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Cellulase enhancing rumen microbiome of Tan sheep indicates plastic responses to seasonal variations of diet in the typical steppe

Hairen Shi[1], Pei Guo[1], Zhen Wang[1], Jieyan Zhou[1], Meiyue He[1], Liyuan Shi[1], Xiaojuan Huang[1], Penghui Guo[1], Zhaoxia Guo[1], Yuwen Zhang[1] and Fujiang Hou[1,2*]

Abstract

Background Climate and geographical changes significantly influence food availability and nutrient composition over time and space, Which in turn affects the selection of microbial communities essential for maintaining gastrointestinal homeostasis and facilitating dietary adaptation. Therefore, it is essential to understand the specific responses of the gut microbiota to dietary and seasonal variations in order to improve animal conservation strategies based on solid scientific knowledge.

Results In summer, due to the higher nutritional quality of forage, Tan sheep exhibited enhanced forage degradation and fermentation. This was reflected by increased populations of key rumen bacteria, including *Bacteroidetes*, *Prevotella_1*, *Prevotellaceae_UCG-003*, *Ruminococcus_1*, *Saccharofermentans*, and *Ruminococcaceae_UCG-014*. Supplementation with cellulase further facilitated these processes, optimizing the utilization of available nutrients. In contrast, during winter, when the nutritional quality of forage decline, we observed lower indicators of forage degradation and fermentation in Tan sheep. Additionally, there was a significant increase in the Firmicutes/Bacteroidetes ratio, microbial diversity, microbial interactions, and metabolic activity.

Conclusions The rumen microbiota adapts to enhance the breakdown of forage biomass and maintain energy balance during periods of inadequate nutritional value. Supplementing the diet with cellulase during these times can help mitigate the reduced digestibility associated with low-quality forage. This study highlights the dynamic adaptation of the rumen microbiota to seasonal variations in forage quality and emphasizes the potential benefits of cellulase supplementation in supporting rumen function and improving animal performance under varying environmental conditions.

Keywords Season, Cellulase, Ruminant, Microbiome

*Correspondence:
Fujiang Hou
cyhoufj@lzu.edu.cn
[1]State Key Laboratory of Herbage Improvement and Grassland Agro-ecosystems, Key Laboratory of Grassland Livestock Industry Innovation, Ministry of Agriculture and Rural Affairs, Engineering Technology Research Center for Ecological Restoration and Utilization of Degraded Grassland in Northwest China, College of Pastoral Agriculture Science and Technology, National Forestry and Grassland Administration, Lanzhou University, Lanzhou 730020, China
[2]Present address: College of Pastoral Agricultural Science and Technology, Lanzhou University, No.768, Jiayuguan West Road, Chengguan District, Lanzhou, Gansu Province, P.R. China

Background

The composition of the gut microbiota is pivotal for sustaining the dynamic equilibrium within the gastrointestinal tract of animals, playing a critical role in their dietary adaptation processes [1, 2]. The variability in feed availability and its nutritional value, influenced by climatic and geographical shifts, presents a challenge to maintaining this balance. Moreover, the dietary needs of animals evolve in response to thermoregulatory demands and life history stages [3, 5]. It is, therefore, imperative to comprehend the specific responses of gut microbiota to dietary and seasonal fluctuations to enhance animal conservation strategies through a foundation of scientific understanding [6, 8]. Empirical evidence from recent research demonstrates that the gut microbiota in wild great apes [9], wild geladas [10], and Tibetan sheep [11] undergoes seasonal adaptations in reaction to changes in diet, highlighting the significant impact of dietary shifts on microbial compositions. Furthermore, studies indicate that variations in gut microbiota among individuals become more distinct due to dietary selections driven by seasonal variations in the nutrient of forage available in grazing regions [10, 12].

Tan sheep (*Ovis aries*), indigenous to the arid and semi-arid steppe grasslands of the Loess Plateau in China, predominantly subsists on flora that is adapted to the local climatic conditions [13]. In the seasons of higher precipitation, their diet is largely composed of a variety of *Poaceae*, *Cyperaceae*, and *Leguminosae*, including both leaves and seeds. Conversely, during periods of reduced precipitation when grass becomes scarce, there is a notable diversification in their dietary intake [10]. Despite this diversification, the harsh conditions and limited availability of forage in the dry season frequently do not suffice to meet their nutritional needs, thereby necessitating the incorporation of supplementary nutritional sources to facilitate the breakdown of fiber. In this context, cellulase enzymes serve as potent biocatalysts. They promote the secretion of endogenous enzymes, addressing the deficit of microbial cellulase which is a common limitation among herbivores, and bolster the activity of other enzymes such as amylase and pectinase [14]. Moreover, the application of cellulase not only enhances the digestibility of fibrous feeds but also improves the overall growth performance of the livestock, leading to enhanced economic returns [15, 17]. Nevertheless, there is an evident knowledge gap concerning how the composition and functionality of rumen microbes adjust to the harsh conditions of extreme dry seasons, particularly in contexts where supplemental sources like cellulase are introduced. This gap underscores the need for further research to thoroughly understand the impact of supplemental enzymes on the rumen's microbial ecosystem under variable seasonal conditions.

Our hypothesis suggests that Tan sheep navigate seasonal fluctuations by maintaining specialized microbial communities, which in turn, augment their efficiency in energy utilization and facilitate their survival amidst extended, severe environmental adversities. We posit that the introduction of cellulase plays a pivotal role in enhancing microbial diversity and vitality, and markedly influences rumen fermentation patterns. The foremost aim of this study is to illuminate the contributions of microflora to the nutrition and metabolism of Tan sheep. Elucidating and modulating the microbial ecosystem within these animals could prove exceptionally advantageous in fostering growth and overall well-being, particularly under circumstances where the animals are confronted with developmental impediments arising from harsh drought conditions.

Methods

Study site, animals and experimental design

Twenty Tan wether sheep (average age = 6 months, average weight = 23.17 ± 0.24 kg) were obtained from the Huanxian Grassland Agriculture Research Station of Lanzhou University (37.1°N, 106.8°E), China. The animals were randomly assigned to two distinct groups, with each group consisting of ten animals. In the cellulase-treated group (**CL**), each wether was orally administered 1 g of cellulase dry powder (10,000 U/g) dissolved in 30 mL of warm water, using a drenching method [18]. To maintain the integrity of the experiment, the control group (**CK**) was administered 30 mL of normal saline daily prior to grazing. The summer grazing phase spanned from early to mid-June through to early to mid-September, whereas winter grazing occurred from early to mid-November until early to mid-January. The predominant vegetation of the rangeland comprises *Artemisia capillaris*, *Stipa bungeana*, and *Lespedeza daurica*, alongside associated species such as *Heteropappus altaicus*, *Dodartia orientalis*, *Potentilla bifurca L*, *Euphorbia esula*, among others, Supplement S1 provides details on pasture grass composition and animal food preference scores. Vegetative growth in this area initiates greening from late March to early April, reaches peak bloom from late June to late August, and begins to yellow by late September each year [19]. Throughout the experimental period, all animals had unrestricted access to water and did not receive any supplementary feeding.

Rumen fermentation parameters

The rumen-fluid samples, each amounting to 20 mL, were collected via an oral stomach tube prior to the morning feeding session at the end of the experiment [20]. To eliminate the risk of cross-contamination, the tube was rigorously sanitized with fresh warm water after each sampling. Furthermore, the first 50 mL of

rumen fluid obtained from each sheep was discarded to mitigate saliva contamination. pH level were measured using a portable pH meter (Model 206-pH2, Testo, Germany). Fresh rumen fluid is briefly filtered and divided into two parts for preservation. One part is injected into a 10 mL sterilized centrifuge tube using a syringe, frozen in liquid nitrogen, and stored at -80 °C in an ultra-low temperature freezer for nucleic acid extraction and subsequent amplification sequencing analysis. The other part is used to determine fermentation function indices (VFAs and NH_3-H) in the rumen fluid. It is injected into a 10 mL sterilized centrifuge tube, centrifuged at 4 °C at 15,000×g for 10 min, and a 3 mL aliquot of the supernatant is transferred into a centrifuge tube containing 0.3 mL of 25% metaphosphoric acid solution. The mixture is gently mixed and stored at -20 °C for later use. The frozen rumen fluid samples were thawed for VFA analysis, which was performed using gas chromatography with a GC-MS522 model [21] (Wufeng Instruments, Shanghai, China), equipped with a capillary column (AT-FFAP: 30 m × 0.32 mm × 0.5 μm). Additionally, the quantification of rumen ammonium nitrogen (NH_3-N) concentrations was carried out using a colorimetric spectrophotometer (UVVIS8500, Tianmei, Shanghai, China) [22].

DNA extraction and high-throughput sequencing

Total DNA was extracted from 2 mL of rumen fluid utilizing the MN NucleoSpin kit (Macherey-Nagel, Germany). The bacterial V3-V4 regions of the 16 S rRNA or ITS genes were amplified employing a Biometra TProfessional Thermocycler (Germany). Subsequent to amplification, the DNA products were subjected to 1.5% agarose gel electrophoresis and purified via gel extraction using the Agencourt AMPure XP Kit, in preparation for library construction. The sequencing libraries were prepared using the TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, United States), following the protocols specified by the manufacturer. The prepared libraries were quantified using the Qubit dsDNA High-Sensitivity Assay Kit (Invitrogen, Carlsbad, CA, United States). Paired-end sequencing was then conducted on an Illumina HiSeq 2500 PE250 platform (Illumina, San Diego, CA, United States), according to the standard procedures provided by Allwegene (Beijing, China).

Bioinformatics analysis

Sequences derived from the Illumina HiSeq platform were initially sorted by their distinct barcodes. These barcodes and primer sequences were then excised using QIIME software (version 1.9.0; http://qiime.org). The process proceeded with paired-end reads merging via FLASH (version 1.2.7), sequence trimming employing Trimmomatic (version 0.33), and chimera detection and removal using UCHIME (version 4.2) to refine the sequences (tags). Low-quality reads were eliminated using QIIME (version 1.7.0). The purified tags were then compared against the Gold database with UCHIME (version 4.2) to further remove chimeric sequences. Sequences that were potentially derived from the mitochondria or chloroplasts of consumed plants were also excluded. The resultant high-quality tags were utilized for subsequent analyses. Clustering of these tags into operational taxonomic units (OTUs) at a 97% similarity threshold was performed using Usearch software, with annotations based on the Silva taxonomy database for bacterial identification. Representative sequences were classified via the RDP classifier (version 2.2) against the SILVA (SSU123) database. This taxonomic profiling generated community structure maps, species distribution heat maps, and detailed taxonomic classifications (phylum, class, order, family, genus, and species) for each sample. For diversity metrics, QIIME (version 1.9.0) computed indices such as Chao1, Shannon, Simpson, ACE, and Good's coverage. Moreover, R software (Version 4.1.1) facilitated the creation of a weighted UniFrac distance-based principal co-ordinates analysis (PCoA) plot, delineating significant discrepancies among samples.

Statistical analysis

Statistical analyses were conducted using IBM SPSS software (version 27.0 for Windows). VFAs, relative abundance of bacteria, and alpha diversity indices were analyzed using a completely randomized design with one-way analysis of variance. All datasets were initially tested for normality and homogeneity of variance. To compare VFAs, alpha diversity indices, and the relative abundance of bacteria between the summer and winter groups, t-tests were applied. In cases where datasets did not meet the assumptions of normality or homogeneity of variance, non-parametric tests (Kruskal-Wallis) were used. Results are presented as the mean ± standard error of the mean, with statistical significance set at $P < 0.05$ for alpha diversity indices. Furthermore, the significance of bacterial composition was assessed using the Benjamini–Hochberg method for false discovery rate adjustment, with $P < 0.05$ considered significant for multiple comparisons. Microbial networks were delineated utilizing Gephi software (version 0.9.2) to explore correlations among predominant taxa. Keystone taxa within these microbial communities were pinpointed based on a composite score reflecting a high mean degree, high closeness centrality, and low betweenness centrality. Pearson correlation coefficients between the relative abundances of rumen bacteria (at the genus level) and short-chain fatty acids were computed using the heatmap package in R software (Version 4.1.1). The R software (Version 4.1.1) and the linkET package were used to perform the Mantel

test for analyzing the correlation between bacteria and fungi on volatile fatty acids. The mantel_test function was first employed to calculate the Mantel test correlation coefficient. Subsequently, the results of the analysis were visualized using the qcorrplot function from the linkET package. The functional pathways of the rumen microbiota were prognosticated employing PICRUSt2 (version 2.3.0_b) software, predicated on 16 S sequencing data. This prognostication was pivotal in forecasting the functional capabilities of the bacterial community within the rumen of Tan sheep across different seasons. Variations in the abundance of KEGG orthology groups at KEGG levels 1, 2, and 3 between the summer and winter seasons were also evaluated.

Results

Ruminal fermentation parameters

An analysis of ruminal fermentation in Tan sheep fed cellulase across different seasons was conducted to assess variations in VFAs, particularly the acetate to propionate ratio, NH_3-N, and pH levels (Fig. 1). While cellulase supplementation increased VFA concentrations ($P>0.05$), season was the determining factor ($P<0.05$), with this trend consistent for acetate, propionate, and valerate concentrations. Conversely, butyrate concentrations were lower in winter compared to summer ($P<0.05$), but cellulase supplementation increased butyrate levels, demonstrating an inverse effect during the summer months. The acetate to propionate ratio was higher in winter, but cellulase supplementation modified this distribution, enhancing the ratio in summer and reducing it in winter. A similar trend was observed for isobutyrate and isovalerate, which decreased in winter compared to summer, but cellulase supplementation reversed this effect across the seasons. Additionally, cellulase supplementation increased ruminal NH_3-N concentrations ($P<0.05$), with a more pronounced increase during summer ($P<0.05$). While pH levels remained largely unchanged across seasons, cellulase supplementation shifted rumen pH towards a more acidic environment, suggesting an influence of cellulase on ruminal fermentation dynamics.

Bacterial species diversity

Figure 2 displays the evaluation of bacterial and fungal alpha diversity indices within the rumen microbiota of sheep under varying treatments. Predominantly, the most elevated alpha diversity indices for bacteria, encompassing Chao1, Observed_species, PD_whole_tree, and Shannon, were documented in the W_CL group. This group was succeeded by the W_CK group and the S_CL group, with the S_CK group demonstrating the lowest diversity levels. An analogous trend was observed in the analysis of fungal diversity.

Comparison of bacterial community composition and otus between treatments

The Venn diagram presented in Fig. 3A and B showcases the distribution of OTUs unique to each treatment, as well as those shared among the different environmental conditions. A total of 1549 bacterial OTUs and 277 fungal OTUs were common across all four environments. Remarkably, the W_CL group displayed the greatest number of unique bacterial OTUs (245), a figure significantly exceeding those observed in the S_CL group with 89 OTUs, the W_CK group with 76 OTUs, and the S_CK group with 66 OTUs. Regarding fungal diversity, winter

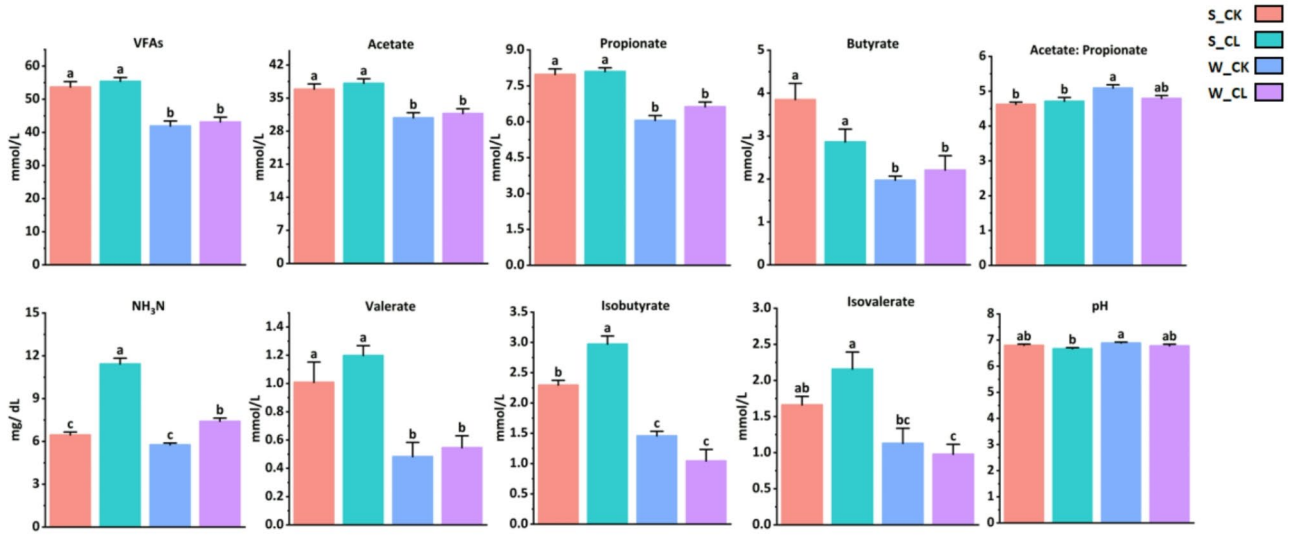

Fig. 1 Ruminal fermentation characteristics of Tan sheep fed cellulase in different seasons. [a, b, c] Boxes with different superscripts differ significantly ($P<0.05$). Abbreviations: S_CK = control group in summer; S_CL = cellulase group in summer; W_CK = control group in winter and W_CL = cellulase group in winter

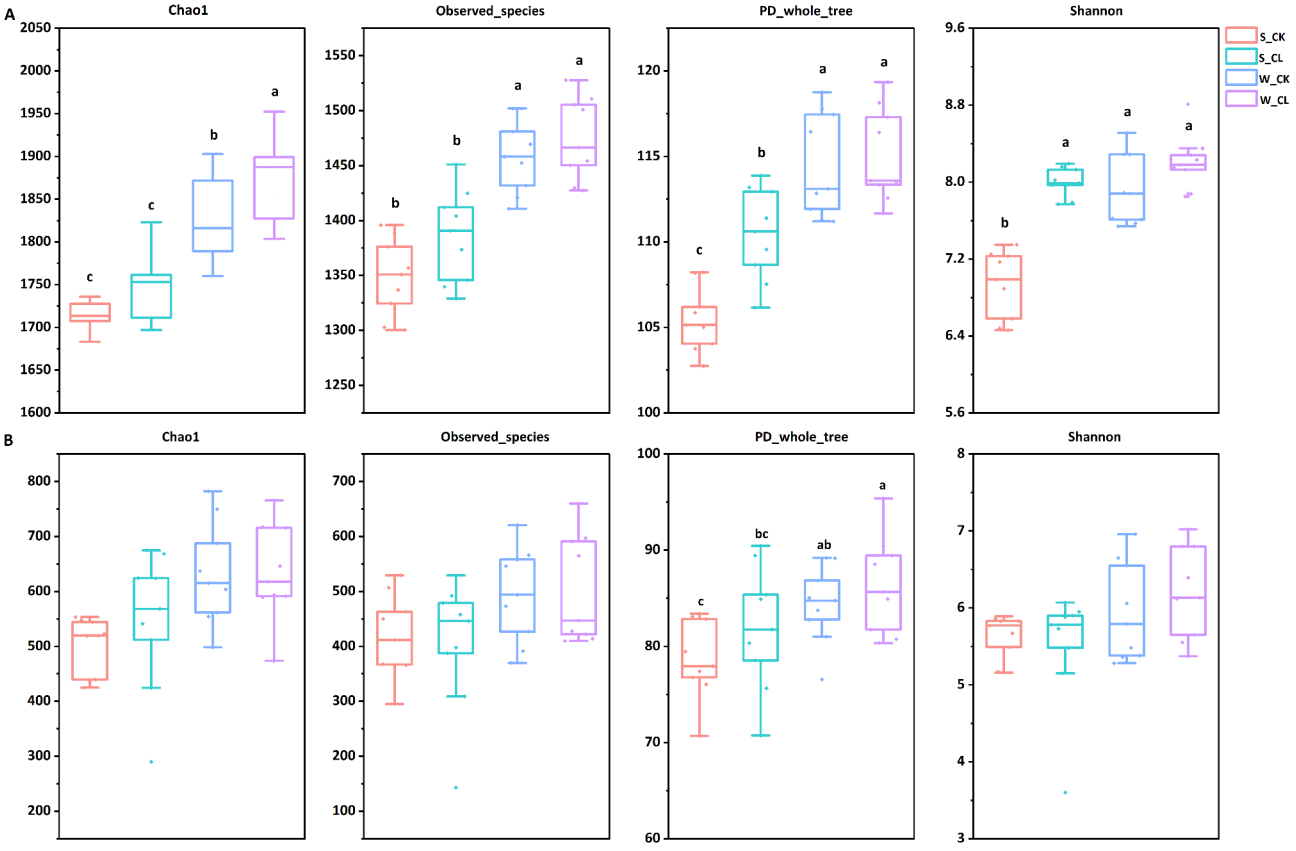

Fig. 2 Bacterial and fungal diversity indices of the rumen microorganisms of sheep fed cellulase in different seasons. [a, b, c] Boxes with different superscripts differ significantly ($P < 0.05$). Abbreviations: S_CK = control group in summer; S_CL = cellulase group in summer; W_CK = control group in winter and W_CL = cellulase group in winter

conditions notably augmented the count of unique fungal OTUs. However, the introduction of cellulase appeared to reduce the abundance of unique fungal OTUs (from 110 in the S_CK group to 71 in the S_CL group, and from 212 in the W_CK group to 80 in the W_CL group). PCoA depicted in Fig. 3C and D highlighted marked distinctions in the microbial communities contingent upon the administration of cellulase across different seasonal settings. In essence, despite the application of various treatments to the sheep, it was the seasonal influences that predominantly shaped the microbial community profiles.

Bacterial and fungal community compositions in rumen

Taxonomic analysis of the sequenced reads identified 21 bacterial phyla within the sheep rumen. The impact of various treatments on the prevalence of specific bacterial phyla (with an average relative abundance ≥ 1%). *Firmicutes* emerged as the most predominant phylum, with its relative abundance significantly increasing with cellulase supplementation across different seasons (S_CK 67.94%, S_CL 71.32%, W_CK 72.40%, and W_CL 81.41%, respectively; $P < 0.05$). Conversely, the proportion of *Bacteroidetes* decreased with cellulase supplementation in different seasons (S_CK 22.58%, S_CL 15.39%, W_CK 15.76%, and W_CL 9.05%, respectively; $P < 0.05$). *Actinobacteria* (S_CK 3.07%, S_CL 6.38%, W_CK 1.54%, and W_CL 2.84%, respectively), *Patescibacteria* (S_CK 1.36%, S_CL 1.77%, W_CK 3.55%, and W_CL 2.60%, respectively), and *Desulfobacterota* (S_CK 1.47%, S_CL 1.14%, W_CK 2.47%, and W_CL 1.66%, respectively) were also identified as dominant phyla. At the genus level, 240 taxa were detected. the *Christensenellaceae_R-7_group* was the most abundant genus, showing variation with cellulase supplementation (S_CK 9.06%, S_CL 11.49%, W_CK 8.30%, and W_CL 9.46%, respectively). Other notable genera include *Rikenellaceae_RC9_gut_group* (S_CK 5.68%, S_CL 4.88%, W_CK 12.48%, and W_CL 6.33%, respectively), *Prevotella* (S_CK 10.78%, S_CL 5.25%, W_CK 1.59%, and W_CL 1.86%, respectively), and *NK4A214_group* (S_CK 5.74%, S_CL 9.22%, W_CK 1.45%, and W_CL 1.40%, respectively). These findings underscore the significant alterations in microbial community composition attributable to cellulase supplementation and seasonal variations, as visually represented in Fig. 4A (for phyla) and Fig. 4B (for genera).

Taxonomic analysis of sequenced reads identified 11 fungal phyla within the sheep rumen. *Ascomycota* emerged as the most abundant phylum, with its relative

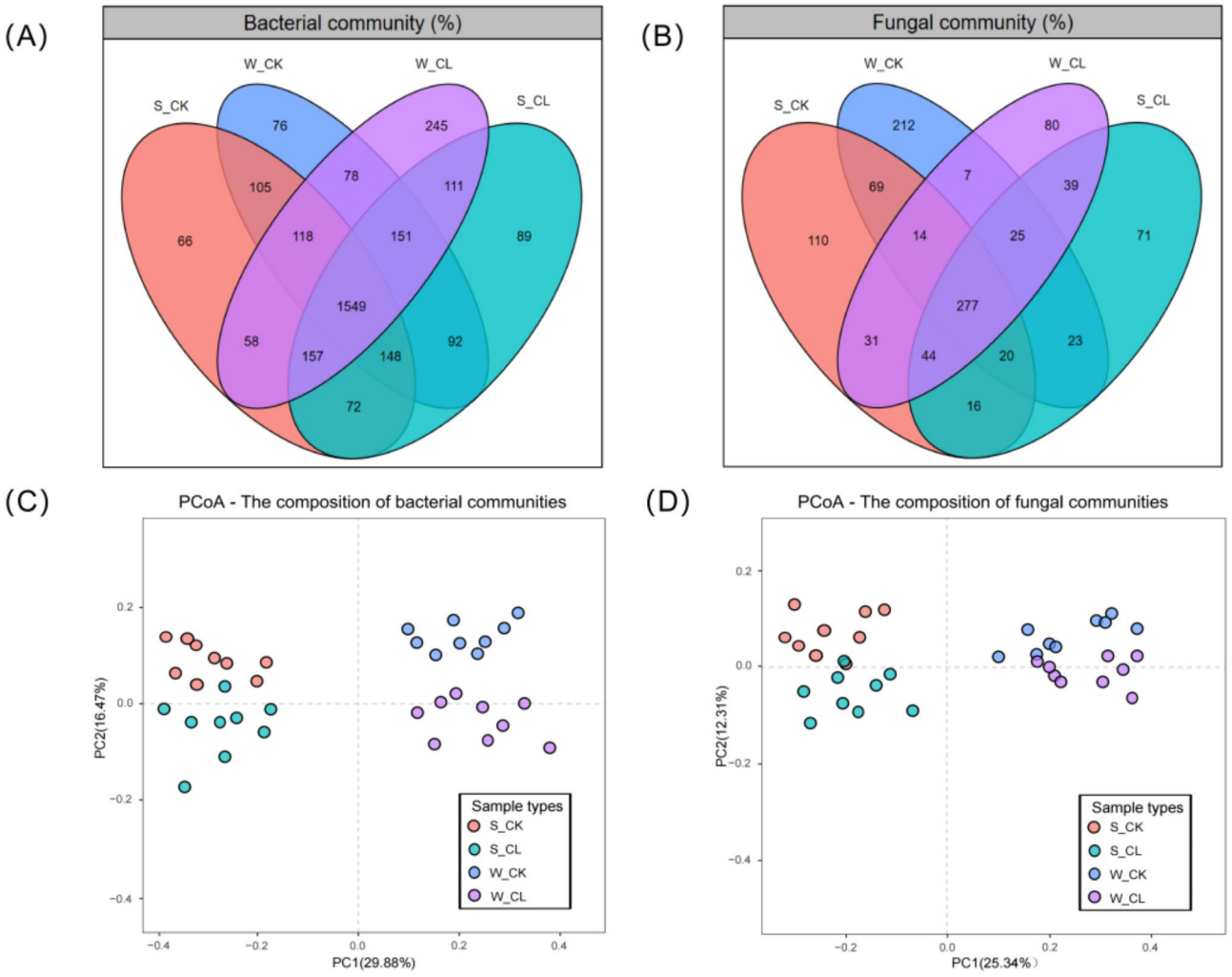

Fig. 3 Differences in community dissimilarities and operational taxonomic units. Venn diagram (**A**, **B**) indicates specific and shared OTUs in both cellulase and seasons. The weighted UniFrac distance (**C**, **D**) was used to calculate the differences in Tan sheep rumen microbiota in the different treatments, and principal co-ordinates analysis (PCoA) was used to calculate the coordinates. Abbreviations: S_CK = control group in summer; S_CL = cellulase group in summer; W_CK = control group in winter and W_CL = cellulase group in winter

abundance significantly increasing in conditions involving cellulase supplementation and during the winter season (S_CK 50.39%, S_CL 57.22%, W_CK 62.68%, and W_CL 68.07%, respectively). Conversely, the proportion of *Neocallimastigomycota* decreased under these same conditions (S_CK 34.11%, S_CL 30.00%, W_CK 23.83%, and W_CL 21.79%, respectively). *Basidiomycota* (S_CK 10.41%, S_CL 11.24%, W_CK 11.29%, and W_CL 9.37%, respectively) and *Chytridiomycota* (S_CK 1.36%, S_CL 1.77%, W_CK 3.55%, and W_CL 2.60%, respectively) also stood out as dominant phyla. At the genus level, 347 taxa were identified. Piromyces was the most prevalent genus across the board, demonstrating an increase in abundance with cellulase supplementation and during winter (S_CK 7.67%, S_CL 10.95%, W_CK 14.12%, and W_CL 16.26%, respectively). Other notable genera include *Thelebolus* (S_CK 8.60%, S_CL 10.42%, W_CK 11.01%, and W_CL 14.00%, respectively), *Acremonium* (S_CK 6.53%, S_CL 9.48%, W_CK 9.87%, and W_CL 12.59%, respectively), and *Didymella* (S_CK 6.98%, S_CL 7.86%, W_CK 8.21%, and W_CL 12.10%, respectively). These findings highlight significant shifts in fungal community composition due to cellulase supplementation and seasonal influences, as visually represented in Fig. 4C (for phyla) and Fig. 4D (for genera).

Correlations between bacterial communities and rumen fermentation parameters

The correlation analysis between the relative abundance of rumen bacterial genera and fermentation parameters (Fig. 5) demonstrated significant associations. The correlations were deemed significant at $P < 0.05$, indicating a substantial relationship between the abundance of specific rumen bacterial genera and the concentrations

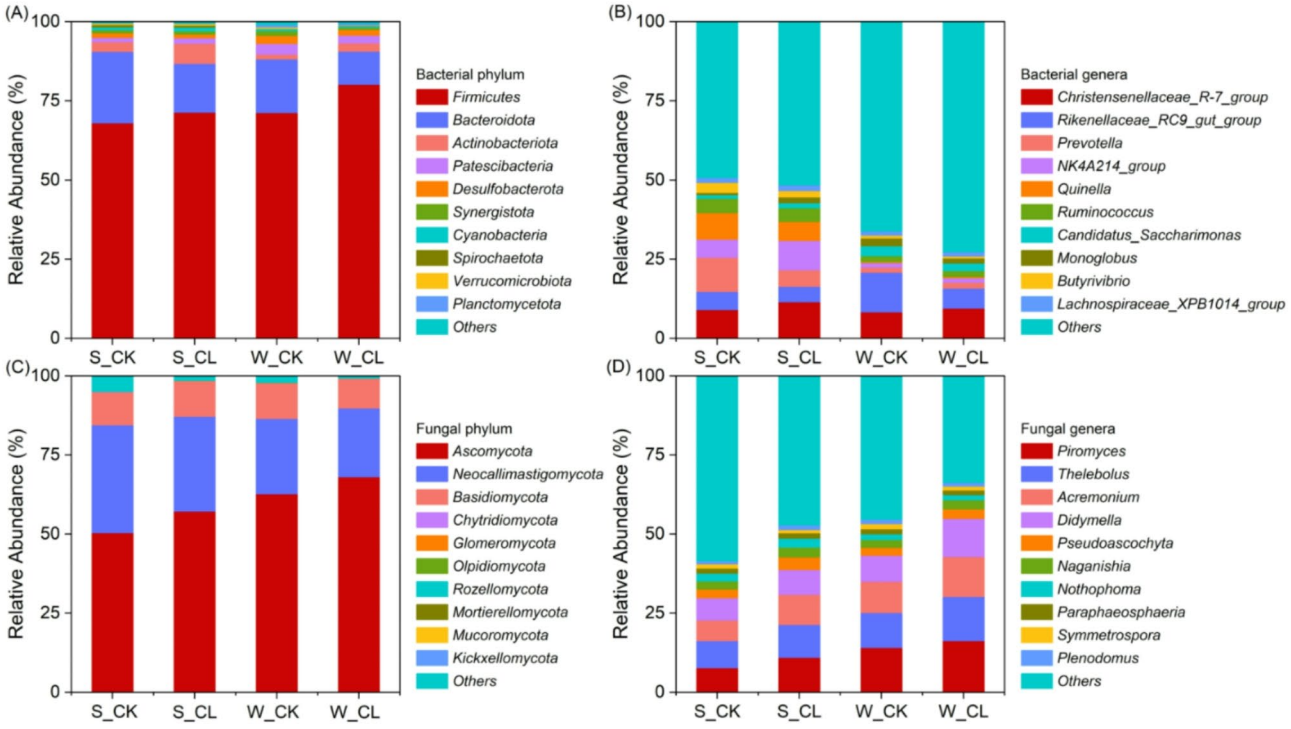

Fig. 4 The relative abundance of dominant bacterial (**A**, **B**) and fungal (**C**, **D**) communities at the phyla and genus level in the different seasons and treatments. Only taxa with an average relative abundance > 0.5% are displayed. Abbreviations: S_CK = control group in summer; S_CL = cellulase group in summer; W_CK = control group in winter and W_CL = cellulase group in winter

of rumen NH_3-N and VFAs. The pH level was directly linked to the relative abundance of the genus *Candidatus_Saccharimonas*, while it exhibited a negative correlation with the abundances of *Prevotella* and *Quinella*. The NH_3-N concentration showed a positive correlation with the abundances of the genera *Selenomonas*, *Lachnospiraceae_NK3A20_group*, and *Quinella*, as well as *Prevotella* and *Butyrivibrio* at a higher significance level ($P < 0.01$). Conversely, it was inversely related to the abundance of *Jeotgalicoccus*, *Planomicrobium*, and *Psychrobacillus*. The concentration of VFAs was positively correlated with the relative abundances of *DNF00809*, *Prevotella*, *Lachnospiraceae_NK3A20_group*, *NK4A214_group*, *Ruminococcus*, *Butyrivibrio*, and *Quinella* at a higher significance level ($P < 0.01$), and negatively correlated with *Carnobacterium*, *Jeotgalicoccus*, *Planomicrobium*, and *Psychrobacillus*. The acetate molar proportion exhibited a positive correlation with the abundance of *Christensenellaceae_R-7_group*, *Prevotella*, *Lachnospiraceae_NK3A20_group*, *NK4A214_group*, and *Ruminococcus*, but was negatively correlated with *Carnobacterium* and *Planomicrobium*. The propionate molar proportion was positively associated with *Prevotella* and *NK4A214_group*. Conversely, the butyrate molar proportion showed a negative association with *Prevotella* abundance. The isobutyrate and valerate molar proportions were negatively correlated with the abundances of *Carnobacterium*, *Jeotgalicoccus*, *Planomicrobium*, and *Psychrobacillus*. Lastly, the isovalerate molar proportion was negatively correlated with the abundance of *DNF00809*, *Lachnospiraceae_NK3A20_group*, *NK4A214_group*, *Ruminococcus*, *Butyrivibrio*, and *Quinella*.

Bacterial and fungal co-occurrence patterns, network complexity, and the stability and synergy

The examination of bacterial and fungal community networks across different habitats revealed unique co-occurrence patterns, as depicted in Fig. 6. Distinct bacterial and fungal interaction networks were identified within varied environments. The complexity of these networks was evaluated using topological parameters such as the number of nodes and edges, along with the degree of interaction, where a higher quantity of nodes and edges combined with a lower degree signifies a more complex network structure. Additionally, the ratio of negative to positive correlations (neg/pos) within the bacterial network served as an indicator of network stability, with a higher ratio denoting enhanced stability. The analysis demonstrated that the most intricate bacterial and fungal interaction networks were present during the winter season, followed by the summer season. Furthermore, the introduction of cellulase was found to increase the complexity of these interactions within both bacterial and fungal communities. Despite the summer ecosystem

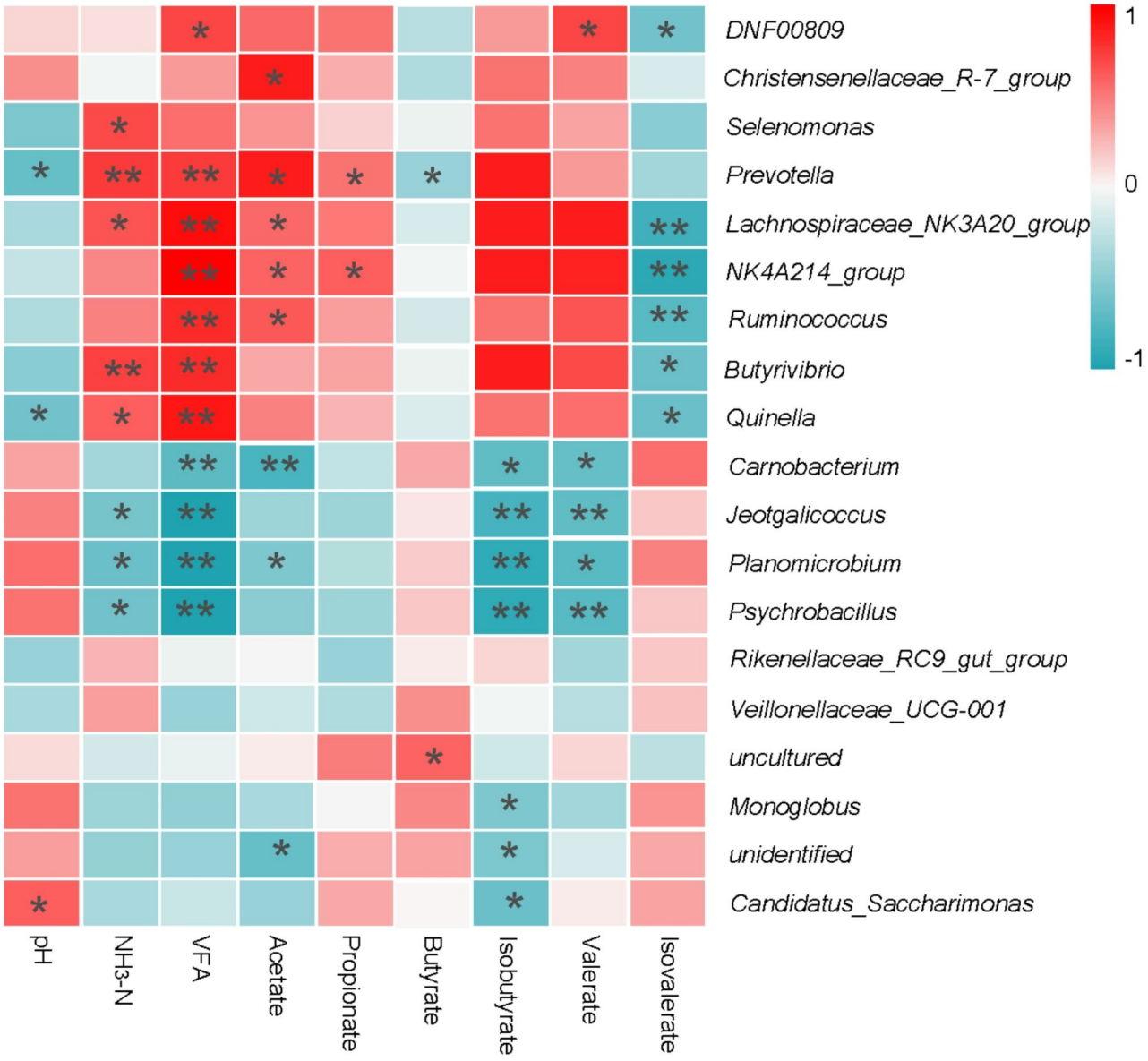

Fig. 5 Relationship among NH_3-N, bacterial Communities, and volatile fatty acids (VFAs). ** and * indicate significance levels at 0.01 and 0.05, respectively

displaying the lowest number of OTUs, diversity, and other metrics, it was noted that the highest stability of bacterial and fungal communities was observed during the summer period. This suggests a notable variation in community dynamics and stability across seasons and with the introduction of cellulase, indicating the influence of environmental conditions and dietary supplements on microbial ecosystem interactions.

Rumen fermentation parameters and microbial composition

Bacterial and fungal communities within the rumen exhibited distinct correlations with various fermentation parameters (Fig. 7). They showed a negative correlation with VFAs and propionate, indicating that as the concentrations of VFAs and propionate increased, the diversity or abundance of these microbial communities tended to decrease. Conversely, a positive correlation was observed with acetate, isobutyrate, the acetate/propionate ratio, pH, and NH_3-N, suggesting that increases in these parameters were associated with increases in the diversity or abundance of bacterial and fungal communities. The strongest correlation was noted with the acetate/propionate ratio. This particular ratio is a critical indicator of the fermentation pattern within the rumen, reflecting the balance between these two major VFAs. A higher acetate/propionate ratio is often associated with a more fibrous diet and is indicative of a rumen environment that supports methanogenesis and fibrolytic bacterial activities. The positive correlation with acetate

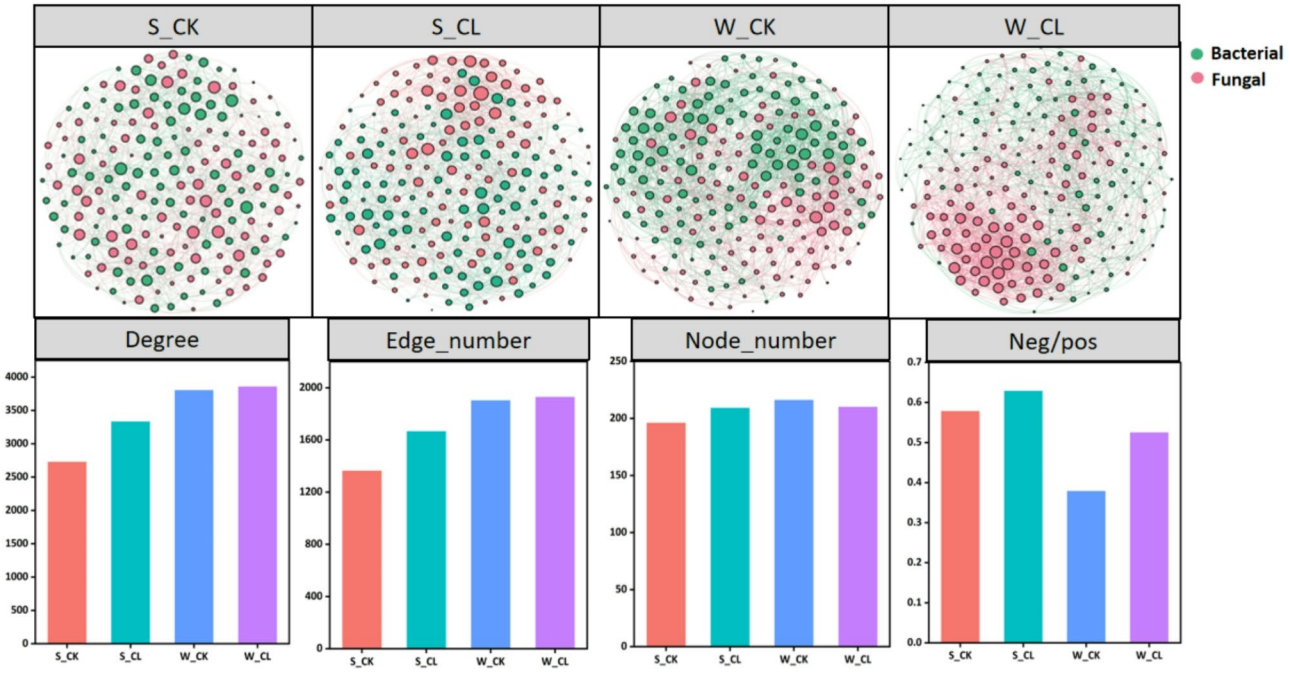

Fig. 6 Co-occurrence network of bacteria and fungi in the rumen microorganism in the different treatment and seasons. The number of nodes and edges and the degree of bacteria and fungi in the different treatment and seasons co-occurrence patterns. Neg/ pos, the ratio of negative correlation to positive correlation. Abbreviations: S_CK = control group in summer; S_CL = cellulase group in summer; W_CK = control group in winter and W_CL = cellulase group in winter

and the acetate/propionate ratio highlights the potential influence of these microbial communities on enhancing fiber degradation and altering rumen fermentation towards a more acetogenic profile. Similarly, the positive correlation with isobutyrate and NH_3-N further underscores the complex interactions between rumen microbiota and the fermentation end-products, which can have significant implications for the host's energy balance and overall health.

PICRUSt2 gene function estimation

Using PICRUSt2 for functional prediction of the rumen microbiota in sheep subjected to different treatments revealed significant insights into the metabolic capabilities of these microbial communities (Fig. 8). The analysis indicated that the biosynthesis of Valine, Leucine, and Isoleucine was the most represented functional pathway across all treatments, accounting for 2.39% of the relative abundance. This was closely followed by D-Glutamine and D-Glutamate metabolism, which represented 2.21%, and the Biosynthesis of vancomycin group antibiotics at 2.16%. These findings highlight the crucial role of the rumen microbiota in amino acid metabolism, which is vital for the nutritional health and growth of the host. The PICRUSt2 analysis further identified 43 predominant pathways with relative abundances exceeding 1% within the level 3 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Of these, 27 pathways exhibited significant differences across the treatments ($P < 0.05$), as illustrated in Fig. 8. This variance underscores the impact of different treatments on the functional potential of the rumen microbiome, reflecting how dietary supplements, seasonal changes, or other environmental factors can influence microbial gene expression related to essential metabolic processes.

Discussion

Ecosystems characterized by high species diversity are acknowledged for their enhanced stability and superior functionality in terms of ecosystem operations [23, 24]. The diversity of interspecies interactions across ecosystems plays a pivotal role in this dynamic, where greater species diversity promotes functional redundancy, thus preserving ecosystem functionalities in the face of species loss [25, 26]. Within the rumen microbial ecosystem of ruminants, a diverse array of microbial communities is responsible for synthesizing distinct digestive enzymes, which are essential for optimizing the digestion and absorption of feed [27]. This microbial diversity is often associated with an augmented metabolic capacity and ecosystem stability [28]. Our research has documented significant seasonal variations in the rumen microbial diversity of Tan sheep, notably identifying a higher diversity during the winter as opposed to the summer. This seasonal fluctuation implies that the rumen microbial community in Tan sheep is potentially more efficient

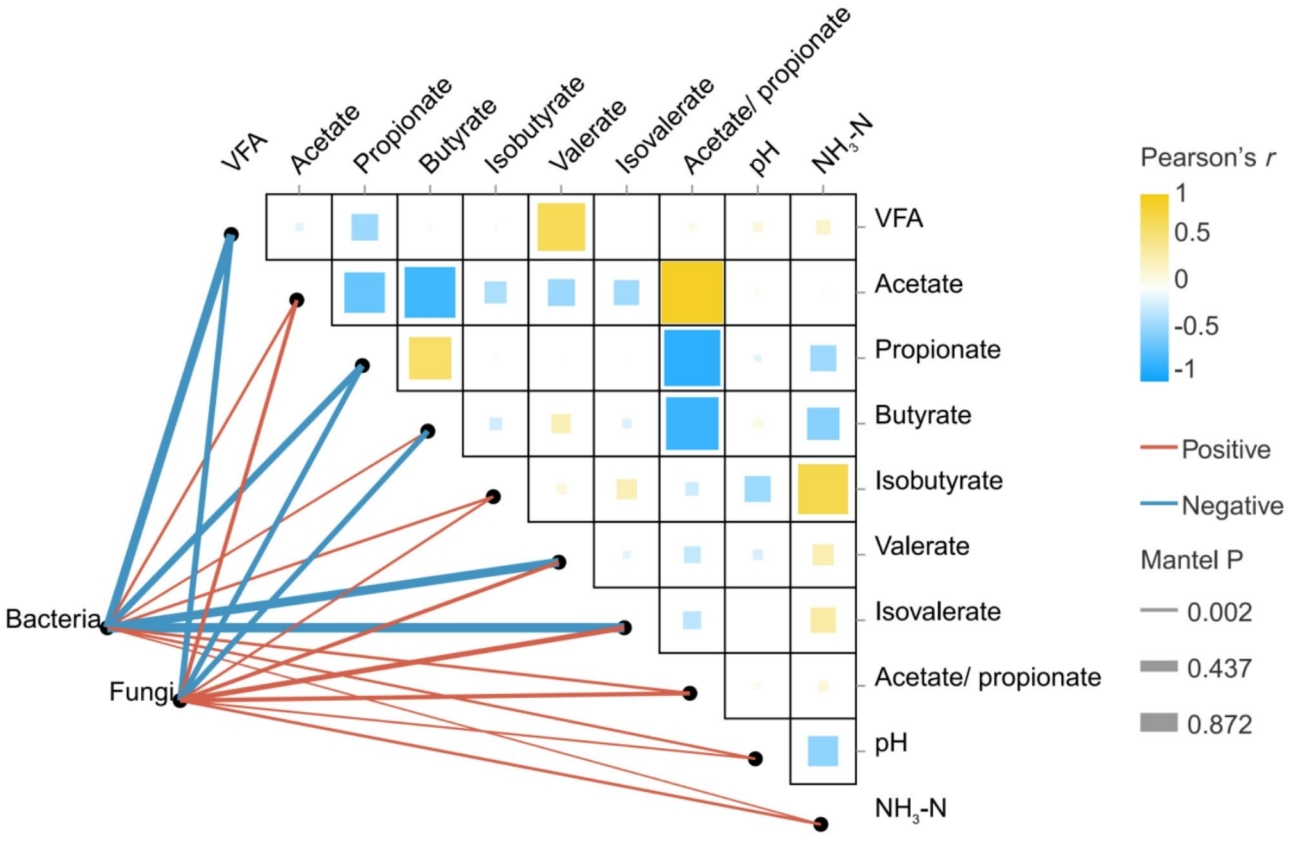

Fig. 7 Interaction networks of the rumen microbiota. 16 S rRNA and ITS gene-based correlation network of the rumen microbiota, displaying statistically significant interactions with absolute value of correlation coefficients > 0.6. The node size was scaled based on the overall abundance of each taxa in the microbiota. A red edge indicates a positive correlation and blue edge indicates a negative correlation

at processing high-fiber feeds during winter droughts, thereby playing a crucial role in fulfilling the energy demands within challenging environments [10, 11]. Furthermore, our findings indicate that the introduction of cellulase contributes to an enhancement in rumen microbial diversity. Previous investigations have demonstrated that cellulase application can elevate the α diversity index in mixed silage samples composed of corn straw and soybean residue, suggesting an increase in microbial flora diversity and richness [29] In contrast, some reported a decrease in the Chao1 index in alfalfa silage samples treated with cellulase [30]. A conceivable explanation for this disparity is that cellulase supplementation introduces additional carbon and nitrogen sources that favor the fermentation processes of lactic acid bacteria, facilitating their proliferation and the accumulation of lactic acid. This, in turn, may suppress the growth of detrimental bacteria, potentially leading to a decrease in microbial diversity [31–32].

The rumen fungal community exhibits remarkable adaptability, enabling it to adjust its diversity and functionality in response to the prevailing environmental conditions effectively [33]. The removal of fungi from the rumen environment has been shown to diminish the degradation rates of DM, NDF, and ADF, along with the activity of carboxymethyl cellulase [34]. Such a notable degradation capability is attributed to the fungi's production of enzymes, including α-amylase and amyloglucosidase, which efficiently break down starch and maltose [35]. This observation is consistent with our findings, indicating that the nutrient quality of forage diminishes in winter, a change that positively correlates with an increase in the proportion of rumen fungi and the fiber content of feed. Moreover, the addition of cellulase to forage has been found to enhance the decomposition process within the rumen, thereby facilitating the synthesis of carbohydrates [36]. These insights highlight the critical role of seasonal variations in shaping rumen microbial diversity and elucidate their consequential impact on the digestive efficiency of ruminants. Specifically, these variations influence the ability of ruminants to extract energy from their diet, underlining the importance of understanding microbial dynamics within the rumen to optimize feed utilization and energy extraction, especially during challenging periods such as winter [10, 37].

Rumen bacteria constitute essential microorganisms within ruminants, with their population and diversity significantly influenced by the diet, breed, and

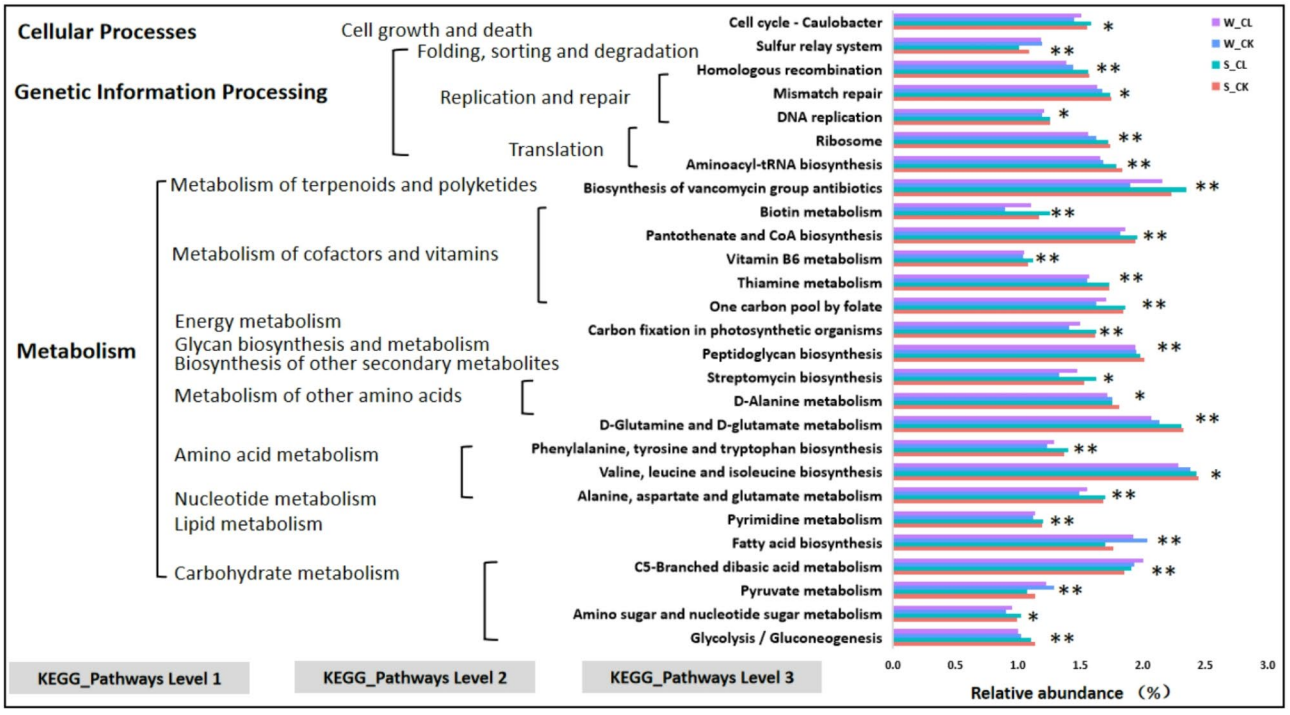

Fig. 8 Relative abundance of predicted functions and significance of KOs (KEGG orthology groups) at KEGG (Kyoto Encyclopedia of Genes and Genomes) level 3 between the summer and winter. Student's t-tests were used to compare the abundance changes two groups; only differences with a $P < 0.05$ are reported. Abbreviations: S_CK = control group in summer; S_CL = cellulase group in summer; W_CK = control group in winter and W_CL = cellulase group in winter

environmental conditions [33]. In our investigation, *Firmicutes* and *Bacteroidetes* were identified as the dominant bacterial phyla in the rumen of Tan sheep, some reported similar average proportions of these bacteria across major livestock species through a 16 S rRNA meta-analysis [38]. These bacterial groups are crucial for energy conversion, carbohydrate degradation, and protein hydrolysis. Notably, the abundance of *Firmicutes* increased during winter, an effect that was further amplified by the addition of cellulase. An elevated *Firmicutes*/*Bacteroidetes* ratio is indicative of enhanced energy utilization and improved resistance to cold stress in the pastures grazed by Tan sheep [11]. The introduction of cellulase might enhance this effect, potentially through synergistic actions with endogenous cellulases [39]. Moreover, the *Firmicutes*/*Bacteroidetes* ratio has been associated with body fat storage and obesity in animals, attributed to the rich metabolic enzyme repertoire of *Firmicutes* facilitating energy absorption and fat deposition [12, 33]. Our previous research revealed that the addition of cellulase did not significantly alter sheep feed intake, suggesting that weight gain was not a result of increased consumption [40]. Rather, it is hypothesized that cellulase might enhance carbohydrate metabolism through the increased abundance of *Firmicutes*, leading to fat deposition [11]. At the genus level, *Christensenellaceae_R-7_group*, *Rikenellaceae_RC9_gut_group*, and *Prevotella* were notably dominant within Tan sheep. *The Christensenellaceae_R-7_group*, implicated in human health and body mass index regulation [40], showed increased abundance with cellulase supplementation, suggesting its potential influence on body mass index through enhanced carbohydrate hydrolysis and fat synthesis [41]. *Rikenellaceae_RC9_gut_group*, associated with body fat and volatile fatty acid production [42], provided a contrast to our findings, possibly due to variances in feeding regimes. *Prevotella*, known for its role in hemicellulose degradation [43, 44], was more prevalent during summer, reflecting the higher nutritional quality and reduced fiber content of summer herbage, thus facilitating digestion. In conclusion, *Christensenellaceae_R-7_group*, *Rikenellaceae_RC9_gut_group*, and *Prevotella* play pivotal roles in the digestion and absorption of carbohydrates within the rumen. The abundance of *Prevotella*, in particular, may serve as an indirect marker for the level of carbohydrate digestion in ruminants.

Fungi play an indispensable role in the degradation of plant biomass into soluble sugars, supplying vital metabolites and nutrients to the host animal [45]. It has been demonstrated that the direct administration of rumen anaerobic fungi can enhance feed intake, milk quality, and yield in ruminants [46, 47]. In our research, the dietary supplementation with cellulase in Tan sheep diets resulted in an increased relative abundance of

Ascomycetes, *Neoflagellates*, and *Basidiomycetes*. This augmentation led to an improvement in the apparent digestibility of NDF and ADF in sheep, corroborating our earlier findings [39]. Some researchers noted a marked reduction in the relative abundance of anaerobic fungi, including *Neotrichobena* and *Pyristobena*, in dairy cows transitioning from coarse to high-concentrate diets, which precipitated subclinical acidosis [48]. In contrast, our study indicated that cellulase supplementation increased the relative abundance of *Pyridinium* in the rumen of Tan sheep, particularly during the winter months as compared to summer. This pattern is consistent with several studies that associate the abundance of anaerobic fungi with the dietary fiber content [45]. Cellulase formulations are recognized for their capacity to trigger the secretion of endogenous enzymes and to supplement any deficiencies therein, thereby influencing their activity and augmenting the abundance of *Piritococcus*, which are key agents in plant cell wall degradation [49]. Furthermore, cellulase has been observed to affect microbial activity [50]. In the context of plant continuous crop disorders, diminished cellulase activity has been linked to *Thelebolus*, a genus associated with the generation of cellulase activity [51]. Our findings propose that the noted increase in the relative abundance of *Thelebolus* may be ascribed to cellulase supplementation. Nonetheless, the precise physiological roles of fungi within the digestive tract, as well as the interactions between anaerobic and facultatively anaerobic fungi, remain elusive. Therefore, delving into these physiological intricacies through metagenomic and metaproteomic methodologies continues to be a vital field of investigation.

Ruminants efficiently absorb VFAs through the rumen wall during the fermentation process, with the dietary composition exerting a significant influence on the levels of VFAs within the rumen [52]. In our investigation, an increase in acetic acid production was observed during the winter, typically linked to fiber degradation processes [53]. This surge in acetic acid production is likely attributable to the enhanced degradation of fiber in colder conditions. Furthermore, the incorporation of cellulase into the diet led to a decrease in acetic acid content and the acetate to propionate ratio, thereby modifying the rumen fermentation profile and augmenting rumen fermentation efficiency [53, 54]. Bacteria such as *Vibriobutyricum* and *Prevotella* exhibited a positive correlation with the concentration of total VFAs, underscoring their pivotal role in VFA production [55, 56]. Additionally, specific bacterial genera, including *Prevotella* and the *Ruminococcaceae_NK4A214_group*, were positively correlated with the concentrations of acetic acid, propionic acid, and total VFAs. These microbial agents are crucial in VFA biosynthesis, with *Prevotella* particularly involved in the conversion of monosaccharides and polysaccharides into propionate [55]. *Ruminococcaceae* are recognized for their fiber-degrading capabilities, facilitating the production of short-chain fatty acids available for host utilization [57]. However, the intricate interactions between bacterial species, encompassing resource competition and cross-feeding mechanisms, present challenges in definitively attributing the production of specific VFAs to particular bacteria [33, 58]. Some reported a higher abundance of key cellulolytic and proteolytic bacteria in the rumen of grazing yak during summer as opposed to winter [59]. This observation aligns with the enhanced nutrient digestibility and average daily weight gain reported in summer for Tan sheep [39]. Consequently, it is hypothesized that an increased presence of functionally significant rumen bacteria during the summer months amplifies feed digestibility, leading to higher concentrations of NH_3-N and VFAs. This enhancement likely contributes to the observed rapid improvements in the growth performance of Tan sheep.

OTUs within the bacterial and fungal metanetworks, categorized across various treatments and seasons, reveal the nuanced response of microorganisms to environmental factors under differing conditions [33]. Our analysis indicated that the complexity of the bacterial and fungal communities was most pronounced during the winter and least evident in summer. In bacterial communities, negative interactions tend to reduce competitive relationships, while positive interactions intensify them [60, 61]. Thus, a greater ratio of negative to positive interactions is indicative of diminished competition among microbial communities, leading to increased stability in the microbial network structure [62]. In our study, the stability of the bacterial and fungal networks was highest in the summer test group, followed by the summer control group, the winter addition group, and was lowest in the winter control group. This observation is consistent with prior studies, which suggest that mature rumen habitats display significant stability [59, 61]. Nutritional interventions often show limited long-term impact, with microbiomes typically reverting to their pre-intervention state. This resilience of mature rumen microbial ecosystems against external factors, including seasonal variations, highlights their intrinsic stability [63] Functional profiling of rumen microbiome genes in Tan sheep revealed a considerable portion of these genes are involved in membrane transport and cellular metabolism, encompassing lipid and protein metabolism, with an overexpression noted during winter drought periods. This suggests that Tan sheep might adjust to the reduced nutritional quality of winter forage through modulating the metabolic functions of their rumen microflora [64]. However, it is critical to acknowledge that these results are predictive, grounded in genomic data, and may not fully elucidate the actual functional dynamics of rumen bacteria in Tan

sheep. Hence, the application of advanced metagenomic techniques is essential for a more comprehensive understanding of how these genes aid the animals' adaptation to harsh environmental conditions.

Conclusion

The diverse and abundant microorganisms within the rumen form a complex ecosystem capable of degrading cellulose into volatile fatty acids, sugars, and proteins, thereby making these nutrients available to the host through synergistic interactions. To date, research into the breakdown of cellulose by rumen bacteria and anaerobic fungi, coupled with the application of omics techniques to investigate the structure, metabolic pathways, and degradation functions of the rumen microbial community, has yielded significant achievements. However, there remains a substantial need for further investigation into the structure and function, metabolism and regulatory mechanisms, and the enzymatic systems and mechanisms underlying the production of cellulase complexes by rumen bacteria and anaerobic fungi. It is anticipated that advancements in technology, particularly in the refinement and expansion of macromolecular combined analysis techniques, will unveil the intricate processes involved in rumen microbial degradation of cellulose. Such insights are expected to inspire novel approaches for the development and exploitation of cellulosic resources, enhancing our understanding of this crucial biological process and potentially leading to innovative applications in bioenergy, animal nutrition, and beyond.

Abbreviations

S_CK	Control group in summer
S_CL	Cellulase group in summer
W_CK	Control group in winter
W_CL	Cellulase group in winter
VFAs	Volatile fatty acids
NH_3-N	Ammonia nitrogen
OTUs	Operational taxonomic units
PCoA	Principal co-ordinates analysis

Supplementary Information

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Supplementary Material 1

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Author contributions

HR, Shi., Writing – original draft, Software, Data curation. P, Guo., Investigation, Data curation. Z, Wang, Data curation. JY, Zhou and MY, He: Software, Data curation. LY, Shi, XJ, Huang and PH, Guo Methodology, Data curation. ZX, Guo Investigation, Data curation. YW, Zhang, Validation. FJ, Hou, Writing – review & editing, Visualization, Validation, Project administration, Conceptualization. All authors contributed to the article and approve the submitted version.

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Data availability

The raw sequencing data reported in this study have been has been uploaded to the National Center for Biotechnology Information platform, with the accession number PRJNA1111033, If the article is accepted, I will immediately release the original sequencing data access.

Declarations

Ethics approval and consent to participate

The animal study was reviewed and approved by Animal Care and Use Committee of Lanzhou University (Protocol number: LZU 201805010). Written informed consent was obtained from the owners for the participation of their animals in this study, No animals were slaughtered or anesthetized during the experiment, and all animals keep on living for other experimental work.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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