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Effects of different fiber levels of energy feeds on rumen fermentation and the microbial community structure of grazing sheep

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Abstract

Background Rumen microbial community structure and stability are very important for ruminant health, growth and development, and livestock product yield. Dietary composition and nutritional structure affect microbial diversity and richness. The purpose of this study was to evaluate the effects of different fiber levels of energy feed on the rumen microflora and fermentation function of grazing sheep in salinized sown pasture, to reveal the response of the main microflora of sheep rumen at the phylum and genus levels to different fiber levels of energy feed and to analyze the internal mechanism to provide a reference for the selection of energy feed and the improvement of the production performance of grazing livestock.

Results The fiber level of energy feed affects the rumen fermentation and rumen microbial community structure of grazing sheep. Low-fiber-energy feeds significantly increased the relative abundance of *Actinobacteria*, while the relative abundances of *Cyanobacteria*, *Ruminococcaceae_UCG_010*, *Ruminococcaceae_NK4A214_group*, and *Elusimicrobium* significantly decreased, adjusting the relationship between the flora toward cooperation. High-fiber-energy feeds significantly increased the concentration of VFAs, significantly decreased the relative abundances of *Proteobacteria*, *Ruminococcaceae_NK4A214_group* and *Rikenellaceae_RC9_gut_group*, adjusted the relationship between the flora to compete, and promoted the enrichment of metabolic pathways such as "Protein Digestion and Absorption," "Nitrogen Metabolism," "Starch and Sucrose Metabolism," and "Degradation of Other Sugars."

Conclusions Supplementary feeding of high and low fiber energy feeds reduced the pH value of rumen fluid and the richness and diversity of microorganisms in grazing sheep, reduced the relative abundance of some harmful microorganisms, affected the metabolic activities of some fiber-digesting bacteria, regulated the interaction and competition between bacteria, increased the content of volatile fatty acids (VFAs) and the relative abundance of metabolic-related microorganisms in the supplementary feeding group, and enriched the metabolic-related pathways. However, further understand the mechanism of the effect of fiber level on the rumen of sheep, it is necessary to conduct in-depth analysis using research methods such as transcriptomics, proteomics and metabolomics.

Keywords Energy feed, Fiber level, Ruminal fermentation parameters, Ruminal microorganisms, Grazing sheep

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Implications

Through an in-depth understanding of the effects of different fiber levels of energy feed on the composition and function of sheep intestinal flora, the response of different rumen flora to fiber levels can be revealed, and a feed formula can be selected and optimized to improve the production performance of sheep in areas limited by the quality and yield of forage resources. The in-depth exploration and practice of this research direction will provide useful guidance and support for the development of ruminant breeding-related industries in salinized sown pasture.

Introduction

By 2050, the global demand for meat will double to meet the changes in resources and eating habits required by the growing population to maintain basic living [1, 2], which will prompt animal husbandry around the world to produce more livestock products [3–5]. Grazing livestock contributes nearly half of the world's livestock products, and improving the production performance of grazing livestock is one of the most effective measures for meeting the increasing demand for livestock products.

Seasonal changes in forage resources and frequent extreme climates (droughts, sandstorms, etc.) restrict the efficient production of livestock in grazing systems, and energy and nitrogen are the main limiting factors [6–8]. Supplemental feeding is a means of increasing livestock production per unit of time by maximizing weight gain [9]. Wheat, an energy feed for small ruminants that integrates the economy and benefits, is the world's second largest grain crop. In recent years, global wheat production has shown a steady growth trend. Wheat bran is a byproduct of wheat seed processing, and the development of the animal husbandry and corn deep-processing industry has led to increasing domestic demand for corn. The price of soaring traditional corn–soybean meal-type feeds has lost cost effectiveness, and wheat and bran can be partially or completely replaced by corn to save feed costs, creating considerable economic benefits for the livestock industry [10–13].

The rumen is the main battlefield for ruminant forage digestion, and the natural fermentation tank has the strongest known ability to degrade fiber materials. The different microbial groups in the rumen cooperate or compete with each other to maintain a stable microbial flora and internal environment [14], and rumen microbial metabolites (VFAs, bacterial proteins, etc.) affect host production performance and product quality [15–17]. The nutritional level of the diet is the main factor affecting rumen microbial diversity, health status, and productivity [18, 19]. An alteration in the host diet influences rumen microbial metabolism, which alters VFAs and methane production, affecting the production of meat, milk, and other livestock products. We used fiber level as the main measure of the difference in nutrient composition between wheat and wheat bran energy feed. Supplementing grazing livestock with wheat and wheat bran will inevitably change the diet structure and thus affect rumen fermentation while improving production performance. The ratio of concentrate to roughage will destroy the rumen environment, reduce the pH, and change the rumen fermentation mode and the type and concentration of metabolites [20–24]. Does the fiber level of energy feed also affect the rumen fermentation of ruminants, and what is the effect and mechanism of fiber level on the microflora in the gastrointestinal tract of ruminants? To this end, we conducted an experiment in a saline-sown pasture in the inland arid zone of Northwest China to study the effects of different fiber levels of added energy feed on the rumen fermentation parameters and the rumen microorganisms of sheep grazing on a sown pasture, which provides insight into the relationships between the rumen microorganisms of sheep and the fiber levels of energy feeds, the optimization of the management of grazing livestock, and the improvement of productivity to meet the ever-increasing demand for livestock products.

Results

Effect of the fiber level of energy feeds on the growth performance of sheep

With the addition of high and low fiber level energy feed, the body weight of grazing sheep increased significantly (Fig. 1). The daily body weight gain (BWG) of WS group and WBS group was significantly higher than that of CON group at 0–15 days, 15–30 days and 0–60 days, and the daily BWG of WS group was significantly higher than that of CON group at 30–45 days and 45–60 days (Table S2). The results of the body weight measurement demonstrated that the body weight of the sheep in the WS group was significantly higher than that of the sheep in the CON group from day 15 to day 60. Furthermore, the body weight of the sheep in the WBS group was also significantly higher than that of the sheep in the CON group from day 30 to day 60 (Fig. 1).

Effect of energy feed fiber level on rumen fermentation parameters in sheep

Compared with CON group, the total VFAs concentration of WS group and WBS group increased by 8.58% ($p > 0.05$) and 20.85% ($p < 0.05$), respectively; acetic acid concentration decreased by 3.63% ($p < 0.05$) and 2.65% ($p > 0.05$), respectively; the concentration of isobutyric acid decreased by 83.87% ($p < 0.05$) and 46.15% ($p < 0.05$), respectively; butyric acid concentration increased by

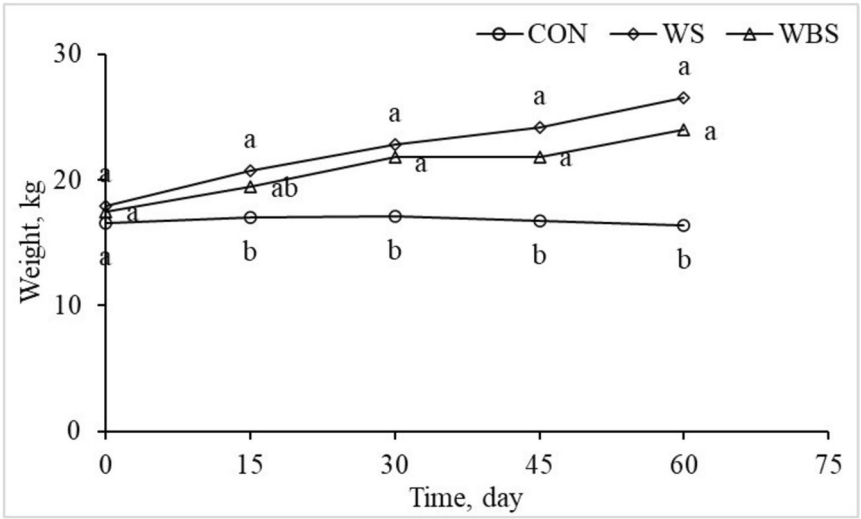

Fig. 1 Effect of high- and low-fiber-level energy feeds on sheep body weight. Note: Different letters in the graph represent significant differences at the 0.05 level at the same sampling time point, and the same letter represents no significant differences

Table 1 Effects of different fiber levels of energy feed on rumen fermentation parameters of sheep

Item	CON	WS	WBS	SEM	*P* value
pH	6.22[a]	5.83[b]	5.89[b]	0.07	0.025
Total VFA	24.12[b]	26.19[ab]	29.15[a]	0.99	0.011
Acetate%	76.81[a]	74.12[b]	74.83[ab]	0.54	0.090
Propionate%	16.92	16.11	18.21	0.42	0.147
Isobutyrate%	1.14[a]	0.62[b]	0.78[b]	0.05	< 0.001
Butyrate%	3.15[b]	7.42[a]	4.68[b]	0.48	< 0.001
Isovalerate%	1.23[a]	0.56[b]	0.77[b]	0.07	< 0.001
Valerate%	0.75[b]	1.17[a]	0.73[b]	0.07	0.008
Acetate/Propionate	4.58	4.69	4.20	0.13	0.315

Different letters represent significant differences in the table, $p < 0.05$ indicated that the difference was significant; $p < 0.01$ indicates that the difference is extremely significant

135.99% ($p < 0.05$) and 48.57% ($p > 0.05$), respectively; the concentration of isovaleric acid decreased by 119.64% ($p < 0.05$) and 59.74% ($p < 0.05$), respectively. In addition, valeric acid concentration increased by 56% in the WS group ($p < 0.05$) and decreased by 2.74% in the WBS group ($p > 0.05$) (Table 1).

Effect of energy feed fiber level on the abundance and diversity of rumen microbial communities in sheep

The number of operational taxonomic units (OTUs)

All rumen fluid samples yielded 750,483 raw reads, with an average of 41,694 reads per sample (largest read: 47,374; smallest read: 37,509). Based on the identification of 97% of the nucleotide sequences from the total reads, 7,299 OTUs were identified (Fig. 2). The percentages of unique OTUs in the CON (2,586 OTUs), WBS (2,445 OTUs), and WS (2,268 OTUs) groups were 119 (4.60%), 29 (1.19%), and 27 (1.19%), respectively (Fig. 2).

Alpha (α) diversity analysis

A diversity index analysis was performed on the CON, WBS, and WS treatment groups (Fig. 3). All the indices of the CON group were significantly greater than those of the WBS and WS groups, and the Chao1 and richness indices of the WBS group were significantly greater than those of the WS group (Fig. 3).

Beta (β) diversity analysis

Principal coordinate analysis (PCoA)1 accounted for 21.21% of the variation in the samples, whereas the PCoA2 axis accounted for 13.22% (Fig. 4). The distribution of samples in each group was relatively concentrated, with no crossover between the groups, and the sample points in the CON group were relatively concentrated and distant from those in the WS group (Fig. 4).

Effect of energy feed fiber level on the composition and community structure of rumen microorganisms in sheep

Analysis of bacterial composition and community structure

Taxonomic analysis of the reads revealed the presence of 28 bacterial phyla. Bacteroidetes, Firmicutes, and Kiritimateellaeota were the most abundant phyla (Fig. 5A). At the genus level, the main genera were *Prevotella_1*, *Unassigned* and *Rikenellaceae_RC9_gut_group* (Fig. 5B).

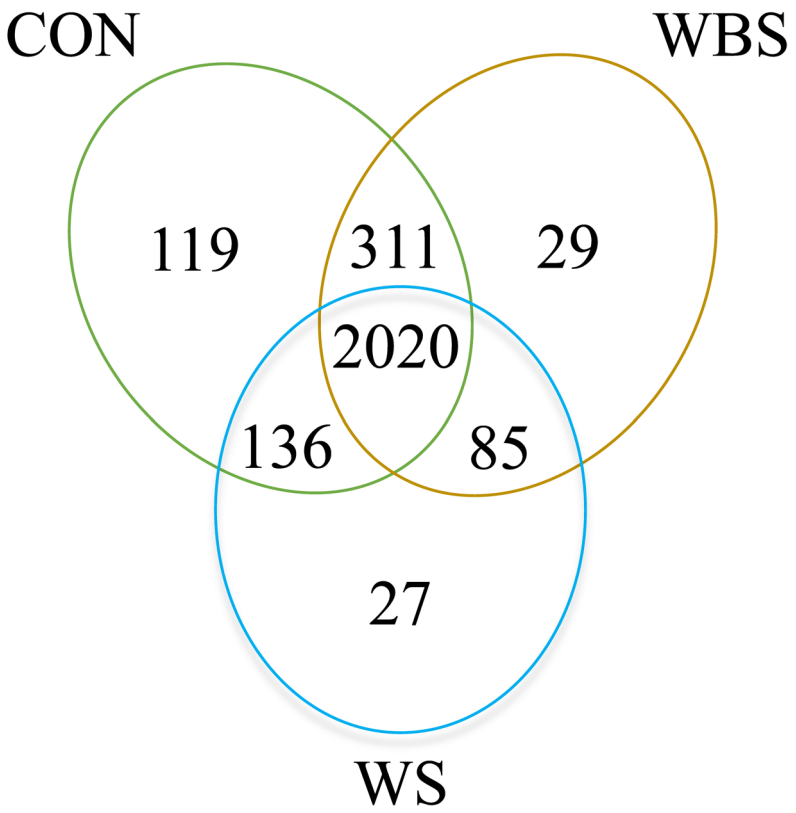

Fig. 2 Venn diagram of rumen fluid flora in different treatment groups

Changes in the relative abundance of major microorganisms

We observed that energy-enriched feed fiber significantly altered the relative abundance of rumen microbes at the phylum and genus levels. At the phylum level, the WBS group had significantly lower relative abundances of Proteobacteria. In the WS group, the relative abundance of Cyanobacteria significantly decreased, and the relative abundance of Actinobacteria significantly increased in the ruminal fluid of sheep. Among the WS and WBS groups, the relative abundance of Actinobacteria in the WS group was significantly greater than that in the WBS group (Fig. 6A).

At the genus level, the WBS treatment significantly reduced the relative abundances of *Rikenellaceae_RC9_gut_group* and *Ruminococcaceae_NK4A214_group*. The WS group had significantly reduced relative abundances of *Ruminococcaceae_NK4A214_group* and *Ruminococcaceae_UCG-010*. The relative abundance of *Rikenellaceae_RC9_gut_group* in the WS group was significantly greater than that in the WBS group (Fig. 6B).

Microbial community differences between the CON, WBS, and WS groups

Using Linear discriminant analysis Effect Size (LEfSe) to detect changes in the composition of microbial taxa, Fig. 7 shows the most significant differences in microbial communities between the different addition levels. A total of 23, 11, and 9 clades were more abundant in the CON, WS, and WBS groups, respectively (Fig. 7). Figure 8 shows the differences in the abundances of the different microbial groups in the CON, WBS and WS groups. Of these, the most differentially abundant microbial genera in the CON group were *Rikenellaceae_RC9_gut_group*, *Ruminococcaceae_NK4A214_group*, *Ruminococcaceae_UCG-005*, *Ruminococcaceae_UCG-010*, *Elusimicrobium*, *Psychrobacter*, and *Prevotellaceae_UCG-001* (Fig. 8). The most differentially abundant microbial genera in the WBS group were *Lachnospiraceae_XPB1014_group* and *Ruminococcaceae_UCG-002*. *Ruminiclostridium*, *Lachnospiraceae_NK3A20_group*, and *Mogibacterium* were more abundant in WS (Fig. 8). Among these, *Rikenellaceae_RC9_gut_group* exhibited the greatest difference among the communities, and the Linear discriminant analysis (LDA) threshold is 3.0 (Fig. 8).

Analysis of microbial interactions and their correlation with fermentation parameters

Heatmaps were constructed for the correlation between genus-level flora and rumen fermentation, yielding a total of 310 correlation coefficients and 59 of these coefficients

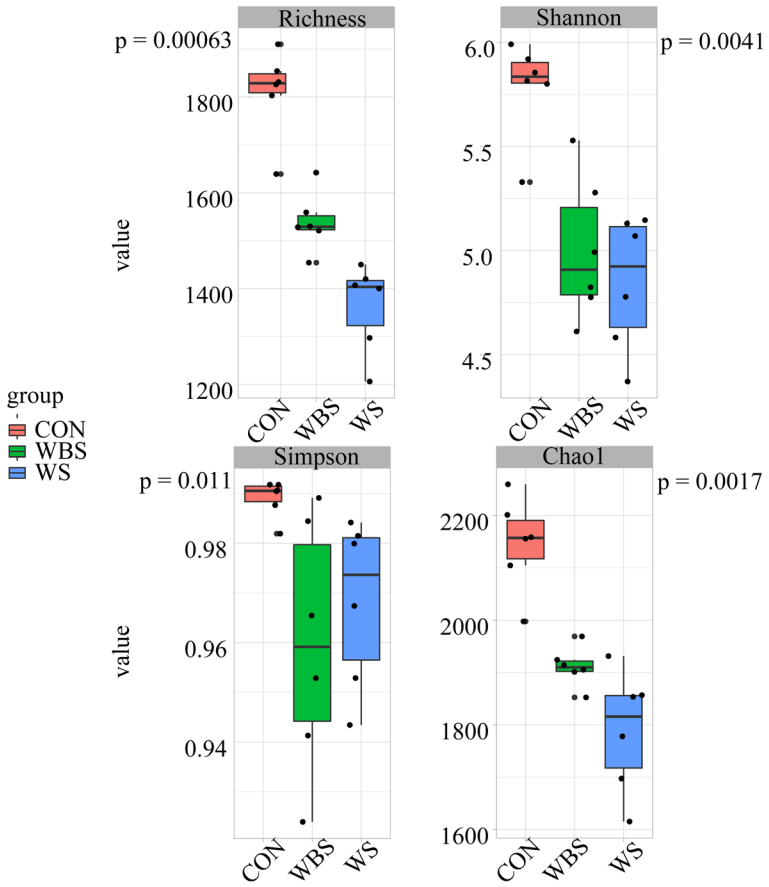

Fig. 3 Alpha diversity indices of rumen bacteria in different treatment groups

showed significant correlations ($p < 0.05$) (Fig. 9). Among several dominant genera with high relative abundances, *Rikenellaceae_RC9_gut_group* was negatively correlated with BWG ($p < 0.05$). *Rikenellaceae_RC9_gut_group* and *Ruminococcaceae_NK4A214_group* were negatively correlated with the total VFAs content of rumen fluid ($p < 0.05$), while significant positive correlations were found with isobutyric acid and isovaleric acid content ($p < 0.05$) (Fig. 9).

We also analyzed the effect of the energy feed fiber level on the rumen microbial community structure and the interrelationship between microorganisms. Low-fiber-energy feeds enhanced the association between microbial groups and promoted a positive correlation between *Rikenellaceae_RC9_gut_group*, *Ruminococcaceae_NK4A214_group*, *Psychrobacter,* and other groups while changing the original negative correlation between microorganisms. The positive correlations between the microorganisms and other groups also changed so that most of the groups showed positive correlations (Fig. 10). High-fiber-energy feeds enhanced the association between *Rikenellaceae_RC9_gut_group* and other flora and enhanced the negative correlation between flora (Fig. 10).

Tax4Fun gene function estimation

At Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway level 1, genes included in the 'Metabolism' and 'Organizational Systems' categories were enriched in the special treatment groups (Fig. 11). At KEGG pathway level 2, genes included in the categories 'Digestive System', 'Energy Metabolism', 'Carbohydrate Metabolism', 'Glycan Biosynthesis and Metabolism', and 'Biosynthesis of Other Secondary Metabolites' were enriched in the special treatment groups (Fig. 11). At KEGG pathway level 3, five pathways showed significant differences among the different treatment groups ($p < 0.05$), and genes included in the categories 'protein digestion and absorption', 'nitrogen metabolism', 'starch and sucrose metabolism', 'other glycan degradation', and 'streptomycin biosynthesis' were more enriched in the WS group than in the WBS group (Fig. 11).

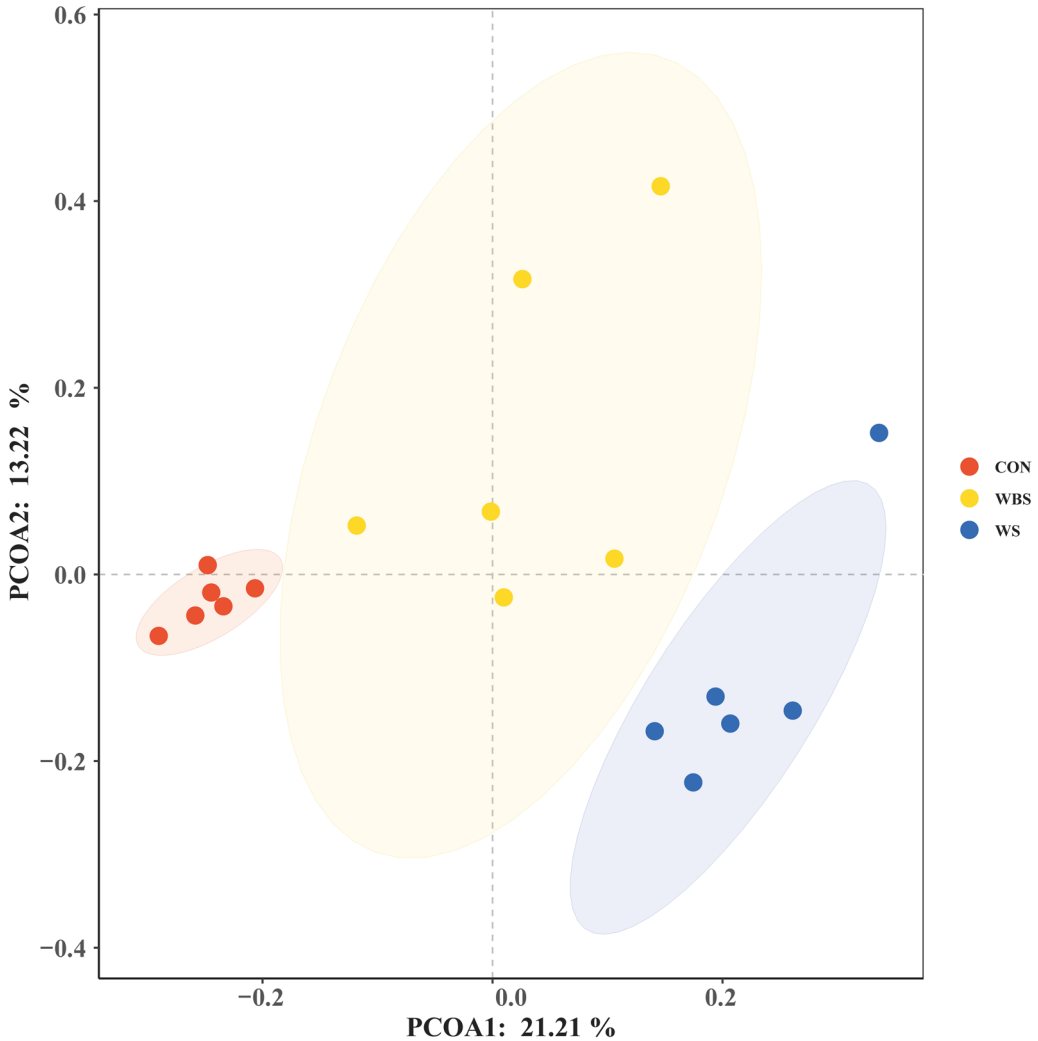

Fig. 4 Principal coordinate analysis of the rumen microbiota in the different treatment groups

Discussion

Weight and BWG are important indicators of livestock growth performance in areas where the livestock industry occupies an important position in economic development. Domestic animals with outstanding growth performance can often bring more economic benefits. Feeding concentrates for yaks grazing in the warm season can improve growth rate, meat quality, and economic efficiency [25–27]. However, supplemental feed concentrated in winter can significantly increase the daily weight gain of Euler-type Tibetan sheep and enhance their ability to overwinter [28]. Feeding concentrated through grazing increases the weight of lambs and other growth and development indicators, such as body height and bust circumference [29–31]. In the present study, energy feeds with different fiber levels significantly increased the body weight of grazing sheep (Fig. 1), Although the fiber in the energy feed is similar to the fiber in the forage, it can stimulate the saliva secretion of ruminants, provide the substrate for rumen microbial fermentation, and produce the VFAs necessary for animals. However, the forage fiber is often more complex than the fiber in the energy feed, and the fermentability is lower, resulting in slower fermentation and more diverse microbial populations in the rumen. The energy feed usually contains more soluble carbohydrates and nutrients that can be rapidly fermented, providing sufficient and easier to use carbon and nitrogen sources for rumen microorganisms, and accelerating microbial proliferation and promote forage digestion and utilization [32]. Tax4Fun functional predictions indicated that 'Metabolism'-related pathways were enriched in the supplementary feeding group (Fig. 11), which also explains why supplemental energy feeds promote the digestion and metabolism of nutrients in grazing sheep.

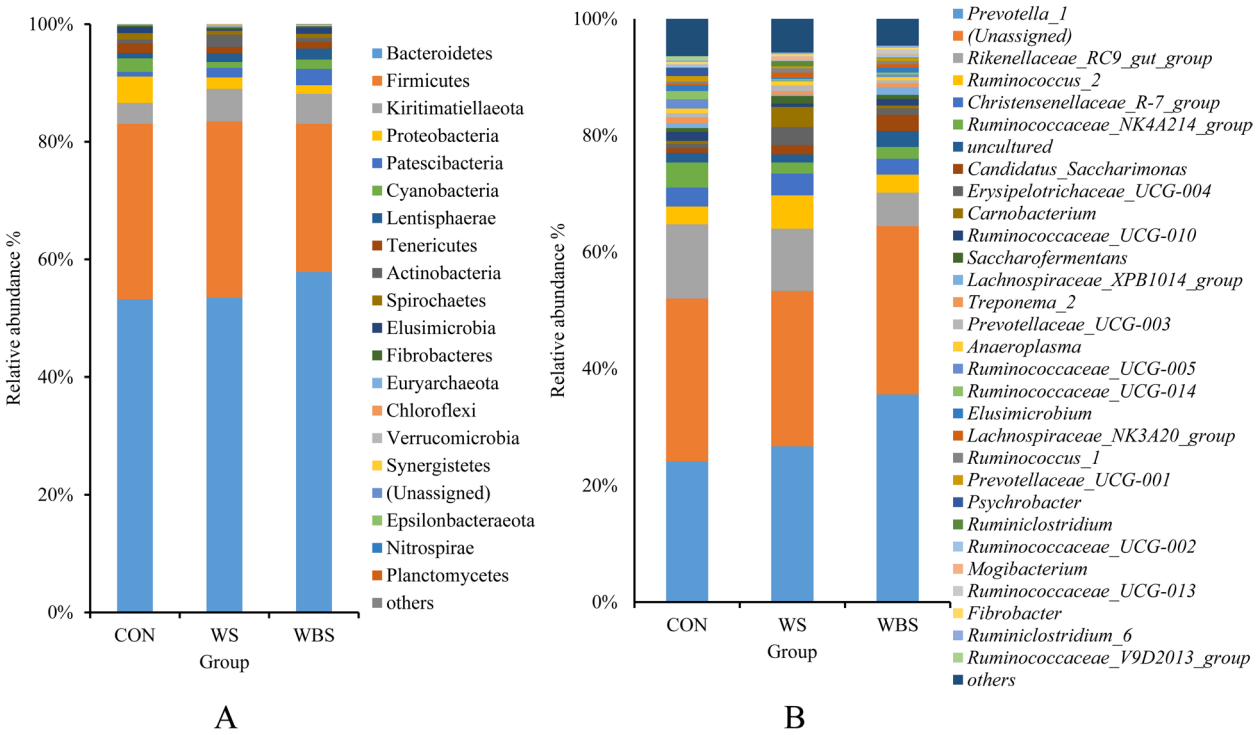

Fig. 5 Bacterial phyla (A) and genera (B) (relative abundance > 1%) in different treatment groups

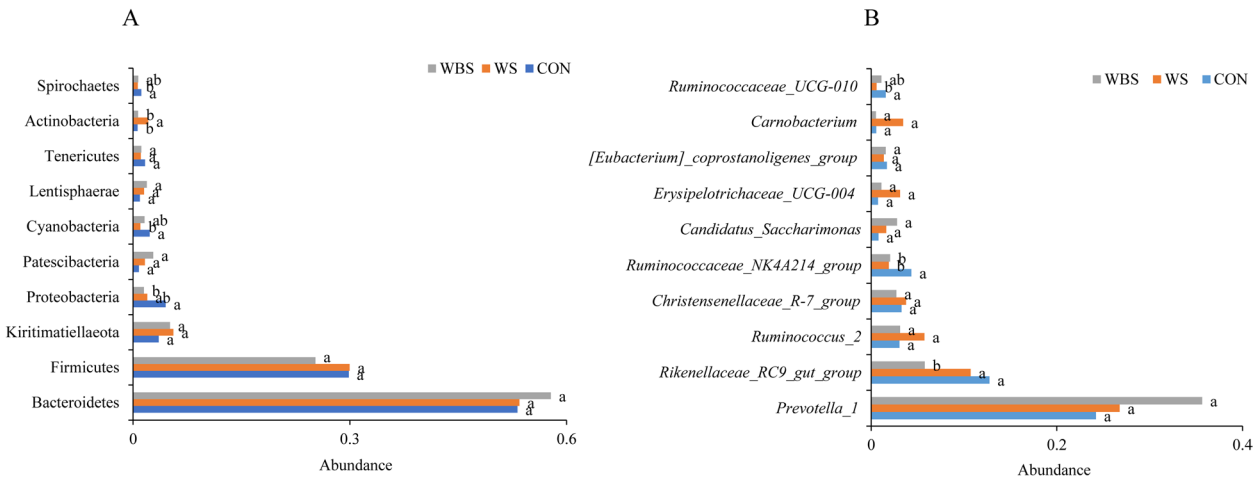

Fig. 6 Changes in the relative abundance of major microorganisms at the phylum level (A) and genus level (B) in the rumen of sheep in different treatment groups

Ruminants provide a suitable internal environment and nutrients for the growth and development of rumen microorganisms, while microorganisms decompose high-fiber feed fermentation products into nutrients for ruminants to absorb and utilize [33, 34]. Energy feed supplementation alters the rumen environment in ruminants, thus affecting rumen fermentation. pH and VFAs are important parameters for measuring rumen fermentation, and different feeds undergo different types of fermentation in the rumen, producing VFAs with different compositions and contents. The rumen pH depends on the rate of synthesis and absorption of VFAs, the secretion of saliva buffer, and the endogenous buffering capacity of feed/digestive juices [35–37]. When a high-concentrate diet with a large number of grains is fed, excessive starch in the rumen fermentation produces

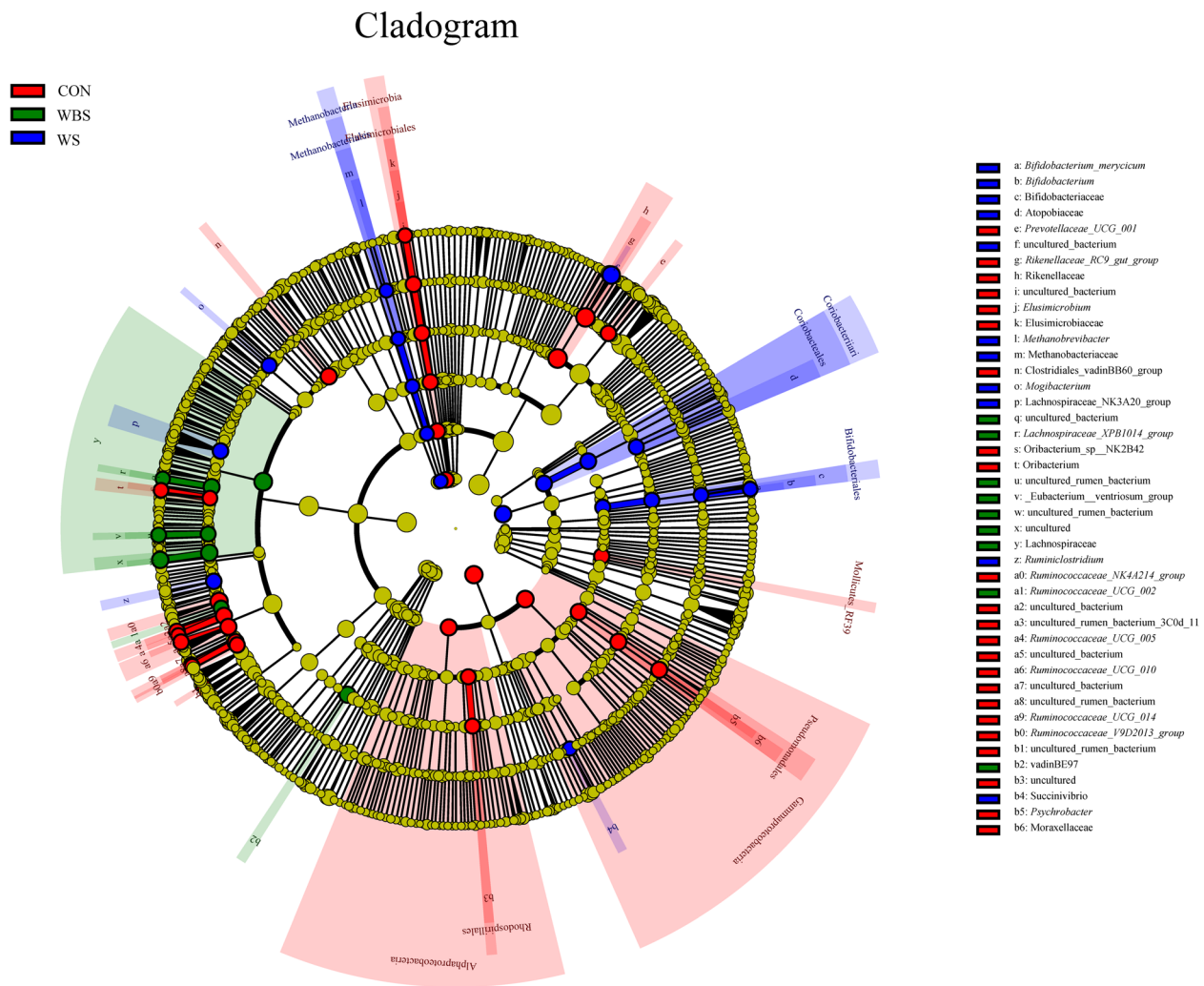

Fig. 7 Linear discriminant analysis effect size (LEfSe) cladogram comparing microbial communities among the different treatment groups

a large amount of VFAs that cannot be used by microorganisms in time to reduce the pH of the rumen fluid, which seriously leads to nutritional metabolic diseases such as rumen acidosis [38, 39]. Feeding high- and low-fiber-of-energy feed reduced the pH of the rumen fluid (Table 2) but did not exceed the appropriate range, indicating that all the grazing sheep maintained a healthy rumen environment. The faster fermentation rate of starch than fiber also resulted in a lower pH in the low-fiber-energy feed group than in the high-fiber-energy feed group (Table 2). VFAs are the primary mode of metabolizing the rumen of ruminants to produce energy [40, 41]. In the ruminant rumen, acetic acid and butyric acid are used to synthesize fatty acids, and propionic acid is the most important substance involved in gluconeogenesis. In this study, both high- and low-fiber-energy diets significantly increased the VFAs concentration in the rumen fluid, and the percentage of acetic acid was significantly greater in the high-fiber-energy diet group than in the low-fiber-energy diet group (Table 2). Cellulose, lignin, and other substances containing a large number of structural carbohydrates decompose slowly in the rumen, and the fermentation products are mainly acetic acid [42, 43]. Starch, monosaccharides, and other substances containing a large number of nonstructural carbohydrates decompose quickly in the rumen, and the fermentation products are mainly propionic acid [42]. Furthermore, increased dietary concentrate consumption can significantly reduce the proportion of rumen acetic acid [44–46], and low-fiber-energy feed significantly reduces the pH of rumen fluid, which is not conducive to the growth of fiber-degrading bacteria. At the same time, the high concentration of glucose produced by rapid starch fermentation has been shown to reduce fiber-digesting bacteria and inhibit the growth and reproduction of related fiber-degrading bacteria by producing

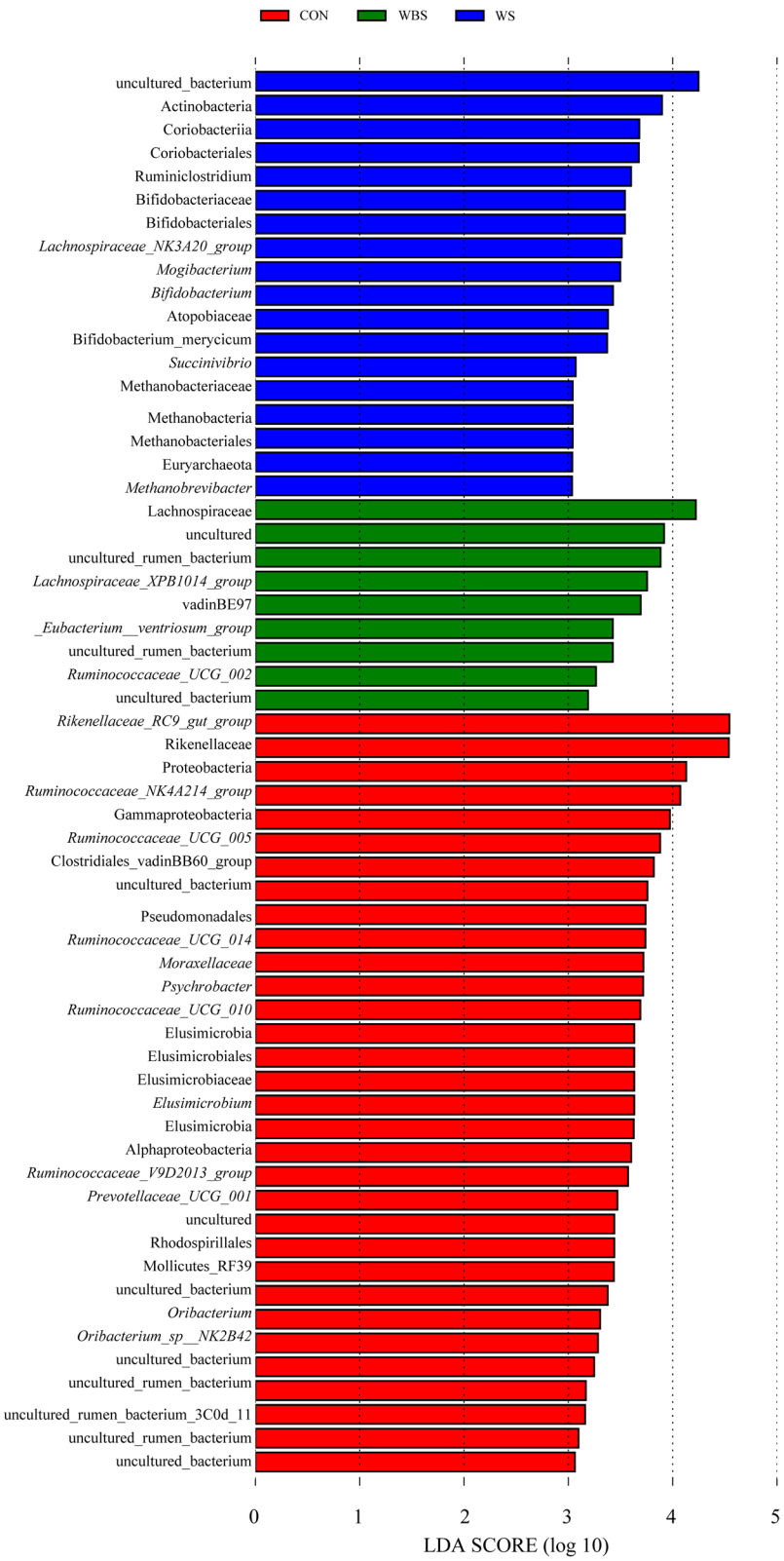

Fig. 8 Histogram of the LDA score calculated for each taxon from phylum to genus

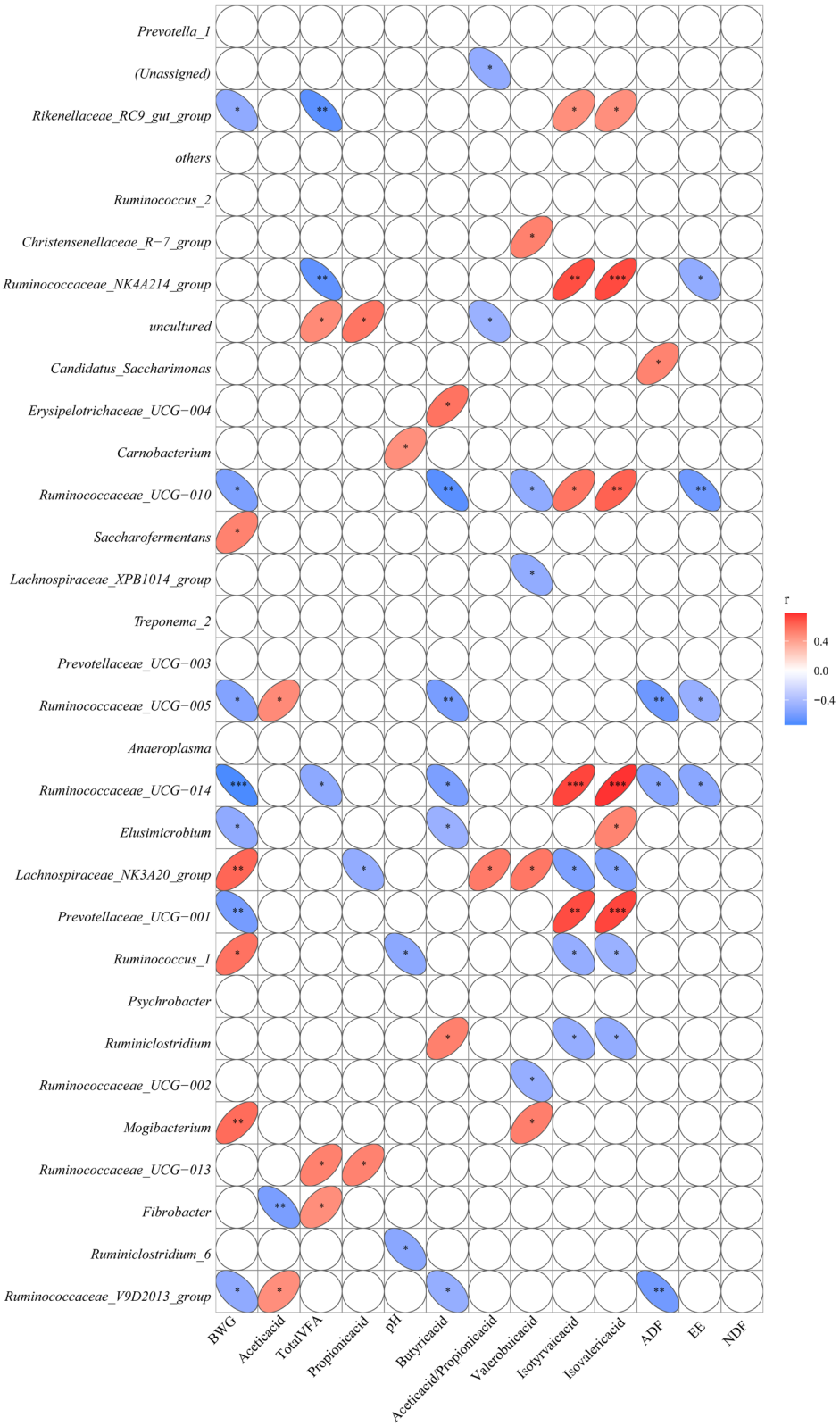

Fig. 9 Spearman correlation and cluster analysis between bacterial abundance and rumen fermentation parameters and body weight gain at the genus level. Note: The area of the ellipse represents the magnitude of the correlation, different colors represent positive (red) or negative correlations (blue), * represents $0.01 < P \leq 0.05$, ** represents $P \leq 0.01$, *** represents $P \leq 0.001$. BWG, body weight gain

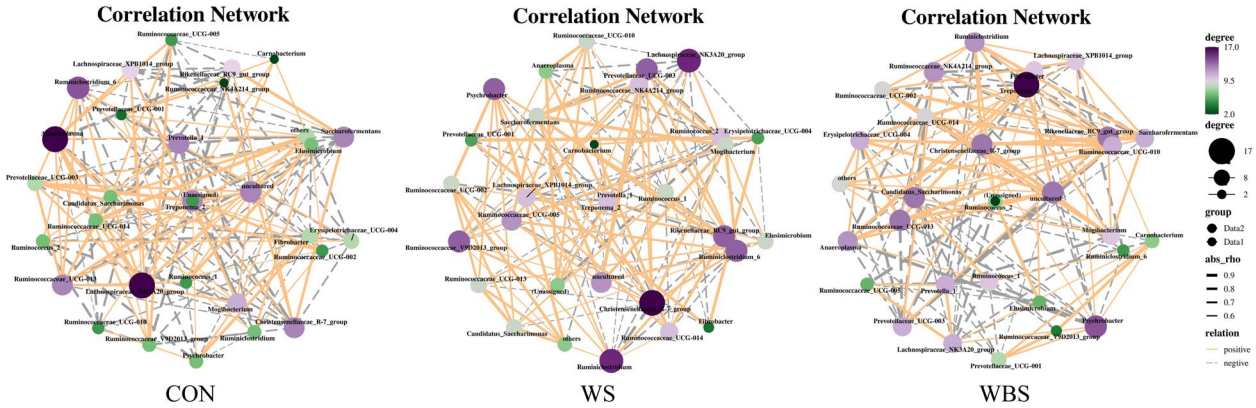

Fig. 10 Response of microbial community structure to energy feed at different fiber levels

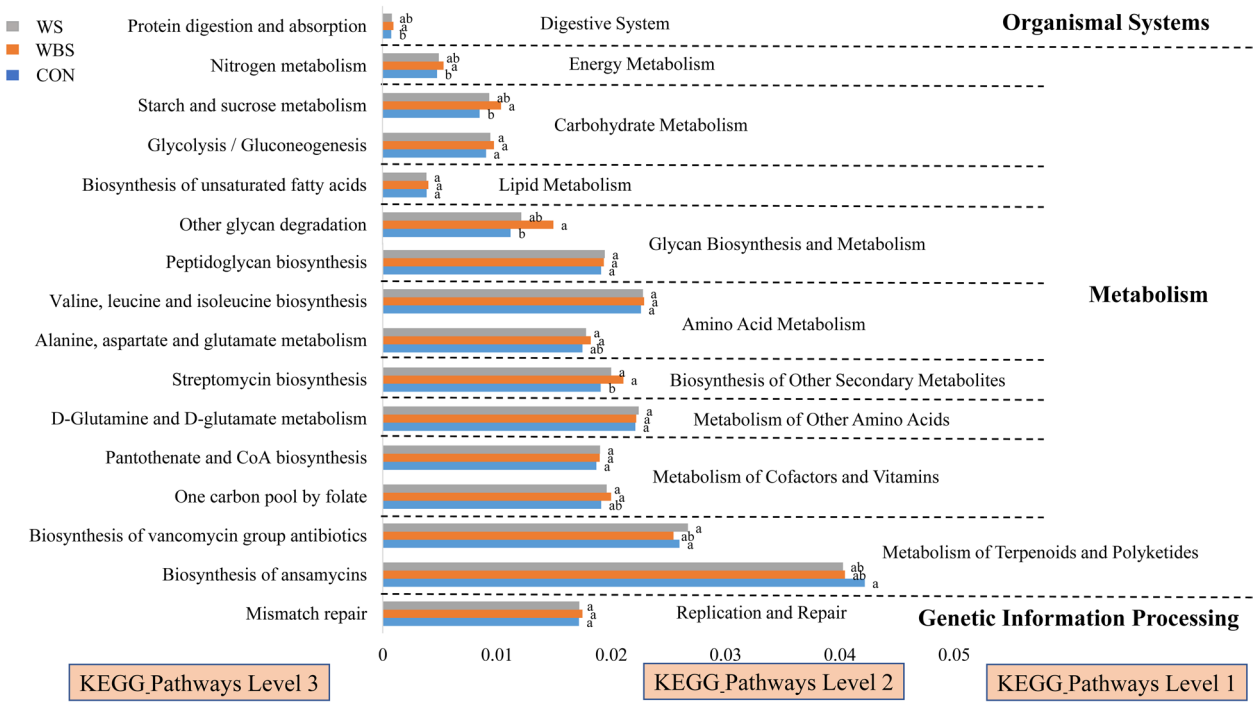

Fig. 11 Functional predictions for rumen microbiota with significantly different KEGG pathways ($P < 0.05$) for the three groups (CON, WS and WBS). Note: KEGG pathways at Level 1, Level 2, and Level 3 are represented. In each group, different lowercase letters indicate a significant difference ($P < 0.05$), while the same letters indicate no significant difference ($P > 0.05$)

inhibitory compounds, leading to a lower percentage of acetic acid [47]. The proportion of propionic acid was different because the rumen fluid VFAs varied greatly in one day and was highly correlated with the amount of food intake. The speed of fiber concentrate feed over the rumen was slower, causing the sensation of satiety and affecting the feed intake of the sheep. Isobutyric acid and isovalerate acid are commonly referred to as conjugate acids, which promote the degradation of food structural carbohydrates [48] and the growth of fiber-degrading microbial species, which is conducive to the digestion of fibers [49, 50].

The structure and stability of the rumen flora play important roles in the health and production performance of ruminants [35, 51] Diet structure affects the species and quantity of rumen microorganisms, which are the main factors affecting the composition of the rumen microflora in ruminants [52]. Supplemental feed leads to a decrease in the diversity and richness of rumen flora in Tibetan lambs and yaks [31, 53]. The

results of this study were similar, as supplementation with high- and low-fiber-energy feeds reduced the richness and diversity of the rumen microflora (Fig. 3). Compared with those in the high-fiber-energy feed group, the rumen microbial diversity and richness in the low-fiber-energy feed group decreased more significantly, indicating that low-fiber-energy feeds have a greater impact on the rumen microbial community structure of sheep, which may be attributed to the large amount of starch that is easier to ferment in low-fiber-energy feeds [54]. In addition, analysis of the rumen bacterial community structure indicated that *Bacteroidetes* and *Firmicutes* were the dominant phyla in the rumen fluid of grazing sheep (Fig. 5) [55, 56], and they dominated the degradation of fiber and starch [57, 58]. Studies have shown that high concentrate ratios increase the relative abundance of Firmicutes and Actinobacteria and decrease the relative abundance of Bacteroidetes and Cyanobacteria in the rumen fluid of dairy cows [59], which is consistent with the results of this study (Fig. 5). Supplementation with high-fiber energy feeds increased the relative abundance of Bacteroidetes and decreased the relative abundance of Firmicutes, possibly because a large number of easily decomposed nonfeed fiber substances provide sufficient substrates for fiber-degrading bacteria, resulting in a large proliferation of fiber-degrading bacteria. In contrast, the supplementation of low-fiber energy feeds results in starch providing a substrate for related starch-degrading bacteria to promote proliferation, and glucose produced by rapid starch degradation inhibits cellulolytic bacteria. Actinobacteria exhibit unparalleled metabolic versatility in the rumen of ruminants and colonize and positively affect the digestive system [60, 61]. Both high- and low-fiber-energy feeds increased the relative abundance of Actinobacteria, and the difference in low-fiber-energy feed was significant (Fig. 5), which also explained the enrichment of 'metabolism' ("protein digestion and absorption", "nitrogen metabolism", "starch and sucrose metabolism", etc.)-related pathways in KEGG function prediction in the supplementary feeding group) (Fig. 10). Both high- and low-fiber-energy feeds reduced the relative abundance of Proteobacteria, and the relative abundance in the high-fiber-energy feed group significantly differed. Proteobacteria contain a large number of pathogenic bacteria, such as *Escherichia coli*, Salmonella, *Helicobacter pylori* and Vibrio cholerae [62–64]. Studies have shown that high-precision diets containing a large number of grains can lead to an increase in the abundance of pathogenic bacteria such as *Escherichia coli* in the rumens of goats and cows [62, 65] and even to nutritional metabolic diseases such as rumen acidosis [59, 66, 67]. This finding is inconsistent with the results of this study, indicating that an appropriate amount of concentrated feed, especially feed with relatively high fiber levels, can reduce the frequency of nutritional metabolic diseases in ruminants.

Prevotella_1 is reported as the dominant genus in the rumen at the genus level, degrading the hemicellulose component of the ration [53, 68]. Previously, Huo et al. (2020) and Chen et al. (2017) reported that increasing the proportion of concentrate significantly increased the abundance of *Prevotella* spp. in the rumen of goats [69, 70]. In our study, both high- and low-fiber-energy feeds tended to increase the relative abundance of *Prevotella_1* (Fig. 5B), but the differences were not significant, probably due to differences in feed sources and test animals. Furthermore, the preference of *Prevotella_1* for substrates varies, and its abundance does not always vary with diet [71, 72]. The *Rikenellaceae_RC9_gut _ group* is related to immunity and can affect intramuscular fat deposition by regulating the production of VFAs [73–75]. Correlation analysis revealed that its relative abundance was significantly negatively correlated with the total VFAs content and significantly positively correlated with the isobutyric acid and isovaleric acid contents (Fig. 9). Similar findings were also reported for Tan sheep and yak [73, 76, 77]. *Ruminococcus* is considered to be the intestinal core microorganism and plays an important role in the degradation and fermentation of polysaccharides [56, 78]. The relative abundance of *Ruminococcaceae* was reduced by supplementation with high- and low-fiber energy feeds (especially low-fiber energy feeds), such as *Ruminococcaceae_NK4A214_group, Ruminococcaceae_UCG-010, Ruminococcaceae_UCG-005, Ruminococcaceae_UCG-014, Ruminococcaceae_UCG-002, and Ruminococcaceae_V9D2013_group,* possibly because carbohydrates, especially starch-rich grains, can cause rumen microbial flora disorders and the production of a large number of abnormal metabolites [79]. *Lachnospiraceae_NK3A20_group* plays a role in fiber degradation and plant fiber fermentation [80–82]. Supplementation with high- and low-fiber energy feeds (especially low-fiber energy feed) increased the relative abundance of the *Lachnospiraceae_NK3A20_group*, possibly because starch provides sufficient energy and carbon sources for rumen microorganisms and promotes the proliferation of the *Lachnospiraceae_NK3A20_group*. Notably, we analyzed the rumen microbial community structure and the relationships between the flora in each treatment group. Compared with those in the control group, the supplementation of low-fiber-energy feed increased the positive correlation in the microbial community, that is, promoted cooperation between the flora, while the supplementation of high-fiber-energy feed increased the negative correlation in the microbial community, that is, promoted competition between the

flora (Fig. 10). It is speculated that the reason is that supplementary feeding changes the dietary nutrition level and affects the rumen microflora of sheep. The low-fiber energy feed is rich in a large amount of carbohydrate starch, which is rapidly fermented into glucose to provide sufficient energy and carbon sources for microorganisms, reducing the competition for resources between the flora; at the same time, a large amount of VFAs produced by glucose decomposition cannot be used in time, resulting in a decrease in rumen fluid pH, resulting in rumen metabolism and microbial flora disorder. The microorganisms with similar decomposition effects have changed from previous competition for the same resource to joint cooperation to decompose a large amount of accumulated resources. High-fiber energy feeds are rapidly decomposed and utilized by microorganisms due to the provision of easily degradable nonfeed fiber materials, which promotes the proliferation of microorganisms in the short term. When easily degradable nonfeed fiber material resources are fully utilized, to survive, it is necessary to compete for the use of refractory feed fibers, thus exacerbating the competition between communities.

Conclusions

Supplementary feeding of high and low fiber energy feeds reduced the pH value of rumen fluid and the richness and diversity of microorganisms in grazing sheep, reduced the relative abundance of some harmful microorganisms, affected the metabolic activities of some fiber-digesting bacteria, regulated the interaction and competition between bacteria, increased the content of VFAs and the relative abundance of metabolic-related microorganisms in the supplementary feeding group, and enriched the metabolic-related pathways. However, further understand the mechanism of the effect of fiber level on the rumen of sheep, it is necessary to conduct in-depth analysis using research methods such as transcriptomics, proteomics and metabolomics.

Materials and methods

Test site

This study was conducted at the Linze Grassland Agricultural Experiment Research Station of Lanzhou University, Linze County, Zhangye City, Gansu Province (100°02′E, 39°15′N). The region is mainly engaged in a specialized intensive cropping production system (SICP) and an extensively integrated crop–livestock production system (EICL) combined with an agricultural system [83]. At an altitude of 1390 m, the average annual precipitation is 121.5 mm, and the average annual temperature is 7.16 °C [84].

Experimental animals and group design

Thirty 6-month-old, healthy male sheep (17.36 ± 3.37 kg) with no significant differences in body weight were selected and subjected to three dietary treatments (n = 10/group) depending on the fiber level of the supplemented energy feed: the control group (CON, no supplementation), low-fiber-energy feed group (WS, 30% wheat), and high-fiber-energy feed group (WBS, 30% wheat bran).

The trial consisted of a prefeeding period (15 days) and a formal period (45 days). The sheep were grazed in a salinized sown pasture (mixed sowing of alfalfa and tall fescue in equal proportions) and allowed to freely feed and drink. The grazing time ranged from 7:00 a.m. to 19:00 p.m. After grazing at night, each sheep was grouped according to the treatment and fed with the corresponding energy feed in the limit bar. The available pasture forage, energy feed formulation and nutrient composition are shown in Table S1. Energy feeds were designed to achieve the same amount of crude protein (CP) and gross energy (GE) to differentiate between different fiber levels, and the daily energy feed supply per sheep was 360 g. All the experimental animals were from Linze Grassland Agricultural Experiment Research Station of Lanzhou University, the experiments were conducted according to the guidelines of experimental field management protocols (files no. 2010–1 and no. 2010–2), which were approved by the Animal Use and Care Committee of Lanzhou University, during the whole experiment, no sheep were euthanized or sacrificed, and no chemical drugs such as anesthetics were used for any sheep. All sheep remained healthy during the experiment and returned to the pre-experiment sheep to continue their lives after the experiment.

Sample collection and processing

The sheep were weighed every 15 days before morning grazing. After the last weighing, six representative sheep with basically the same health status and body condition were selected from each group, and rumen fluid was collected before morning grazing at the end of the experiment. According to the description of Fan et al. (2020) [76], an oral stomach tube was used to collect rumen fluid samples. The sheep were controlled in the limit bar, the left hand of the sampler controlled the opening and closing of the mouth, and pressed the tongue, the right hand slowly extended the sampling tube (the outer shell was a stainless-steel elastic hose, the inner was a transparent latex tube) into the rumen through the mouth and throat, and the other sampler carefully extracted the rumen fluid using an external syringe. The original 50 ml rumen fluid was discarded to avoid containing saliva, gastrointestinal

mucosal secretions, and residual distilled water. Each sheep collected 50 ml of rumen fluid samples, filtered through four layers of medical gauze, and immediately measured pH with a pH meter (Model 144 PB-10, Sartorius Co., Germany), and then a portion of the rumen fluid was placed in a 5 ml (2 portions) sterilized centrifuge tube and stored at −80 °C for Deoxyribonucleic acid (DNA) extraction. The remaining rumen fluid was stored at −20 °C for analysis of rumen fermentation parameters. The VFAs concentrations were determined with a GC 3420 gas chromatograph fitted with HP-INNO capillary column (30×0.32 mm) [85].

Sample analysis

Extraction of DNA and 16S rDNA sequencing

DNA was prepared and extracted from rumen fluid using the TINamp Stool DNA Kit (TIANGEN, Beijing, China) as a template for 16S rDNA sequencing analysis, where the quality and concentration of the extracted DNA were checked with a Thermo NanoDrop One. The V3-V4 region of the 16S rDNA gene was amplified using the primers 806R (5-GGACTACHVGGGTWTCTAAT-3) and 515F (5-ACTCCTACGGGAGGCAGCA-3). A barcode of the unique eight-base sequence of each sample was added to each primer for sample identification and determination [35].

The Polymerase chain reaction (PCR) mixture consisted of 1 μL of primer R, 25 μL of 2×Premix Taq, 1 μL of primer F, 50 μL of nuclease-free water, and 50 ng of DNA. The PCR conditions were as follows: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s, and 72 °C for 10 min. PCR products were extracted from amplicons by electrophoresis on a 1%-(w/v)-agarose gel. The proteins were excised and purified using the E.Z.N.A.® Gel Extraction Kit (Omega, USA). Sequence libraries were constructed with the NEBNext ® Ultra ™ DNA Library Prep Kit for Illumina ® (New England Biolabs, Ipswich, MA, USA) and sequenced with the Illumina Nova 6000 platform (Guangdong Magi Genetic Biotechnology Co., Ltd., Guangzhou, China) [35].

Sequencing data processing and data analysis

Sequence quality trimming was performed with FASTP (version 0.14.1) based on the unique sample barcode of the original sequence [86]. Primers were removed using Cutadapt software (version 1.14) based on primer information at both ends of the sequence. Noncompliant sequences were filtered with USEARCH-FASTQ-MERGEPAIRS (version 10.0.240) [87] to obtain the original spliced sequences. Sliding window quality clipping was performed on the raw tag data using FASTP (version 0.14.1) to obtain clean tags for subsequent analysis [87]. The tags were clustered with 97% similarity using UPARSE in USEARCH 10 (version 10.0.240) software to obtain OTUs [88]. We annotated the OTUs for classification and used the RDP classifier (version 11.5) to assign representative sequences to organisms based on the SILVA (version 138) database [89, 90].

The alpha (α) diversity index, relative abundance of flora in rumen fluid and fermentation parameters were analyzed by one-way analysis of variance (ANOVA) using a completely randomized design (significant differences at $p < 0.05$). α diversity analysis was performed using USEARCH-ALPHA_DIV (version 10.0.240) to calculate the richness, Chao1, and Shannon_2 indices. Principal coordinate analysis (PCoA) and correlation heatmap analysis were performed using the vegan package and heatmap package of R software (https://www.omicstudio.cn/tool), respectively, where PCoA utilizes a distance algorithm (weighted unifrac distance) to assess differences in bacterial communities between samples. Lefse and LDA were performed using R software for OTU abundance tables with Kruskal–Wallis rankings and tests, followed by false discovery rate (FDR) correction, and then the significance of species differences between groups was analyzed based on homogenized abundance tables for each species category using LEfse software. The functional pathways of the rumen microbiota were predicted based on 16S sequencing data using Tax4Fun software with reference to information from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [35].

Abbreviations

VFAs	Volatile fatty acids
OTUs	Operational taxonomic units
PCoA	Principal coordinate analysis
LEfSe	Linear discriminant analysis Effect Size
LDA	Linear discriminant analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes
BWG	Body weight gain
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction

Supplementary Information

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Supplementary Material 1.

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Declaration of generative AI and AI-assisted technologies in the writing process

There was no use of AI or AI-assisted technologies in the writing process.

Authors' contributions
FH and XZ designed the experiments and authored the main manuscript, while XL, KX, YP, and FL contributed to sampling and data collation. XZ also prepared the figures and tables and conducted analysis and discussion. All authors have read and approved the final version of the manuscript.

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Data availability
The data sets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: NCBI; PRJNA855246. Submission ID: SUB11738613 and BioProject ID: PRJNA855246, available online at http://www.ncbi.nlm.nih.gov/bioproject/855246.

Declarations

Ethics approval and consent to participate
The experiments were conducted according to the guidelines of experimental field management protocols (files no. 2010–1 and no. 2010–2), which were approved by the Animal Use and Care Committee of Lanzhou University.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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