degrade fiber to allow them to adapt to the plateau environment. Additionally, the results showed that consuming a high concentrate diet may be more favorable to *Isotricha*. It was also reported that *Isotrichidae* can rapidly use soluble sugars such as glucose, fructose, and sucrose and convert them into glycogen for storage (33). Besides, *Ophryoscolecidae* was a functionally rich population, and the results indicated that *Metadinium* and *Eudiplodinium* belonging to this order may ingest and degrade plant fibers.

The study investigated fungal community composition and abundance using the internal transcribed spacer (ITS) region, a sequence widely used in such previous studies. Anaerobic fungi, classified as phylum *Neocallimastigomycota*, were crucial in the degradation of lignocellulosic plant fiber in the rumen (4, 5, 34, 35), and they were highly abundant in CFG in the present study. This may be the result of fungi adjusting their community and mechanism to adapt to a change in diet type. In previous work, 11 genera of anaerobic fungi were isolated and cultured (36), and some of these common anaerobic fungi were detected in the present study. Similar to Griffith et al. (37) and Guo et al. (38), the results showed that *Orpinomyces* was the main anaerobic fungal genus in the rumen of yak. In addition to, *Cyllamyces*, *Anaeromyces*, and *Neocallimastix* were relatively high in abundance, but only the rhizoidal genus *Neocallimastix* was higher in abundance in CFG. This genus functions in the degradation of complex lignocellulosic plant biomass using highly active enzymes (39), but it can also secrete amylases involved in the degradation of easily fermentable carbohydrates (40). Similar to this genus, other genera such as *Cyllamyces* can also rapidly colonize substrate surfaces of different carbon sources and thereby participate in the degradation of different diets (36). The results seemed to suggest that each genus had many functions and activities. Therefore, anaerobic fungi may widely participate in diet degradation in yak. In recent years, more and more results non-strictly anaerobic fungi have been identified in yak rumen samples (20, 38, 41). The non-strict anaerobic fungi *Ascomycota* and *Basidiomycota* were also found to be the dominant fungi in yak. This may be the result of a combination of environmental factors and host selection. However, most of these fungi came from the environment, and there has been no immediate concern about non-strict anaerobic fungi in the rumen of ruminants. Therefore, their metabolism and functions in the rumen ecosystem remain unclear.

Ruminant metabolites are needed for ruminant growth and health, and the proliferation of microorganisms is also closely related to metabolites. A change in feed type can alter the fermentation substrates of microorganisms, which in turn changes the metabolites in the rumen. VFAs are closely related to microbial fermentation and energy metabolism in the host.

In this study, a high-fiber diet produced more acetate and high total VFA. One of the reasons may be the changes of bacteria (the genus *Ruminococcus 2* and *Bacteroidales BS11 gut group*) in the rumen mentioned in the previous article (21), and the other was the result of fungi changes. In this study, *Thermoascus* and *Cystofilobasidium* were more abundant in yaks fed the concentrate, in the meanwhile, the genera were negatively correlated with acetate and propionate concentrations. Previous study showed that *Thermoascus* had the ability to secrete highly active and thermostable CAZymes (42). And scientists have isolated a strain of the genus *Cystofilobasidium* capable of producing cold-active enzymes that degrade pectin (43). These suggested that *Thermoascus* and *Cystofilobasidium* were probably involved in carbohydrate metabolism. In addition, the concentration of branched-chain volatile fatty acids was also affected by the feed type. Branched-chain volatile fatty acids (valerate, isobutyrate, isovalerate) are important sources of carbon skeleton for microorganisms, are derived from branched-chain amino acids. The result showed the lower level of the isobutyrate and isovalerate concentrations in RFG. *Chryso sporium*, *Cladosporium* had the same changes as bacteria (the genus *Prevotellaceae UCG-003*), which were negatively correlated with branched volatile fatty acids. The inference was that the microbes in the CFG made more use of nutrient metabolites to meet their needs.

Previous studies have proved that feed type significantly affects protein digestion and absorption, and purine metabolism and other metabolic pathways in yak (21). In the present study, the results showed that correlations between different microorganisms and metabolites in the two groups were significantly different, indicating that rumen metabolism was closely related to feed type. Amino acids were among the metabolites significantly affected by feed type in the present study. In the rumen, amino acids mainly come from the degradation of dietary protein and microbial protein by the rumen microbial community. The higher concentration of  $\text{NH}_3\text{-N}$  and amino acids in CFG indicated that more protein may be utilized and degraded. Fungi such as *Neocallimastix* possess proteolytic activity (44), and there are significant differences in protease activity between different anaerobic fungi (45). This may explain why *Neocallimastix* showed a strong positive correlation with individual amino acids. Meanwhile, protozoa cannot use ammonia and must ingest microbes to obtain certain nutrients (46). Similar to *Neocallimastix*, we found that *Thermoascus* and *Isotricha* were potentially related to protein metabolism, through degrading and/or ingesting for themselves. However, we still cannot ignore that bacteria play a major role in protein degradation. Fungi may form a consortium with bacteria that attaches to a feed particle, as well as protozoa regulates bacterial nitrogen turnover by gobbling up large molecules (47). Moreover, we observed that L-leucine, L-phenylalanine, L-tyrosine, and L-tryptophan were negatively associated with *Ascomycota*. Branched amino acids

and aromatic amino acids are involved in many physiological activities. It has been reported that L-leucine can produce a large amount of isovalerate in the rumen, which can be further metabolized into acetoacetate and acetyl-CoA via the citric acid cycle as potential ketogenic substances (48). It is also an essential amino acid preferentially used by protozoa in the rumen (49). In addition, L-phenylalanine, L-tyrosine, and L-tryptophan are important glucogenic and ketogenic amino acids, and low levels may limit the growth and fermentation rate of rumen microorganisms (50). These Observation may have been the results of amino acids playing important roles in the growth and proliferation of microbe. Specifically, the amino acid  $\beta$ -alanine, which mainly forms carnosine and is not actually involved in protein formation, is a breakdown product of pyrimidine and normally metabolized into acetate (51). In the present study,  $\beta$ -alanine was negatively associated with the high abundance of certain genera in RFG. Therefore, it speculated that the degradation of  $\beta$ -alanine in the rumen of RFG yaks with lower starch and available carbohydrate levels may be one of the reasons for the higher acetate concentration.

Purine metabolism was also associated with the activity of some microbes in this study. Guanine and adenine are DNA and RNA bases that are catabolized to produce hypoxanthine and xanthine, and hypoxanthine can be further oxidized to xanthine, eventually producing uric acid. These molecules are combined with ribose or deoxyribose to form guanosine and adenosine. Adenosine, guanosine, guanine, adenine, xanthine, and hypoxanthine degradation products were detected in the present work. Similar to Ametaj et al. (52) and Zhang et al. (53), it found that the rumen environment of yaks fed concentrate may be more prone to microbial nucleic acid degradation. Furthermore, it was reported that anaerobic fungal genomes had an extremely high adenine-thymine (AT) content (54). Thus, we should pay more attention to the role of fungi in purine metabolism. Nowadays, most scientists use the purine derivative content of ruminant urine to calculate microbial protein production (17). However, in this study, changes in the concentrations of these metabolites in the rumen indirectly reflected the effects of diet on the internal environment.

The results of correlation analysis between oleic acid/palmitic acid and microbes indicated the effects of feed type on lipid metabolism. Palmitic acid is saturated, while oleic acid is monounsaturated, and it plays an important role in regulating the proliferation and differentiation of preadipocytes (55, 56). The composition of unsaturated fatty acids has an important positive effect on beef flavor (57), but they are rarely used in a free form in hosts. One of the reasons is that unsaturated fatty acids consumed from feed reach the rumen and are converted to saturated fatty acids by microbial hydrogenation, which are deposited in body fat. Previous studies showed that protozoa had no direct role in the hydrogenation of unsaturated fatty acids (58), but may be able to modulate the biohydrogenation. It reported that rumen protozoa can



maintain pH by swallowing starch particles to avoid the negative transformation of biohydrogenated intermediates, as well as the unsaturated fatty acids combined with the protozoa membranes, avoiding the biohydrogenation of bacteria (59). In addition, rumen fungi catalyze biohydrogenation, but the hydrogenation activity was much lower than that of bacteria (60). Although the specific role of these fungi and protozoa in lipid metabolism in the rumen is still unclear, this correlation suggested that they may be involved in this process.

In addition, diet changes also affected the concentrations of other typical metabolites. D-maltose is a product of starch degradation by amylase. High-concentrate diets contain a large amount of easily fermentable carbohydrates, of which starch is the most important. This may explain the significant positive correlation between the D-maltose concentration and highly abundant genera in CFG. This also indicated that these fungi and protozoa may participate in the process of degrading unstructured carbohydrates. Niacin and choline are indispensable B vitamins in ruminants. Niacin acts as a direct precursor of important coenzymes NAD and NADP, and it is involved in the metabolism of nutrients in the body (61). It can be synthesized from tryptophan (62). The results showed that the change in L-tryptophan concentration was consistent with that of niacin. Thus, our study results revealed a strong association between affected microbiota and metabolites in important pathways; they not only showed the effects of feed type on rumen metabolism, but also that protozoa and fungi were closely related to metabolic processes. However, most microbiome eukaryotes remain poorly studied, hence we can't give precise conclusion.

## 5. Conclusion

In our study, feed with a high fiber content was more beneficial to protozoa growth and proliferation in the yak rumen, while fungi remained stable in high- and low-fiber diet groups. Feed type affected the microbiota, and altered some rumen metabolite concentrations. Both protozoa and fungi were found to be extensively involved in various metabolic processes, but fungi seem to be more active than protozoa in most processes. However, no individual rumen metabolite is independent of others, and the actions of multiple microbes may be important. This required further investigation. Regardless, this study contributes to a more comprehensive and complete understanding of rumen microbial composition and function, as well as important metabolites in yak. It provides more in-depth information on the yak rumen, and a reference for subsequent production and research. The findings also provide

new avenues for exploration related to rumen microbes and rumen metabolism.

## Data availability statement

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found at: SRA, NCBI; PRJNA665783.

## Ethics statement

The animal study was reviewed and approved by College of Animal Science and Technology, China Agriculture University, Beijing, P. R. China (permit number DK1402006).

## Author contributions

The study was designed by SL, SC, QM, and ZZ. Sample processing was carried out by HW. Data analysis was performed by XC. The manuscript was written by XC and modified by YL. All authors read and approved the final manuscript.

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## Conflict of interest

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

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