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Lycium barbarum Byproducts Modulate Rumen Fermentation, Enhance Digestive Enzyme Activity, and Improve Immune and Antioxidant Status in Grazing Sheep

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ABSTRACT

The incorporation of functional plants rich in bioactive components or secondary metabolites as alternatives to growth promoters, such as antibiotics, in ruminant production is gaining momentum. Diet composition and structure are principal determinants of rumen microbial community structure, function, and the health status of ruminants. This study aimed to assess the impact of dietary *Lycium barbarum* byproducts on rumen fermentation parameters, digestive enzyme activity, microbial composition and proportion, and rumen health status in grazing sheep on sown pastures. The findings revealed that, compared to the control group (CON), the *Lycium barbarum* seed (LBS) and residue (LBR) groups exhibited increased proportions of acetic acid and propionic acid, while the proportions of valeric acid and isovaleric acid decreased. Activity of rumen digestive enzymes, including pepsin, pyruvate dehydrogenase (PDH), urease, aminopeptidase (Aps), and cellobiase increased, and rumen microbial diversity was altered (increased richness but not diversity). The relative abundance of beneficial bacteria such as *Prevotella* and *Ruminococcaceae_NK4A214_group* increased, whereas that of *Desulfovibrio* decreased. Furthermore, levels of total protein (TP), albumin (ALB), immunoglobulin (GLB), superoxide dismutase (SOD), and glutathione peroxidase (GSH-PX) increased, while uric acid (UA), blood urea nitrogen (BUN), and malondialdehyde (MDA) levels decreased. The active ingredients in the byproducts, including polysaccharides, polyphenols (flavonoids), and vegetable oils, exert beneficial effects by directly inhibiting pathogen proliferation, modulating intestinal microbiota composition, enhancing immune function, and mitigating oxidative stress.

1 | Introduction

Ruminants are crucial net protein providers, converting inedible biomass into nutritious food (Weimer 2022; Liu, Wu, et al. 2019; Liu, Xu, et al. 2019). Rumen, their unique digestive organ, houses a complex microbial ecosystem essential for feed fermentation (Ma et al. 2019), body development (Sommer and Backhed 2013), immune regulation (Martin et al. 2010), and energy balance (Shabat et al. 2016). The rumen microbiome is central to nutrient digestion and utilization, producing volatile fatty acids as the primary nutrient source for the host and thereby influencing productivity (Mizrahi et al. 2021; Seradj et al. 2014). Notably, the structure and stability of the microbial community are mainly regulated by dietary components (Zhang, Du, et al. 2025; Zhang, Ran, et al. 2025).

With the surging global meat demand, scientists are actively seeking green additives to replace antibiotics in animal

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husbandry (Wemette et al. 2021). Bioactive compounds from natural plants show potential in enhancing livestock productivity and reducing environmental impacts via rumen fermentation modulation (Elghandour et al. 2018; Khiaosa-ard and Zebeli 2013). Plant active ingredients, particularly phytochemicals such as tannins, essential oils, and other bioactive compounds significantly influence rumen fermentation. In vitro, certain essential oils can reduce the acetic-to-propionic acid ratio and boost volatile fatty acid concentrations (Lv et al. 2011). Tannins, when added to ruminant diets, alter the rumen's microbial community, binding proteins and affecting fiber fermentation, which modifies volatile fatty acid production and cuts methane emissions (Díaz Carrasco et al. 2017). Specific plant extracts also reshape the rumen microbiome (Seradj et al. 2014). Flavonoids from alfalfa and mulberry leaves promote beneficial bacteria, reducing methanogens and methane (Zhan et al. 2017; Oskoueian et al. 2013). Functional polysaccharides from various sources regulate flora, cut pathogens, and ease acidosis, enhancing feed efficiency (Li et al. 2018, 2019; Jin et al. 2021). These plant compounds impact serum immunity and digestive enzymes too. Supplements like postbiotics from *Lactobacillus plantarum* RG14 improve ruminal papillae growth and modulate immune-related genes, while dietary inclusions can affect enzymes such as pancreatic α-amylase (Izuddin et al. 2019; Lee et al. 2020). This indicates that plant-derived compounds can not only affect rumen fermentation but also have downstream effects on digestive processes in the intestines.

Lycium barbarum, a globally distributed plant, is an economic crop with both medicinal and culinary value and has gained increasing popularity worldwide, accompanied by an expansion of its cultivation areas. Preliminary statistics indicate that during processing in China, approximately 5%–10% of residual *Lycium barbarum* seeds and residues, along with over 200,000 tons of *Lycium barbarum* twigs are generated annually (Masci et al. 2018; Hou et al. 2019; Ma et al. 2022; Gonçalves et al. 2017). The seeds, residues, and twigs of *Lycium barbarum* are rich in various compounds, including polysaccharides, carotenoids, flavonoids, alkaloids, amides, peptides, anthraquinones, coumarins, lignin, terpenes, steroids, organic acids, anthocyanins, essential oils, and glycolipids (Gao et al. 2017; Liu et al. 2014). The efficacy of plant extracts in modulating rumen fermentation, microbial communities, and digestive enzyme activity varies with their composition—single or mixed ingredients. Single compounds like rosemary essential oil exert targeted, predictable effects, such as enriching fiber-degrading bacteria (e.g., *Fibrobacter succinogenes*) to enhance fermentation efficiency (Cobellis et al. 2016), yet their scope remains limited within the complex rumen ecosystem. In contrast, blended formulations—e.g., tannins with essential oils—often produce synergistic effects, enabling broader regulation of microbial composition and metabolic pathways, as evidenced by improved fermentation and suppressed methanogens (Rabee et al. 2024). These advantages, along with potential improvements in stability and bioavailability, position mixed extracts as a more integrated strategy to enhance feed efficiency, animal performance, and environmental sustainability. Further studies are warranted to clarify interaction mechanisms and optimize combinatorial designs for ruminant nutrition. Given the potential of *Lycium barbarum* byproducts, which are rich in diverse bioactive compounds, we hypothesized that polysaccharides and flavonoids in *Lycium barbarum* byproducts could enhance fiber-degrading enzyme activities and improve host antioxidant and immune functions by modulating rumen microbial community structure. Thus, this study aimed to further investigate the impact of *Lycium barbarum* by-product supplementation on rumen fermentation parameters, digestive enzyme activities, microbial composition and ratios, and overall rumen health status in grazing sheep on sown pastures.

2 | Materials and Methods

2.1 | Statement of Institutional Animal Care and Use Committee (IACUC)

The experiments were conducted according to the guidelines of experimental field management protocols (Files No: 2010-1 and 2010-2) and were approved by the Animal Use and Care Committee of Lanzhou University. All procedures for handling and caring for animals conform with China's regulations on the protection and use of laboratory animals and are approved by the Chinese Zoological Soc.

2.2 | Test Site

This experiment was conducted from July to September 2021 at Linze Pratacultural Research Station of Lanzhou University (100°02′ E, 39°15′ N; 1390 m asl) in Gansu Province, China, which focuses on specialized intensive cropping (SICP) and integrated crop-livestock (EICL) systems. The region has an annual rainfall of 121.5 mm and a mean temperature of 7.16°C.

2.3 | Experimental Animals and Group Design

Twenty-four healthy F1 hybrid rams (Small-tailed Han × Hu sheep; 6 months old, 34.1 ± 1.2 kg BW) were stratified by initial weight and randomly assigned to four groups ($n = 6$) using a block randomization method to minimize individual variation: CON (50 g soybean meal), LBS (2.5% *Lycium barbarum* seeds + 50 g soybean meal), LBR (7.5% *Lycium barbarum* residue + 50 g soybean meal), LBT (2.5% *Lycium barbarum* twigs + 50 g soybean meal) (Zhang, Du, et al. 2025; Zhang, Ran, et al. 2025).

Lycium barbarum byproducts were sourced from Ningxia Saishang Hongbaozhu Agricultural Technology Co. Ltd. (Zhongwei, Ningxia, China). The selection of these byproducts was based on their availability and potential nutritional value. The specific proportions of *L. barbarum* byproducts (seeds, residue, and twigs) were determined through preliminary in vitro experiments conducted prior to the main study (Xiaoyun Zhang, Lijuan Ran, Kaili Xie, Fujiang Hou. unpublished data). The inclusion rates were determined based on the Chinese Mutton Sheep Feeding Standard (NY/T 816-2021) to achieve a target daily dry matter intake of 900 g. Based on this target, daily supplementation levels were calculated as follows: 22.5 g of LBS, 67.5 g of LBR, and 22.5 g of LBT. To ensure complete consumption of the supplements, each *Lycium barbarum* by-product was mixed with 50 g of soybean meal. The CON group received 50 g of soybean meal only. The nutritional composition (including

TABLE 1 | Nutritional level of the sown pasture forage and *Lycium barbarum* seeds, *Lycium barbarum* residue, and *Lycium barbarum* twigs (DM basis).

Nutritional level (%, as DM)	*L. barbarum* seed (LBS)	*L. barbarum* residue (LBR)	*L. barbarum* twigs (LBT)	Forage
OM	96.82	93.69	93.73	88.42
CP	14.67	18.17	5.48	13.84
EE	16.23	7.07	3.4	3.43
NDF	45.08	17.4	73.53	55.94
ADF	22.03	3.46	47.48	33.31
GE (MJ/kg, DM)	19.36	16.8	16.99	16.75
Functional ingredient (%, as DM)				
Polysaccharide	2.84	3.64	1.93	/
Total flavonoids	0.09	0.13	0.26	/
Betaine	0.02	0.02	0.33	/

Abbreviations: ADF, acid detergent fiber; CP, crude protein; EE, ether extract; GE, gross energy; NDF, neutral detergent fiber; OM, organic matter.

dry matter, crude protein, neutral detergent fiber, acid detergent fiber, ether extract, ash) and key functional ingredients (polysaccharides, total flavonoids, and betaine) of each *Lycium barbarum* by-product and the sown pasture forage are presented in Table 1. The methods used to determine the concentrations of polysaccharides, total flavonoids, and betaine are described in the Section 2.5.

The trial spanned 75 days, comprising a 15-day adaptation period followed by a 60-day experimental phase. Throughout the study, sheep were permitted to graze daily from 07:00 to 19:00 on cultivated pastures consisting of an equal proportion of alfalfa and tall fescu, with ad libitum access to forage and water.

Upon returning to the enclosure post-grazing, sheep were individually provided with group-specific *Lycium barbarum* by-product supplements at designated feeding stations. Access to free movement within the enclosure was granted only after complete consumption of the allocated supplements.

2.4 | Sample Collection and Processing

On the final day of the trial, prior to morning grazing, all sheep were weighed, and weights were recorded; 2–3 blood samples (5 mL each) were collected from each lamb via jugular venipuncture using heparinized vacutainer tubes. Samples were immediately centrifuged at 3000 *g* for 15 min at 4°C to separate anticoagulated plasma. The supernatant was aliquoted and stored at −80°C for subsequent analysis of immune indices (e.g., immunoglobulin profiles, cytokine levels).

Rumen fluid sampling was conducted approximately 2 h post-grazing. A sterilized oral stomach tube was gently inserted orally into the rumen, and fluid was aspirated using a 150 mL sterile syringe. To minimize salivary contamination, the initial 50 mL of rumen fluid was discarded, and the rumen fluid was filtered through four layers of sterile medical gauze to remove particulate matter. The pH of the clarified rumen fluid was measured in triplicate using a calibrated portable pH meter (Model 150, IQ Scientific Instruments, USA). The fluid was then aliquoted into 5 mL centrifuge tubes, with one portion stored at −20°C for volatile fatty acid (VFA) and digestive enzyme activity analyses, and another portion preserved at −80°C for microbial assessment.

2.5 | Sample Analysis

2.5.1 | Fermentation Parameters

Volatile fatty acid (VFA) concentrations were determined by gas chromatography. Rumen fluid was diluted 5:1 with 25% metaphosphoric acid, centrifuged at 10,000 *g* for 10 min, and the supernatant collected. VFAs were then analyzed using a DB-FFAP capillary column (30 m × 0.32 mm × 0.25 μm).

2.5.2 | Digestive Enzyme Activities

Digestive enzyme activities were quantified using an enzyme-linked immunosorbent assay (ELISA) kit provided by Meibiao Biotechnology Co. Ltd. (Yancheng, China). These assays are predicated on the enzymatic reactions that occur under defined substrate conditions, subsequent to which the concentration of the resultant end product is ascertained.

2.5.3 | Serum Immune and Antioxidant Indices

Serum immune and antioxidant indices were performed by BeijingJinHaiKeYu Biological Technology Development Co. Ltd.

2.5.4 | Chemical Composition and Bioactive Compound Analysis

The proximate chemical composition (crude protein, crude fat, crude fiber, ash, and neutral detergent fiber) of *Lycium barbarum*

byproducts and forage was determined using standard forage analysis methods (AOAC 2006). Bioactive compounds were quantified via high-performance liquid chromatography (HPLC) with column-specific configurations for each target analyte.

Polysaccharide content was analyzed using an Agilent Eclipse XDB-C18 column (4.6 mm × 250 mm, 5 μm; Agilent Technologies, Santa Clara, CA, USA). The mobile phase consisted of acetonitrile and 0.1% phosphoric acid (v/v, 25:75) at a flow rate of 1.0 mL/min, with detection at 280 nm. Method validation followed protocols described in Yang et al. (2024).

Total flavonoids were determined using a Zorbax SB-C18 column (4.6 × 250 mm, 5 μm; Agilent Technologies, Santa Clara, CA, USA). The mobile phase comprised methanol and 0.5% acetic acid (v/v, 70:30) at 1.0 mL/min, with UV detection at 360 nm. Analytical conditions were optimized based on Li et al. (2024).

Betaine levels were measured using a Partisil SCX-10 cation-exchange column (4.5 mm × 250 mm, 10 μm; Grace Davison, Broxburn, UK). The mobile phase was a mixture of methanol, acetonitrile, and 2 mM heptanesulfonic acid (pH 2.0, 50:20:30 v/v) at 1.0 mL/min, with detection at 240 nm. The method adhered to the protocol outlined in Chendrimada et al. (2002).

All HPLC analyses were performed on an Agilent 1260 Infinity II system (Agilent Technologies, Santa Clara, CA, USA), with quantification based on external standard calibration curves.

2.5.5 | Extraction of DNA and 16S rDNA Sequencing

Genomic DNA was extracted from rumen fluid samples using the TINamp Stool DNA Kit (TIANGEN, Beijing, China) combined with cetyltrimethylammonium bromide (CTAB). DNA purity and integrity were evaluated via spectrophotometric analysis (NanoDrop One, Thermo Fisher Scientific, USA) and validated by 1% agarose gel electrophoresis. Extracted DNA was diluted with sterile water to a final concentration of 1 ng/μL for subsequent PCR amplification.

The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified using universal primers 515F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). PCR reactions were performed under the following conditions: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s (denaturation), 52°C for 30 s (annealing), and 72°C for 30 s (extension), with a final extension at 72°C for 10 min. PCR products were purified using 1% agarose gel electrophoresis and quantified using a Qubit Fluorometer (Thermo Fisher Scientific, USA).

Purified amplicons were processed for library construction using the NEB Next Ultra DNA Library Prep Kit (E7645S, New England Biolabs, USA), with dual index barcoding to enable multiplex sequencing. Libraries were quantified via Qubit 4.0 Fluorometer (Thermo Fisher Scientific) and pooled equimolarly. Paired-end sequencing (2 × 250 bp) was performed on an Illumina NovaSeq 6000 platform (Magigene Biotechnology Co., China), generating a minimum output of 50,000 reads per sample. Raw FASTQ files were deposited in the NCBI Sequence Read Archive (BioProject ID: PRJNA1150173).

2.6 | Sequencing Data Processing and Data Analysis

Raw paired-end reads were processed using FLASH (version 1.2.7; Magoč and Steven 2011) to merge overlapping sequences into raw tags, incorporating primer and barcode information for sample identification. Subsequent quality filtering removed low-quality sequences (quality score < 20), truncated reads shorter than 200 bp, and chimeric sequences. High-quality tags were clustered into operational taxonomic units (OTUs) at a 97% sequence similarity threshold using UPARSE (Edgar 2013) within the USEARCH v10.0.240 software package. Representative OTU sequences were taxonomically classified using the SILVA SSUrRNA database (Quast et al. 2013) and annotated at the species level.

Alpha diversity metrics, including observed species richness, Chao1 estimator, Shannon diversity index, and Simpson's index, were calculated using the USEARCH-ALPHA_DIV tool. Beta diversity analysis was performed via principal coordinates analysis (PCoA) based on weighted UniFrac distances, implemented in the vegan package (version 2.5-7) in R (R Core Team 2021).

Differential abundance analysis was conducted using LEfSe (Segata et al. 2011) and linear discriminant analysis (LDA) effect size (LDA > 4.0). The Kruskal–Wallis's rank sum test was applied to the OTU abundance table, followed by false discovery rate (FDR) correction ($\alpha = 0.05$) to identify differentially abundant taxa between groups. Taxonomic biomarkers were further validated using LEfSe, which integrated LDA scores and Kruskal–Wallis's statistics.

Functional predictions of the rumen microbiota were inferred from 16S rRNA gene data using Tax4Fun (Aßhauer et al. 2015), mapping OTUs to Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs and pathways. Heatmaps visualizing bacterial relative abundance and functional profiles were generated using the pheatmap package in R.

Statistical comparisons of bacterial community composition and alpha diversity indices were performed using one-way analysis of variance (ANOVA) in a completely randomized design. All statistical tests were considered significant at $p < 0.05$.

3 | Results

3.1 | Growth Performance

Compared with the CON group, sheep in the LBS, LBR, and LBT groups exhibited significantly higher average daily gain

(ADG) ($p < 0.001$), with the LBS group showing a significantly greater ADG than both the LBR and LBT groups ($p < 0.001$) (Table 2).

3.2 | Ruminal Fermentation Parameters

Lycium barbarum byproducts did not significantly affect the pH levels or total VFA content in the rumen fluid ($p > 0.05$) (Table 3). Compared to the CON group, acetate and propionate concentrations in the rumen fluid showed a tendency to increase in the LBS, LBR, and LBT groups, with significant differences observed in the LBT group ($p < 0.05$). Conversely, isovalerate and valerate concentrations exhibited a decreasing trend, with significant differences in the LBT and LBR groups as compared to the CON group ($p < 0.05$).

3.3 | Ruminal Digestive Enzyme Activity

Compared to the CON group, the pepsin concentration in the LBT groups increased by 48.14% ($p < 0.05$). The PDH content in the LBS and LBR groups increased by 24.55% ($p < 0.05$) and 25.48% ($p < 0.05$), respectively. The carboxymethyl cellulase (CMC) content increased by 17.39% ($p < 0.05$) in the LBS group. Urease content in the LBS group increased by 13.83% ($p < 0.05$). The aminopeptidase (AP) content in the LBS group increased by 52.75% ($p < 0.05$). Similarly, the cellobiase content in the LBS group increased by 52.57% ($p < 0.05$) (Table 4).

3.4 | Serum Immune Indexes and Antioxidant Indexes

Compared to the CON group, TP and ALB content increased by 2.87% ($p < 0.05$) and 9.92% ($p < 0.05$) in the LBR group, respectively. GLB content increased by 8.49% ($p < 0.05$) and 7.88% ($p < 0.05$) in the LBS and LBR groups, respectively. UA content decreased by 16.12% ($p < 0.05$) in the LBS group. BUN content decreased by 21.88% ($p < 0.05$) and 24.77% ($p < 0.05$) in the LBS and LBT groups, respectively. SOD content increased by 6.67% ($p < 0.05$) in the LBR group. MDA content was reduced by 12.37% ($p < 0.05$) in the LBS group (Table 5).

3.5 | Number of OTUs in the Ruminal Microbiota

High-throughput sequencing was performed on rumen fluid samples, and the resulting raw sequences underwent splicing and filtering to enhance quality. After the elimination of low-quality reads, a total of 1,798,421 V3-V4 16S rRNA sequence reads were obtained for analysis, averaging 74,934 sequence reads per sample (Figure 1). Based on nucleotide sequence identity of 97%, 16,193 OTUs were identified, with 659 OTUs common to all four

TABLE 2 | Effect of *Lycium barbarum* byproducts on growth performance of sheep.

	Group					
Item	**CON**	**LBS**	**LBR**	**LBT**	**SEM**	***p***
Initial BW, kg	29.85	29.98	28.64	29.17	0.82	0.942
Final BW, kg	31.54	34.04	33.31	31.83	0.81	0.675
ADG, g/day	78.72^{c}	139.94^{a}	111.28^{b}	106.33^{b}	5.72	< 0.001

Note: a, b and c means within the same row with different letters are significantly different ($p < 0.05$).

TABLE 3 | Effect of *Lycium barbarum* byproducts on rumen fermentation in sheep.

	Group					
Item	**CON**	**LBS**	**LBR**	**LBT**	**SEM**	***p***
N	6	6	6	6		
pH	7.14	7.09	6.94	7.16	0.046	0.339
Total VFA	84.39	94.05	88.85	97.07	2.374	0.249
Acetate%	67.56^{b}	68.11^{b}	68.36^{ab}	69.26^{a}	0.195	0.01
Propionate%	16.99^{b}	18.01^{ab}	18.02^{ab}	18.22^{a}	0.176	0.044
Isobutyrate%	1.64	1.70	1.68	1.74	0.159	0.181
Butyrate%	8.65	8.77	8.79	8.71	0.146	0.989
Isovalerate%	2.50^{a}	1.76^{a}	2.00^{a}	0.94^{b}	0.154	< 0.001
Valerate%	2.66^{a}	1.66^{ab}	1.15^{b}	1.14^{b}	0.227	0.047
Acetate/Propionate	3.98	3.79	3.80	3.81	0.036	0.182

Note: Total VFA, total volatile fatty acids; a and b means within the same row with different letters are significantly different ($p < 0.05$).

TABLE 4 | Effect of *Lycium barbarum* byproducts on the activity of digestive enzymes in the rumen of sheep.

Item	Group				SEM	*p*
	CON	LBS	LBR	LBT		
N	6	6	6	6		
AMS, U/L	93.85	73.37	99.48	130.41	8.661	0.114
Pepsin, U/L	89.41^{b}	100.98^{b}	94.69^{b}	132.45^{a}	6.004	0.016
Cellulase, IU/L	2.33	2.73	6.11	5.91	0.792	0.177
LPS, U/L	16.70	28.41	30.96	45.23	4.931	0.250
PDH, U/L	106.80^{b}	133.02^{a}	134.01^{a}	119.01^{ab}	4.222	0.036
CMC, IU/L	239.46^{b}	281.10^{a}	263.97^{ab}	231.45^{b}	7.143	0.019
Pectinase, U/L	54.57	55.07	59.72	48.70	9.941	0.416
BCAAD, U/L	20.36	20.92	22.28	20.46	0.878	0.895
Urease, IU/L	565.41^{b}	643.58^{a}	640.63^{ab}	570.35^{ab}	14.390	0.049
Aps, U/L	199.34^{b}	248.52^{a}	232.26^{ab}	215.00^{ab}	6.908	0.032
SDH, IU/L	1403.67	1384.33	1346.86	1389.16	27.928	0.931
Cellobiase, IU/L	32.87^{b}	50.21^{a}	38.51^{b}	34.51^{b}	2.212	0.001
EGases, IU/L	74.51	55.34	75.49	76.66	3.630	0.093
MDH, IU/L	158.45	180.73	160.57	159.64	11.503	0.918
Xylanase, IU/L	23.93	23.39	26.10	24.16	0.691	0.600
LDH, IU/L	23.75	29.92	43.17	24.21	4.250	0.372
Chymosin, IU/L	584.30	696.25	614.15	536.03	31.372	0.365

Note: a and b means within the same row with different letters are significantly different ($p < 0.05$).
Abbreviations: AMS, amylase concentration; Aps, aminopeptidase; BCAAD, branched-chain amino acid dehydrogenase; CMC, carboxymethyl cellulase; EGases, endoglucanase egases; LDH, lactate dehydrogenase.; LPS, lipase; MDH, malate dehydrogenase; PDH, pyruvate dehydrogenase; SDH, succinate dehydrogenase.

TABLE 5 | Effect of *Lycium barbarum* byproducts on serum immunological and antioxidant indices in sheep.

Item	Group				SEM	*p*
	CON	LBS	LBR	LBT		
N	6	6	6	6		
TP, g/L	64.87^{b}	65.90^{ab}	66.73^{a}	64.97^{b}	0.252	0.017
ALB, g/L	30.34^{b}	31.71^{ab}	33.35^{a}	30.91^{b}	0.358	0.009
GLB, g/L	39.46^{b}	42.81^{a}	42.57^{a}	40.65^{ab}	0.506	0.044
GLU, mmol/L	5.32	5.58	5.47	5.54	0.087	0.747
UA, μmol/L	17.72^{a}	15.26^{b}	16.27^{ab}	16.20^{ab}	0.290	0.016
BUN, mmol/L	9.47^{a}	7.77^{b}	8.47^{ab}	7.59^{b}	0.269	0.042
TG, mmol/L	0.66	0.71	0.75	0.82	0.035	0.460
SOD, U/mL	95.57^{b}	100.98^{ab}	101.94^{a}	98.39^{ab}	0.916	0.050
MDA, nmol/mL	5.36^{a}	4.77^{b}	5.04^{ab}	5.07^{ab}	0.074	0.031
GSH-PX, U/mL	800.60	807.44	819.02	803.45	7.058	0.826

Note: a and b mean within the same row with different letters are significantly different ($p < 0.05$).
Abbreviations: ALB, albumin; BUN, blood urea nitrogen; GLB, globulin; GLU, glucose; GSH-PX, glutathione peroxidase; MDA, malondialdehyde; SOD, superoxide dismutase; TG, triglyceride; TP, total protein; UA, uric acid.

groups. The number of bacterial OTUs unique to the sheep in the CON, LBS, LBR, and LBT groups was 5544, 5193, 5226, and 5643, respectively (Figure 1).

3.6 | Microbial Diversity and Relative Abundance

3.6.1 | Alpha (α) Diversity Analysis

The outcomes of the α-diversity analysis revealed that the Chao1 and ACE richness estimators for the LBT and LBR groups were significantly higher compared to the CON group ($p < 0.05$). In contrast, no significant differences were observed in the Shannon and Simpson diversity indices across the groups (Figure 2).

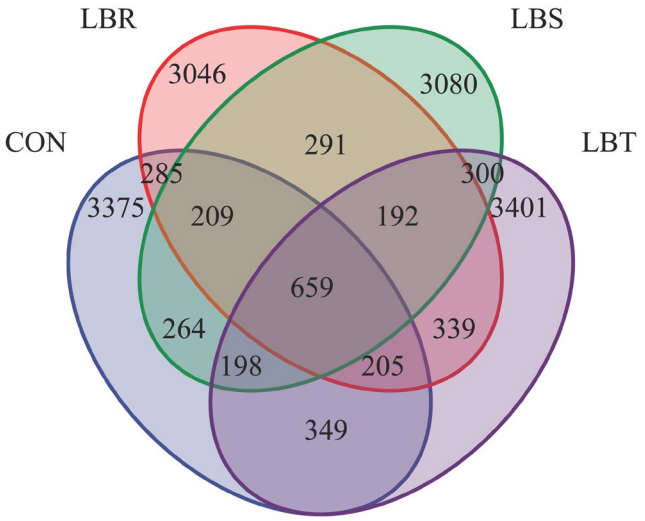

FIGURE 1 | Venn diagram of rumen fluid flora.

3.6.2 | Beta (β) Diversity Analysis

Principal coordinates analysis (PCoA) was conducted on the samples, revealing that the first and second principal components explained 15.90% and 11.73%, respectively, of the total variance (Figure 3). The representative ellipses for each group showed significant overlap, with the LBS and LBR groups exhibiting more concentrated sample distributions compared to the CON and LBT groups, which displayed a more dispersed pattern (Figure 3).

3.6.3 | Analysis of Bacterial Composition and Community Structure

The top 10 bacterial communities at both the phylum and genus levels were analyzed. At the phylum level, Firmicutes (51.08%) and Bacteroidetes (38.33%) were the predominant bacteria (Figure 4A). The relative abundance of Desulfobacterota in the LBR group and Proteobacteria in the LBT group was significantly lower compared to the CON group ($p < 0.05$).

At the genus level, the dominant bacteria were *Prevotella*, *uncultured rumen bacterium*, and *unclassified_F082* (Figure 4B). The relative abundance of *Prevotella* in the LBS, LBR, and LBT groups increased by 82.40% ($p < 0.05$), 89.85% ($p < 0.05$), and 47.20% ($p > 0.05$), respectively, compared to the CON group. The relative abundance of *Ruminococcaceae_NK4A214_group* in the

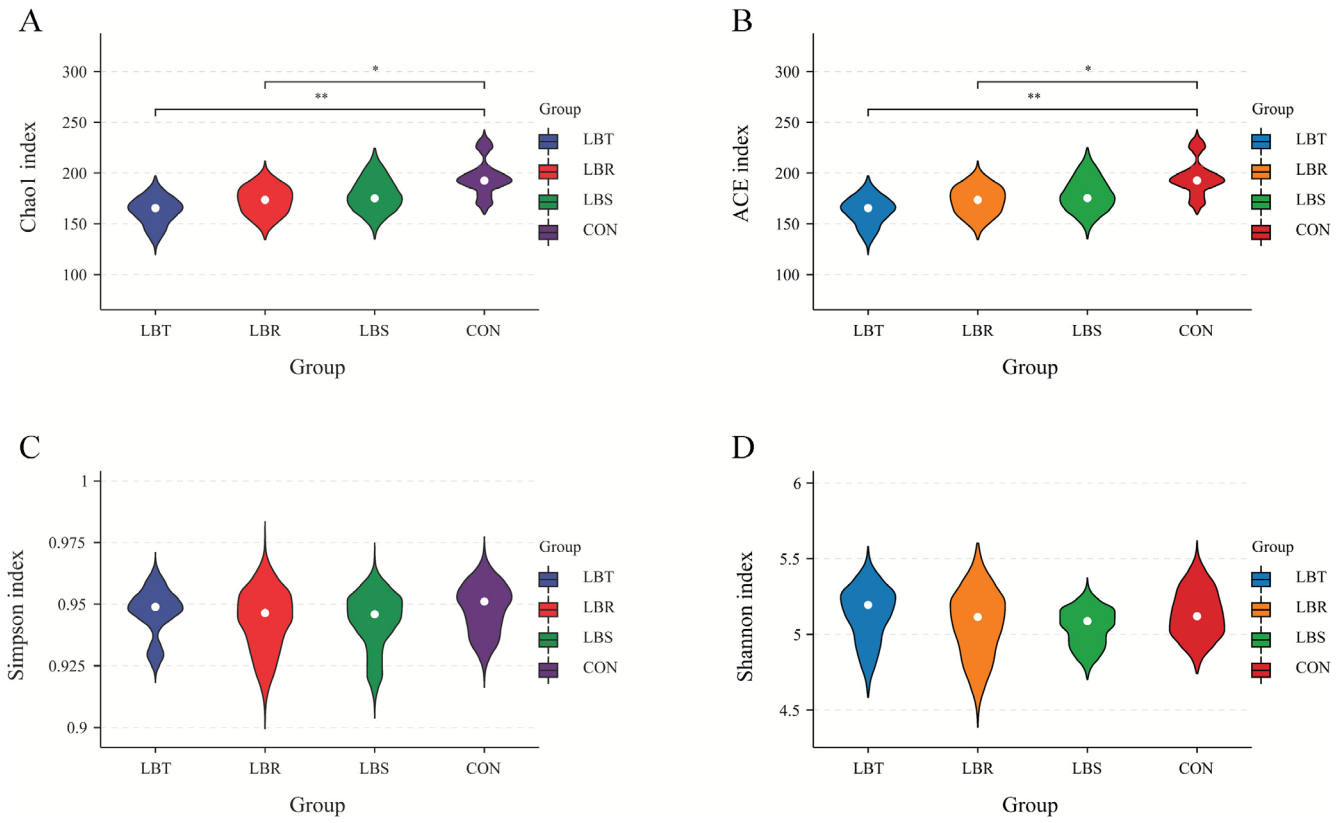

FIGURE 2 | Alpha diversity indices of rumen bacteria in sheep in the three groups.

LBS and LBR groups increased by 27.74% ($p > 0.05$) and 33.75% ($p > 0.05$), respectively, while it decreased by 23.75% ($p > 0.05$) in the LBT group. The relative abundance of *Desulfovibrio* in the LBS, LBR, and LBT groups was significantly lower than that in the CON group, with reductions of 47.97% ($p < 0.05$), 62.73% ($p < 0.05$), and 56.56% ($p < 0.05$), respectively.

3.7 | Microbial Community Differences Between the CON, LBS, LBR, and LBT Groups

LEfSe was employed to identify shifts in bacterial taxa composition, presenting a representative cladogram of the predominant microbiome structure and highlighting the most substantial taxonomic differences across various supplementation levels. Specifically, three branches were predominantly enriched in the CON group, one branch in the LBR group, two branches in the LBS group, and one branch in the LBT group (Figure 5). The general exhibiting significant differences within the CON group were *Rikenellaceae_RC9_gut_group* and *Prevotella*, while the genus showing significant differences within the LBT group was *Butyrivibrio*, with an LDA score threshold set at 4.0 (Figure 6).

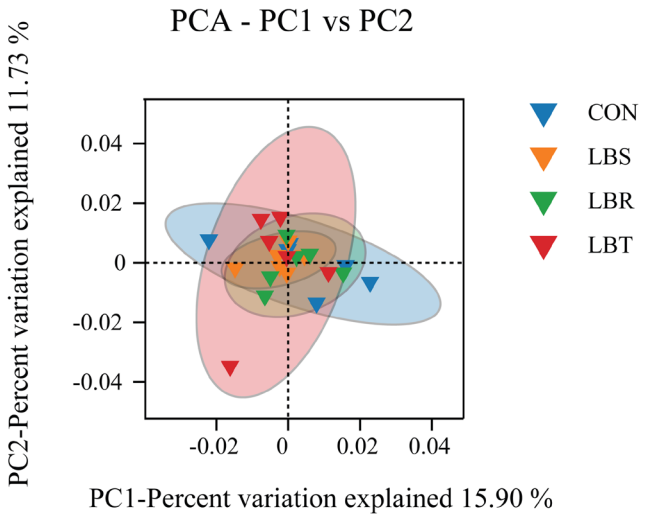

FIGURE 3 | Principal coordinate analysis (PCoA) of rumen microbial communities in different treatment groups.

3.8 | Analysis of Microbial Interactions and Their Correlation With Fermentation Parameters

At the genus level of bacterial taxonomy, the rumen fluid pH showed a positive correlation with the relative abundance of *Prevotella* ($p < 0.05$), and the proportion of acetate in the rumen fluid was positively associated with the relative abundances of *Prevotella* and *Rikenellaceae_RC9_gut_group* ($p < 0.05$) (Figure 7). The concentration of isobutyrate was positively correlated with the relative abundances of *unclassified_F082*, *Rikenellaceae_RC9_gut_group*, and *unclassified_Lachnospiraceae* ($p < 0.05$). The concentration of valerate was positively correlated with the relative abundances of *unclassified_F082*, *Ruminococcaceae_NK4A214_group*, and *Desulfovibrio* ($p < 0.05$). The concentration of isovalerate was positively correlated with the relative abundances of *Ruminococcaceae_NK4A214_group*, *Christensenellaceae_R_7_group*, *Desulfovibrio*, and *Butyrivibrio* ($p < 0.05$). Additionally, the proportions of propionate and the acetate-to-propionate ratio were positively correlated with the relative abundance of *Butyrivibrio* ($p < 0.05$) (Figure 7).

The TVFAs in rumen fluid exhibited a positive correlation with the concentrations of propionate and isobutyrate ($p < 0.05$)

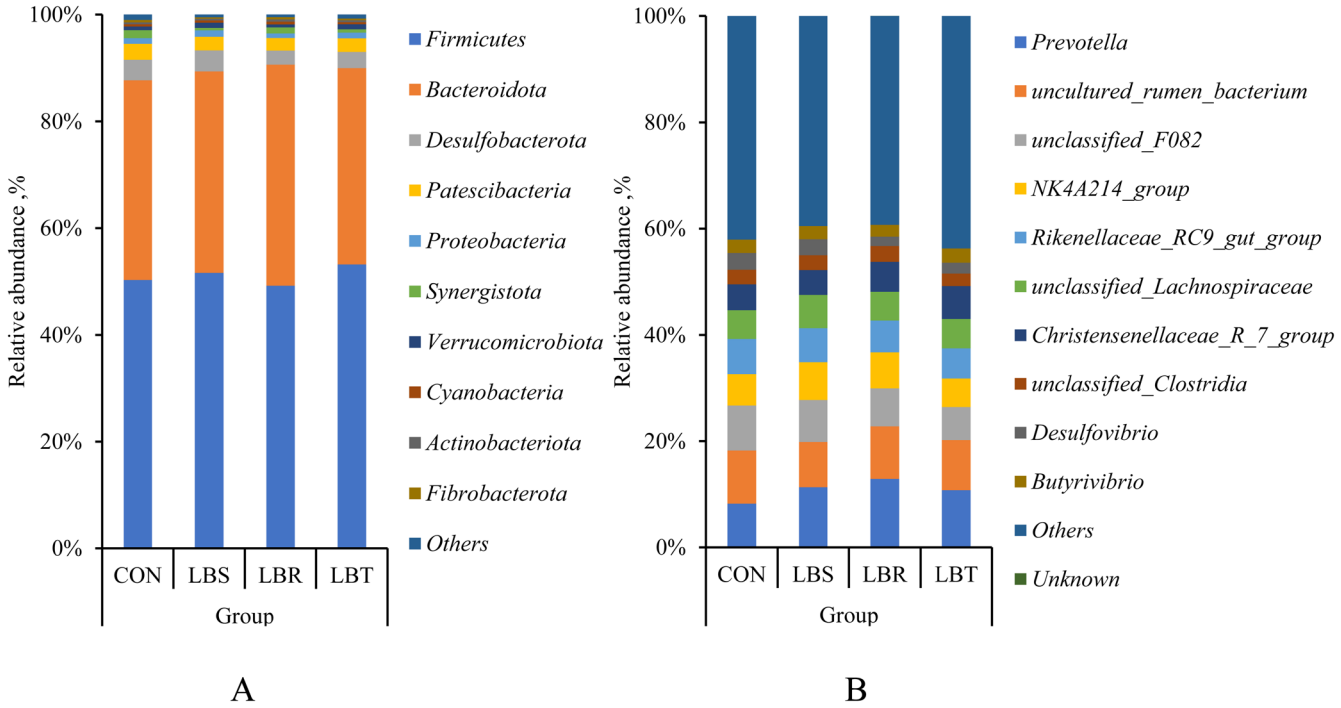

FIGURE 4 | Histogram of the horizontal species distribution of bacterial phyla (A) and genus (B) in the different treatment groups.

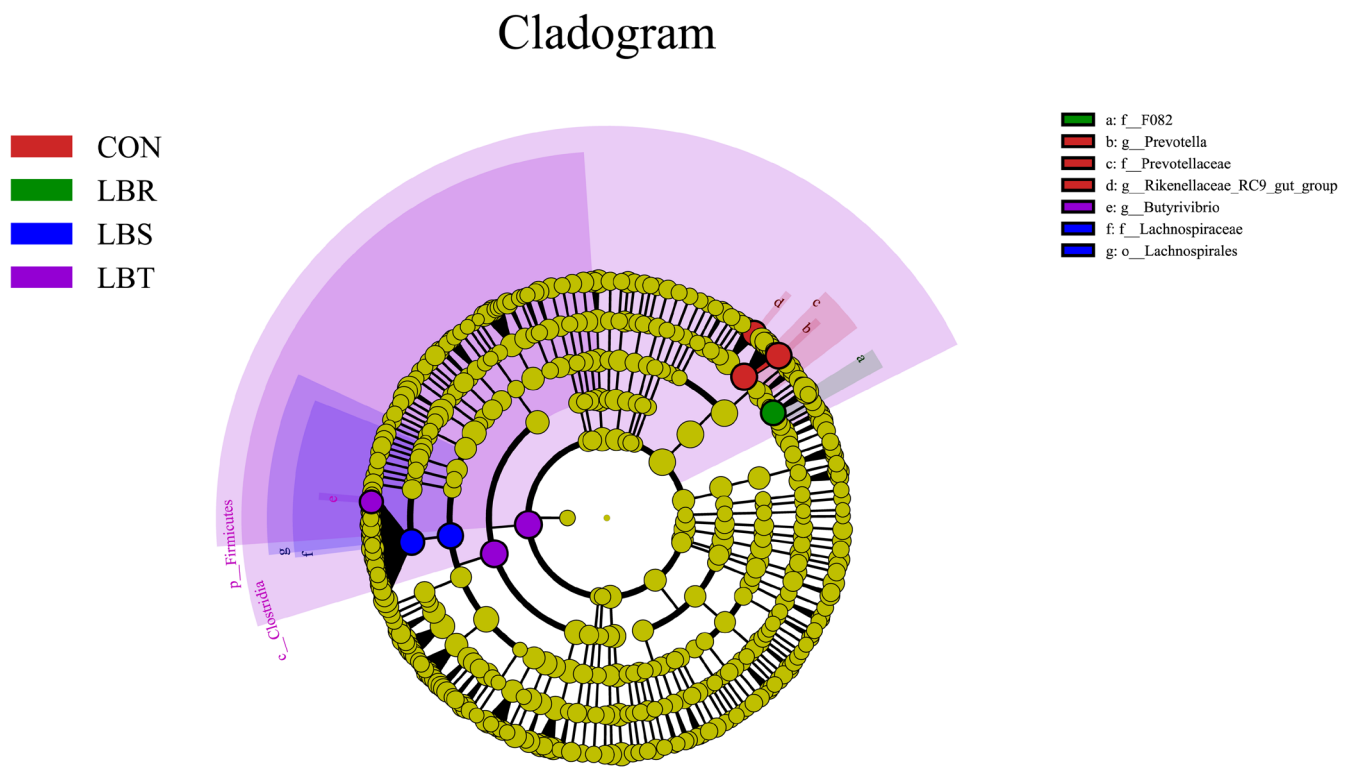

FIGURE 5 | Linear discriminant analysis effect size (LEfSe) cladogram comparing microbial communities among the different treatment groups.

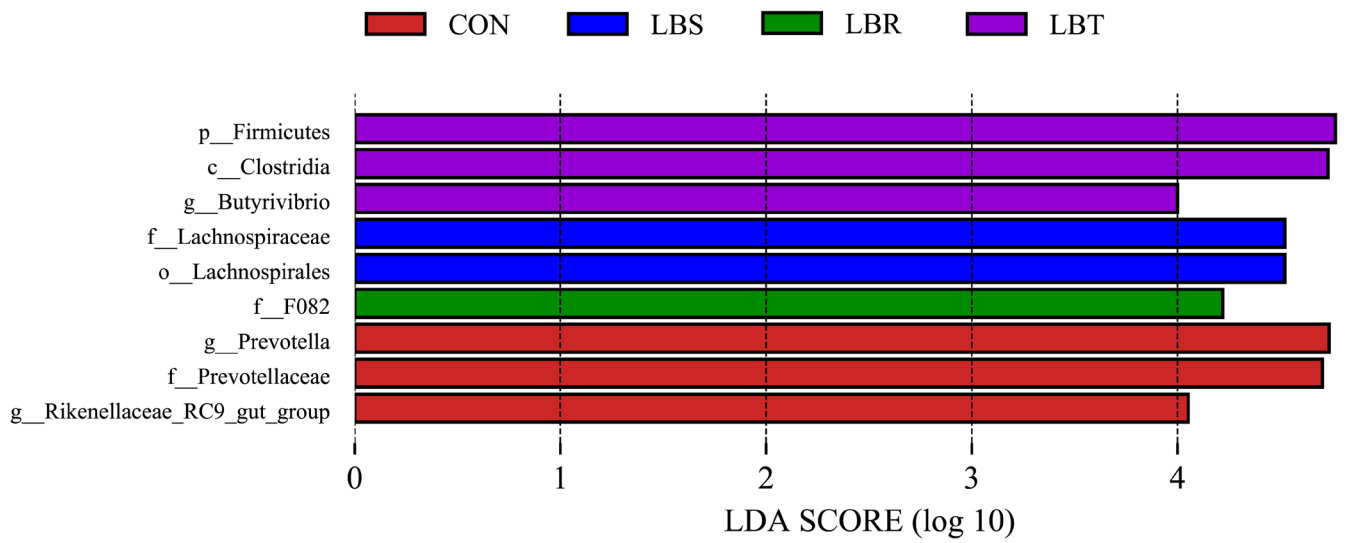

FIGURE 6 | Histogram of LDA scores calculated for each taxon from phylum to genus.

(Figure 7). Conversely, the concentration of acetate showed a negative correlation with the concentrations of isovalerate and valerate ($p < 0.01$). The ratio of butyrate was found to be negatively correlated with the ratio of valerate ($p < 0.01$). Furthermore, the ratio of isovalerate demonstrated a positive correlation with both the ratio of valerate and the acetate-to-propionate ratio ($p < 0.05$). Similarly, the ratio of valerate was positively correlated with the acetate-to-propionate ratio ($p < 0.05$) (Figure 7).

3.9 | Tax4Fun Gene Function Estimation

The Tax4Fun software was utilized to predict the functional profiles of the rumen microbial community in sheep, with the top 15 functions selected for detailed analysis (Figure 8). The predominant functions identified were "Metabolic pathways," "ABC transporters," and "Biosynthesis of secondary metabolites," which are considered core to the rumen microbiome. Supplementation with *Lycium barbarum* byproducts induced functional variations

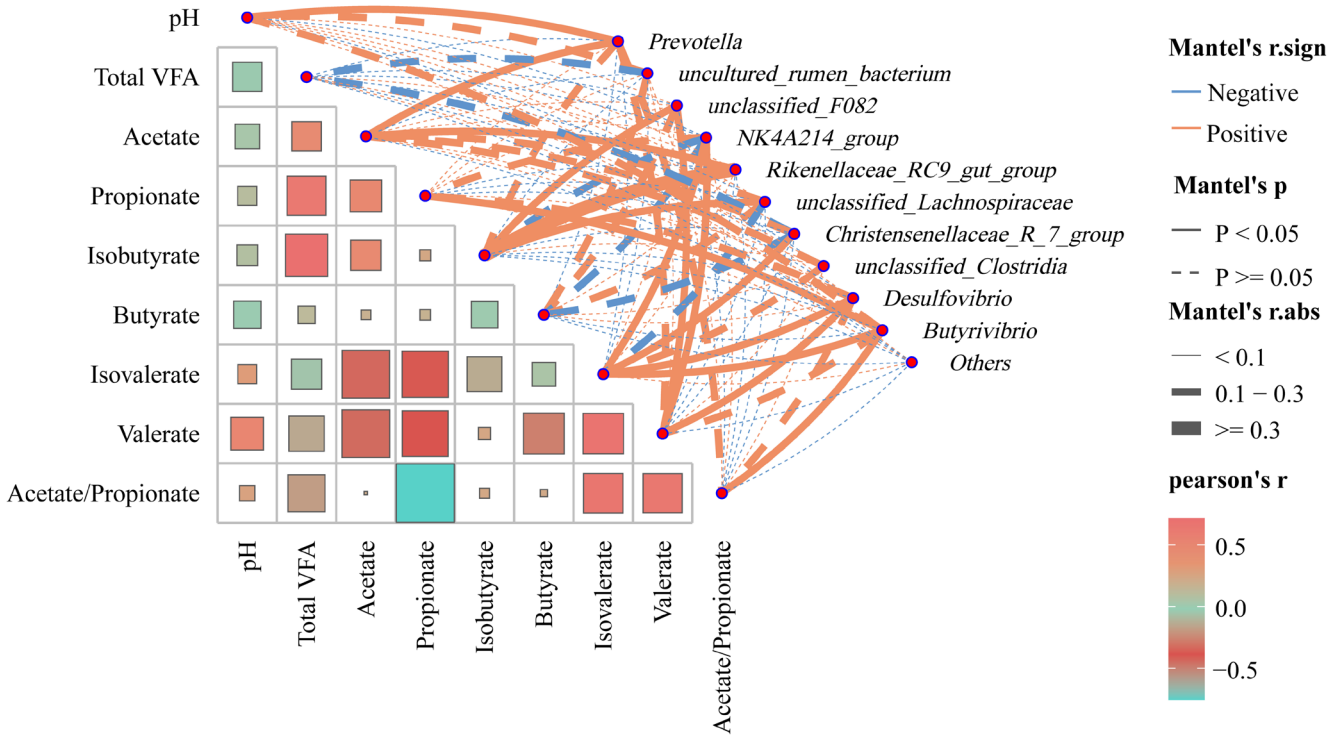

FIGURE 7 | Spearman correlation and cluster analysis between bacterial abundance and rumen fermentation parameters at the genus level.

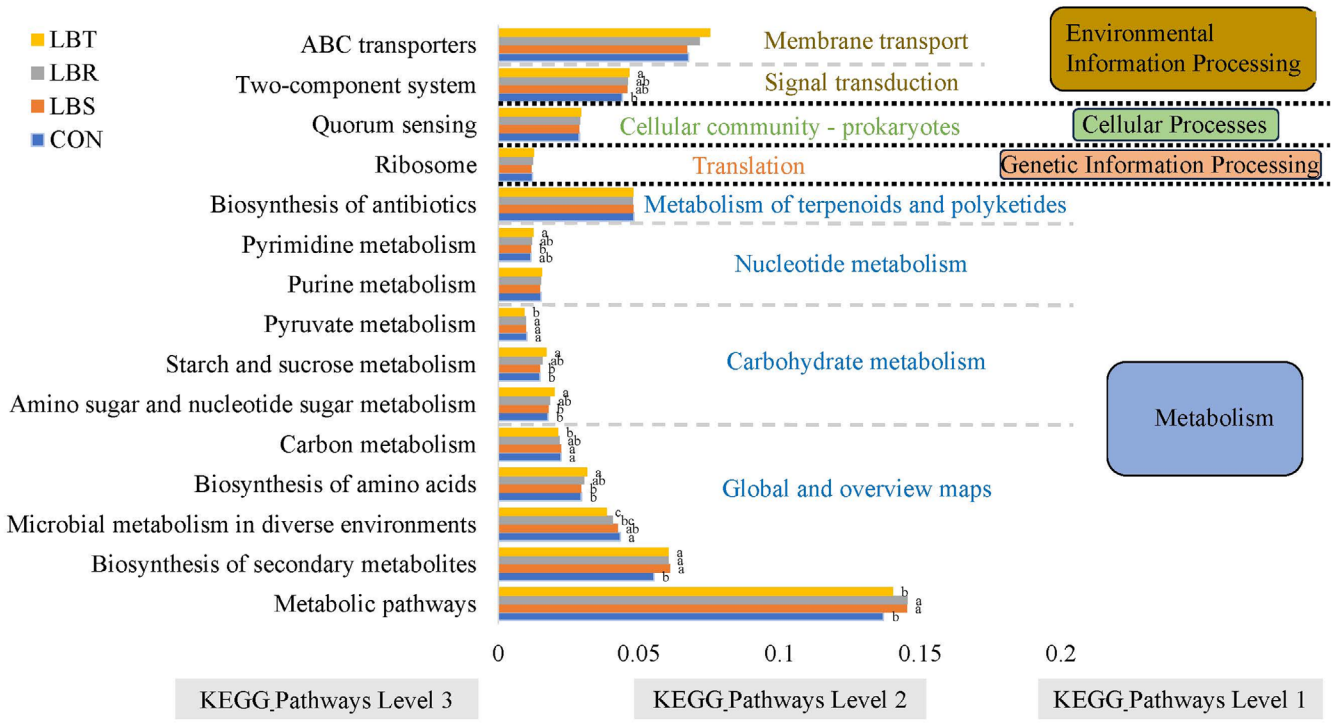

FIGURE 8 | Functional predictions for rumen microbiota with significantly different KEGG pathways ($p < 0.05$) for the different treatment groups.

among the groups. The "metabolic pathways" genes were found to be enriched in the LBS and LBR groups ($p < 0.05$). Furthermore, an analysis of functional genes associated with "Secondary Metabolite Biosynthesis" revealed an enrichment of these genes within the LBS, LBR, and LBT groups ($p < 0.05$). Conversely, functional genes related to "Microbial Metabolism in Diverse Environments" exhibited a marked decrease in enrichment in the LBR and LBT groups ($p < 0.05$). A subsequent analysis identified an overrepresentation of functional genes associated with "amino acid biosynthesis," "carbohydrate and nucleotide metabolism,"

"starch and sucrose metabolism," and "two-component systems" within the LBT group ($p < 0.05$). Concurrently, there was a decline in the enrichment levels of functional genes related to "carbon metabolism" and "pyruvate metabolism" ($p < 0.05$) (Figure 8).

4 | Discussion

Bacteria represent the most numerous and metabolically active microbial population in the rumen of ruminants. They encode and secrete a variety of digestive enzymes (Stewart et al. 2019; Hess et al. 2011), converting indigestible fiber into essential nutrients, such as amino acids and proteins, through rumen fermentation, thereby meeting the nutritional needs of the host (Mullins et al. 2013; Li et al. 2021). The structure of the diet regulates the activity of digestive enzymes and the stability of the rumen environment, which in turn affects the microflora and fermentation function. In this study, compared to the control group, the proportions of acetic acid and propionic acid in the rumen fluid of sheep supplemented with *Lycium barbarum* byproducts increased, while the proportions of valeric acid and isovaleric acid decreased; this change is attributed to the rich nutrients and active ingredients found in *Lycium barbarum* byproducts. Structural carbohydrates, such as cellulose, lignin, and polysaccharides, are slowly degraded in the rumen, with acetic acid being the main product (Wang 2020). In contrast, non-structural carbohydrates, such as starch and monosaccharides, are degraded rapidly, primarily yielding propionic acid (Wang 2020), this may explain why the total volatile fatty acids, as well as the contents of acetic acid and propionic acid, are highest in the rumen fluid of sheep in the LBT group. Studies have shown that plants rich in polysaccharides can stimulate the growth of fiber-degrading bacteria and increase the concentration of volatile fatty acids such as acetic acid, propionic acid and butyric acid (Rabee et al. 2024). We also found that the activity of carboxymethyl cellulase (CMC) increased in the LBS and LBR groups. CMC was shown to be able to synergize with xylanase, salicylate and other complex enzymes to convert fiber substances into glucose, which is an important indicator of the ability of rumen microorganisms to degrade fibrous materials (Hao et al. 2021; Thapa et al. 2023). Additionally, vegetable oils have been shown to reduce ruminant methane emissions, a process that is often accompanied by an increase in propionic acid production (Cobellis et al. 2016). *Lycium barbarum* seeds and residues provide *Lycium barbarum* seed oil and *Lycium barbarum* polysaccharides, flavonoids, betaine and other active ingredients as fermentation substrates (Abdallah et al. 2019; Nur Atikah et al. 2018). These readily fermentable substrates promote glycolysis and fatty acid metabolism in sheep, with pyruvate dehydrogenase (PDH) playing a crucial role in this process. The enzyme catalyzes the oxidative decarboxylation of pyruvate into acetyl-CoA and carbon dioxide, subsequently participating in the tricarboxylic acid cycle and generating ATP through the oxidative phosphorylation of acetyl-CoA to supply energy to cells (Škerlová et al. 2021; Kolobova et al. 2001). This explains why PDH activity was higher in both the LBS and LBR groups in this study. Pepsin and APs decompose proteins into small peptides and amino acids, participating in protein synthesis and degradation processes (Hao et al. 2021; Plaizier et al. 2012). Additionally, APs play a role in cellular metabolism and immune regulation (Harmon and Swanson 2020). The increased content of these two enzymes with the addition of *Lycium barbarum* byproducts indicates a promotion of protein degradation-related bacterial abundance in the rumen and an enhancement of microbial activity and enzyme secretion capacity. Urease, involved in the urea cycle and a crucial component of amino acid metabolism, catalyzes urea hydrolysis to maintain nitrogen balance and promotes microbial crude protein synthesis (Zhang et al. 2020; Jin et al. 2018). The increased urease production due to *Lycium barbarum* byproducts suggests a heightened ability of the rumen microbiota to synthesize microbial crude protein post-addition.

Physical health and resistance to immune diseases are crucial for the grazing and management of livestock in fluctuating climates. Polysaccharides have demonstrated the ability to enhance immunoglobulin production in animal serum and bolster immunity (Islam et al. 2020; Sun et al. 2021; Zhou et al. 2024). The supplementation of *Lycium barbarum* byproducts improved the immune status of sheep, with *Lycium barbarum* polysaccharides potentially playing a significant role. Additionally, the aforementioned increase in AP levels, which are involved in immune regulation, may have facilitated the conversion of specific immune factors or substances. ALB, a key transporter in the bloodstream, not only binds and carries insoluble organic small molecules and inorganic ions but is also an essential nutrient (Belinskaia et al. 2021). The observed increase in serum ALB content may reflect an increase in various digestive enzymes, promoting the breakdown and synthesis of nutrients. Excess nutrients must be rapidly transported to different cells and organs for utilization, indirectly stimulating metabolic processes and hastening the excretion of waste products, which aligns with the observed reductions in UA and BUN levels. Oxidative stress is intricately linked to animal diseases (Lykkesfeldt and Svendsen 2007), and enhancing the activity of antioxidant enzymes is vital for promoting animal health. Studies have confirmed that plant-derived active ingredients such as flavonoids, thyme essential oil, and allicin possess antioxidant properties (Wang et al. 2024). The addition of *Lycium barbarum* byproducts increased the levels of SOD and GSH-PX, likely due to the antioxidant activity of flavonoids and *Lycium barbarum* seed oil, which scavenge free radicals and decrease serum malondialdehyde levels.

Diet structure and feeding methods have been shown to alter microbial diversity, effectively safeguarding livestock health (Henderson et al. 2016). Alpha (α) diversity analysis results indicated a reduction in bacterial diversity within the supplementary feeding group. This shift in microbial composition, rather than a simple loss of diversity, may indicate a strong selection for a more efficient and specialized microbial community adapted to the new substrates, a phenomenon that has been associated with improved performance in some studies. Research indicates that reduced diversity correlates with an increased dominance index of *Prevotella_1* and superior growth performance in sheep (Liu, Wu, et al. 2019; Liu, Xu, et al. 2019). Buffaloes with lower bacterial diversity, as measured by the α diversity index, showed superior milk yield, fat-corrected milk yield, and milk protein content (Li et al. 2020). The bacterial phyla Bacteroidetes and Firmicutes are dominant and play crucial regulatory roles at the phylum level in the rumen of ruminants (Fan et al. 2020; Cui et al. 2019; Singh et al. 2012; Oliveira et al. 2013; Myer et al. 2015). The inclusion of *Lycium barbarum*

byproducts has been observed to reduce the relative abundance of Desulfobacterota, a class of bacteria within the Proteobacteria phylum that oxidizes lactic acid, pyruvic acid, and other compounds (Kim et al. 2022). Under heat stress, the rumen experiences an increase in lactic acid bacteria and a decrease in acetic acid bacteria (Zhao et al. 2019). The biologically active substances in *Lycium barbarum* byproducts, such as *Lycium barbarum* seed oil and polysaccharides, can effectively alleviate heat stress (Abdallah et al. 2019; Nur Atikah et al. 2018; Chandrasekharaiah et al. 2015), leading to a decrease in lactic acid bacteria, a reduction in Desulfobacterota-available lactic acid, and a lower relative abundance. Proteobacteria, including pathogenic bacteria like *Escherichia coli* and Salmonella, are dominant in the spoilage process of samples (Edgar 2010). The addition of *Lycium barbarum* byproducts decreased the relative abundance of Proteobacteria, likely due to the combined effects of dominant bacteria competing for nutrients, the inhibition of harmful bacteria by active ingredients such as flavonoids, polysaccharides, and vegetable oils in the byproducts, and the enhancement of immunity and antioxidant capacity (Kumar et al. 2014).

Prevotella plays a central role in carbohydrate and hydrogen metabolism, and its high abundance in the rumen of ruminants is closely associated with a healthy microbiome (Kim et al. 2017; Dodd et al. 2010; Kabel et al. 2011). *Prevotella* was identified as the dominant bacterial genus in this study's sheep, as well as in cattle (Holman and Gzyl 2019; Furman et al. 2020), buffalo (Aguilar-Marin et al. 2020), goats (Wetzels et al. 2015; Lv et al. 2019), yaks (Su et al. 2022; Guo et al. 2020; Zhao et al. 2022), deer (Gruninger et al. 2014), and other animals. *Prevotella* is widely distributed across various physical environments, including humans, livestock, rodents, and insects (Tett et al. 2021; Accetto and Avguštin 2019; Portincasa et al. 2022; Könönen and Gursoy 2021; Richter et al. 2022; Thomas-White et al. 2018). Supplementation with *Lycium barbarum* byproducts increased the relative abundance of *Prevotella*, particularly from *Lycium barbarum* seeds and residues, suggesting a positive impact on maintaining a healthy microbiome. This increase may be attributed to the utilization of byproduct nutrients by microorganisms, with *Prevotella* being capable of decomposing various polysaccharides (Portincasa et al. 2022; Dietary et al. 2022). As the dominant bacterium with the highest abundance, *Prevotella* outcompetes other bacteria for resource utilization. Additionally, the increase in rumen juice digestive enzyme activity due to byproduct addition and the inhibition of harmful bacteria by active ingredients may also contribute. It is hypothesized that the active flavonoids present in *Lycium barbarum* byproducts modulate the rumen microflora, thereby altering the digestion and metabolism of nutrients. This hypothesis is supported by studies where Holstein calves were fed silage mulberry leaves and mulberries rich in flavonoids, which led to similar conclusions (Kong et al. 2019; Balcells et al. 2012). Metabolic analysis of the rumen microbiome revealed an enrichment of carbohydrates, amino acids, lipids, vitamins, and energy, among other metabolic effects (Tett et al. 2021; Accetto and Avguštin 2019; Kovatcheva-Datchary et al. 2015; Aguilar-Marin et al. 2020; Takahashi and Yamada 2000). *Lycium barbarum* byproducts, including polysaccharides, seed oil, polyphenols, and other bioactive components, are known to be metabolized by *Prevotella* species (Zhang et al. 2022; Gong et al. 2022). Correlation analysis indicated a positive correlation between the proportion of acetic acid in rumen fluid and the abundance of *Prevotella*. The additional nutrients from supplementary feeding led to rapid reproduction of dominant bacteria and increased production of volatile fatty acids. Tax4Fun gene function analysis results suggested an enrichment of "metabolism"-related functions in the byproduct supplementation group, and the "environmental information processing" function was also found to be enriched. *Prevotella* has been shown to benefit the effective biosynthesis of nutrients in ruminants and significantly mitigate the negative environmental impacts of rumen metabolism (Betancur-Murillo et al. 2023), we speculate that supplementing with *Lycium barbarum* byproducts may reduce the excretion of unused nutrients (such as fecal nitrogen and urinary nitrogen) into the environment by enhancing rumen digestive function. It is important to note that these functional profiles were predicted from 16S rRNA gene data using Tax4Fun and represent functional potential rather than actual metabolic activity. Future research using metagenomics or metatranscriptomics is needed to confirm these predicted functional shifts. *Uncultured_rumen_bacterium* and *unclassified_F082* were identified as dominant bacteria at the genus level in sheep in this study, and variations in rumen microorganisms among different ruminant species were observed, potentially related to breed, diet, and other factors. The functions of *Uncultured_rumen_bacterium* and *unclassified_F082* remain unclear, with some studies suggesting their involvement in fatty acid formation, directly or indirectly promoting the synthesis of polyunsaturated fatty acids and inhibiting their biohydrogenation to saturated fatty acids (Wang, Li, et al. 2022; Wang, Zeng, et al. 2022). Some studies have also postulated that the dominant bacterial genus *unclassified_F082* (belonging to the Rikenaceae family) is a rumen bacterium involved in carbohydrate degradation, and its abundance changes may be related to the fermentation of dietary fiber components (Wang, Li, et al. 2022; Wang, Zeng, et al. 2022; Bensoussan et al. 2017). The *Ruminococcaceae_NK4A214_group*, involved in the degradation of fibrous materials, primarily acts through the production of complex enzymes (Israeli-Ruimy et al. 2017; Bensoussan et al. 2017). Supplemental feeding with *Lycium barbarum* seeds and residues increased the relative abundance of the *Ruminococcaceae_NK4A214_group*, possibly due to the presence of active ingredients such as unsaturated fatty acids, polysaccharides, and flavonoids. Functional polysaccharides have been shown to positively regulate the structure of the rumen flora. Hericium erinaceus polysaccharides can maintain rumen health and prevent rumen acidosis in goats (Li et al. 2019), while *Artemisia annua* polysaccharide can increase the proportion of Firmicutes and Fibrobacteres in the rumen and reduce pathogenic bacteria (Jin et al. 2021). Flavonoids, known for their anti-inflammatory, antioxidant, and antibacterial properties (Polumackanycz et al. 2019; Balcells et al. 2012), have significantly increased the abundance of cellulose-decomposing bacteria in the rumen of fattening beef cattle and enhanced rumen fermentation (Li et al. 2017). Supplementing with *Lycium barbarum* twigs reduced the abundance of the *Ruminococcaceae_NK4A214_group*, possibly due to low polysaccharide and flavonoid content in branches and leaves, which do not reach the threshold for regulatory function. Additionally, the more difficult degradation of fiber substances in woody plants compared to herbaceous plants can lead to reduced feed intake and, consequently, a decrease in the population size of fiber-degrading bacteria. *Desulfovibrio* species, classified within the

phylum Proteobacteria, are well-recognized as pathogenic bacteria (Huisingh et al. 1974). The supplementation of *Lycium barbarum* byproducts has been observed to decrease the abundance of *Desulfovibrio*, potentially attributable to the presence of bioactive components within the byproducts that possess antibacterial activities, such as polysaccharides and flavonoids. It is important to acknowledge a limitation in the current study's design: the diets were not formulated to be isocaloric or isonitrogenous. The treatment groups received additional nutrients (e.g., protein, energy) from the *Lycium barbarum* byproducts in addition to the soybean meal base. Consequently, some of the observed benefits, such as improved average daily gain and enhanced immune status, may be partially attributed to this increased nutritional intake rather than solely to the specific bioactive compounds. Future studies should employ isocaloric and isonitrogenous diets to fully isolate the effects of the bioactive components.

5 | Conclusions

Supplementing grazing sheep with *Lycium barbarum* byproducts enhances growth performance, improves rumen function and host health status. The bioactive components present in these byproducts, such as polysaccharides, polyphenols (flavonoids), and vegetable oils, may have contributed to the observed results through mechanisms such as directly inhibiting pathogen proliferation, modulating the intestinal microbiota, bolstering immune function, and mitigating oxidative stress. This study provides evidence for the valorization of *Lycium barbarum* processing wastes as functional feed supplements, offering a sustainable strategy to improve sheep production and reduce reliance on antibiotics.

Author Contributions

Conceptualization: F.H. and X.Z.; data curation: F.H.; formal analysis: X.Z.; funding acquisition: F.H.; methodology: F.H. and X.Z.; software: X.Z.; supervision: F.H.; writing – original draft: X.Z. and F.H.; writing – review and editing: X.Z., F.H., W.D., and K.X. All authors have read and agreed to the published version of the manuscript.

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Ethics Statement

The experiments were conducted according to the guidelines of experimental field management protocols (Files No. 2010-1 and 2010-2), which were approved by the Animal Use and Care Committee of Lanzhou University.

Consent

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

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 below: NCBI; PRJNA1150173. Submission ID: SUB14675436 and BioProject ID: PRJNA1150173. https://www.ncbi.nlm.nih.gov/sra/PRJNA1150173.

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