Supplementation of Lycium barbarum residue improves the animal performance through the interaction of rumen microbiota and metabolome of Tan sheep

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Abstract

Background

Lycium barbarum residue (LBR), a by-product of *Lycium barbarum* processing, contains bioactive components which can be used as a plant-derived feed additive in animal husbandry. This study aimed to investigate the effects of LBR on the rumen fermentation parameters, ruminal microbes and metabolites, and growth performance of Tan sheep.

Methods

Sixteen Tan sheep were fed with a basal diet (CON; $n = 8$) or a basal diet supplemented with 5% of LBR (LBR; $n = 8$). The experiment lasted for 70 d, with 10 d adaptation period and 60 d treatment period. After slaughter, rumen fluid samples were collected and analyzed by 16S rRNA sequencing and untargeted metabolomics to elucidate the alternations of rumen microbial community and rumen metabolites.

Results

Data showed that LBR enhanced the average daily feed intake, average daily gain, and total volatile fatty acids ($P < 0.01$) but decreased ammonia-nitrogen concentration ($P < 0.01$) and rumen pH ($P < 0.05$). Additionally, LBR supplementation improved the relative abundances of *Prevotella*, *Succiniclasticum*, *Ruminococcus*, *Coprococcus*, *Selenomonas*, and *Butyrivibrio* ($P < 0.05$) and reduced the abundances of *Oscillospira* and *Succinivibrio* ($P < 0.05$). Metabolomics analysis revealed that the LBR altered the ruminal metabolome ($P < 0.01$) by increasing the abundances of ruminal metabolites involved in amino acids (e.g., L-proline, L-phenylalanine, L-lysine, and L-tyrosine), pyrimidine metabolism (e.g., uridine, uracil, and thymidine), and microbial protein synthesis (e.g., xanthine and hypoxanthine). Correlation analysis provided potential relationships between major microbiota and metabolites in the rumen.

Conclusions

In conclusion, supplementation of LBR affects rumen fermentation by altering the rumen microbiome and metabolome, thereby improving the growth performance of Tan sheep.

Introduction

With a ban on antibiotics as feed additives in many countries worldwide [1], there is growing interest worldwide in developing functional feed additives that could maintain health and improve the performance of animals [2]. Plant-derived bioactive compounds have shown promise as alternatives to growth-promoting antibiotics in ruminant production [3].

Lycium barbarum is a well-known traditional Chinese herbal medicine and edible plant species [4], commonly used as a supplement in Eastern and Western diets. *Lycium barbarum* has various biologically active components, including *Lycium barbarum* polysaccharides (LBPs), polyphenols, pigments, flavonoids, amino acids, vitamins, and trace elements [5], which can exert antioxidant, anti-inflammatory, anti-aging [6], gastrointestinal protective [7], cytoprotective as well as immunomodulatory [4] properties. The properties suggest that *Lycium barbarum* may be used as an alternative supplement for improving animal performance [8]. Previous studies found that dietary supplementation of *Lycium barbarum* could reduce pre-weaning mortality, improve feed conversion ratio (FCR) and growth rate [9]. *Lycium barbarum* promotes the growth of probiotics, such as *Ruminococcaceae* and *Lachnospiraceae*, alters the Firmicutes:Bacteroidetes

ratio in the gut microbiota and modulates gastrointestinal microbiota composition and cecal fermentation in rabbits [10]. *Lycium barbarum* affects the secretion of leptin, insulin, and glucose, improving energy homeostasis in rabbits [11]. Additionally, *Lycium barbarum* residue (LBR), a by-product of *Lycium barbarum* processing, contains bioactive components of *Lycium barbarum* and is available as a feed additive. LBR as a dietary supplement improved animal performance and product quality [11, 12]. The addition of LBPs to pig diets improved growth performance [13], immune function [14], and gut microflora [15]. Moreover, the diet supplemented with LBPs improved body weight, average daily gain (ADG), average daily feed intake (ADFI), and FCR in broiler chickens [16]. As a popular functional food, LBPs can stimulate the growth of beneficial gut bacteria, inhibit the growth of *E.coli*, and enhance the immune status and antioxidant activity, thereby improving host growth performance [12].

Up to now, the effects of LBR on livestock animals have mainly been studied in monogastric animals, with few in ruminant animals. Feeding regimens and diets considerably impact on rumen microorganisms [17]. Rumen fermentation and rumen microorganisms have critical roles in utilizing dietary fibers and other components, thereby modulating host health [18]. In a recent study in lambs, the supplementation of a Chinese medicine polysaccharide comes from the mixture of *Lycium barbarum* and *Astragalus membranaceus* enhanced animal growth performance by changing rumen fermentation and bacterial community [19].

Thus, we hypothesized that there are LBR associated rumen microbes and the ruminal microbial communities and function contributed to the growth performance of Tan sheep. Therefore, this study investigated the effects of LBR on the ruminal microbiome and metabolome and revealed the interaction between them, thereby improving the rumen inner environment and growth performance of Tan sheep.

Materials And Methods

Animal ethics statement

The experimental protocols and treatments were approved by the Institutional Animal Care and Use Committee of Ningxia University (NXUC20200618).

Animals, diets, and experimental design

This study was conducted on a large-scale breeding farm in Ningxia, China. Sixteen ram Tan sheep (*Ovis aries*) at the age of 4 months old (local breed; 20.8 ± 0.34 kg) were randomly divided into 2 experimental groups, including the control and *Lycium barbarum* residue groups (n = 8 in each group). The sheep in the control group (CON) were fed with a basal diet, while those in the LBR groups were fed with a basal diet supplemented with 5% LBR. A 70 d feeding experiment (the first 10 d is the pre-trial period) was then carried out accordingly. The basal diets were offered to the Tan sheep twice at 08:00 and 16:00 daily with free access to drinking water. The ingredients and nutritional composition of the basal diet are listed in Table 1. The chemical compositions of the diets were analyzed based on the AOAC [20]. The metabolizable energy content was formulated based on the NRC [21]. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were assessed following the method described by Van Soest et al. [22]. The experimental design and workflow are summarized in Fig. 1.

Table 1
Ingredients and chemical composition of the basal diet (on a dry matter basis).

Ingredients	Diets[1]	
	CON	LBR
Corn (%)	54.8	49.8
Soybean meal (%)	12.0	12.0
Linseed (%)	3.33	3.33
Flaxseed meal (%)	4.25	4.25
Soybean oil (%)	3.40	3.40
Salt (%)	0.42	0.42
Baking soda (%)	0.42	0.42
Premix (%)[2]	4.25	4.25
Bone meal (%)	2.13	2.13
Straw (%)	15.0	15.0
Lycium barbarum residue (%)	0	5.00
Chemical composition		
DM (%)	81.7	80.4
DE ($MJ \cdot Kg^{-1}$)	12.9	12.7
CP (%)	10.2	10.2
EE (%)	12.4	12.0
NDF (%)	37.0	36.7
ADF (%)	19.1	19.0
Calcium (%)	0.23	0.23
Phosphorus (%)	0.30	0.30

[1] CON = control group; LBR = *Lycium barbarum* residue group.

[2] contained in each kilogram of premix: 100000 IU vitamin A, 20000 IU vitamin D3, 60 IU vitamin E, 1000 mg Fe, 1000 mg Mn, 780mg Zn, 270 mg Cu, 12 mg Se, 10 mg I.

DM = dry matter; DE = digestible energy; CP = crude protein; EE = ether extract; NDF = neutral detergent fiber; ADF = acid detergent fiber.

Sample collection and measurements

On d 70, the rumen fluid was collected, filtered with 4 layers of sterile gauze, and divided into 10 mL sterile tubes. Each rumen fluid had 4 replicates and was stored at -80°C. The pH of the rumen fluid was measured immediately by a pH meter. Total volatile fatty acids (TVFAs) were determined using gas chromatography (Agilent 8890 GC, CA, USA) [23]. The ammonia-nitrogen (NH_3-N) concentration was measured using an enzyme-labeled instrument (Multiskan FC, Thermo

Fisher, Beijing, China) [24]. During the trial, the body weight (BW) and leftover feed were measured daily for the calculation of ADG and ADFI. The gain feed ratio (F/G) was calculated by ADFI/ADG.

DNA extraction and sequencing

The QIAamp DNA Stool Mini Kit was used to extract the total DNA of rumen fluid samples. The DNA quality was determined by 1.2% agarose gel electrophoresis. The primers were designed, and PCR was used to amplify the V3 and V4 fragments of the 16S rRNA sequences. After purification, quantification, and homogenization of the PCR amplification products, high-throughput sequencing was performed by Illumina Miseq. The raw sequence data were demultiplexed using the demux plugin followed by primers cutting with the cutadapt plugin [25] and subjected to quality filtered, denoised, merged, and chimera removed using the DADA2 plugin. Taxonomy was assigned to amplicon sequence variants (ASVs) using the classify-sklearn naive Bayes taxonomy classifier in the feature-classifier plugin [26] against the SILVA Release 132 database. The raw sequence data have been uploaded to NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA837041).

Bioinformatics for rumen microbiota analysis

Sequence data analyses were mainly performed using QIIME2 and R packages (v3.2.0). The alpha diversity index (Shannon, Simpson, Chao1, and Good's coverage) of the rumen microbiota based on ASV level was analyzed using the ASV table in QIIME2 (version 2019.4) and visualized as box plots. Beta diversity (principal coordinates analysis, PCoA and nonmetric multidimensional scaling analysis, NMDS) was also conducted through QIIME2 (version 2019.4) and was assessed using Bray-Curtis dissimilarity. The Kruskal-Wallis sum rank test detected differentially abundant taxa between the groups. The differential bacteria were analyzed through linear discriminant analysis effect size (LEfSe) software (http://huttenhower.sph.harvard.edu/galaxy/). Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) was conducted to perform a functional prediction analysis of differential microbiota. The analysis was operated using the PICRUSt software package. The databases of MetaCyc (https://metacyc.org/) were conducted to map the enriched pathways of different levels.

Metabolite extraction and LC-MS/MS analysis

A 100 μL of rumen fluid sample was transferred to an EP tube. Each sample was mixed with 400 μL extract solution (acetonitrile:methanol = 1:1, v/v, containing isotopically-labeled internal standard mixture). Then, the mixture was vortexed for 30 s, sonicated for 10 min in an ice-water bath, and incubated for 1 h at -40°C. After that, the samples were centrifuged at 13,000 × g at 4°C for 15 min. The supernatant was pipetted into a sample bottle. Each sample with an equal volume of approximately 20 μL was mixed thoroughly to be a quality control (QC) sample [27].

A UHPLC system (Vanquish, Thermo Fisher Scientific) with a UPLC BEH Amide column (2.1 mm × 100 mm, 1.7 μm) coupled to Q Exactive HFX mass spectrometer (Orbitrap MS, Thermo) was utilized for the detection and analysis of metabolites in rumen fluid. The mobile phases were 25 mM ammonium acetate and 25 mM ammonium hydroxide in water (pH = 9.75, solvent A) and acetonitrile (solvent B). The auto-sampler temperature was 4°C, and each injection volume was 3 μL. The QE HFX mass spectrometer was used to acquire MS/MS spectra on information-dependent acquisition (IDA) mode in the control of the acquisition software (Xcalibur, Thermo).

Metabolomic data preprocessing and analysis

The original data were converted to the mz XML format by Proteo Wizard and processed by R packages of XCMS software to identify peaks and remove noise [28]. Then an in-house MS2 database (BiotreeDB) was applied in metabolite annotation. The cutoff for annotation was set at 0.3. The positive and negative data were combined. The data were conducted using Metaboanalyst 5.0 for principal component analysis (PCA), orthogonal partial least squares discriminant analysis (OPLS-DA), and pathway enrichment analysis. Based on the OPLS-DA result, the differential metabolites with the variable importance in the projection (VIP) > 1.0 and the Student's *t*-test ($P < 0.05$) were identified as the significantly

different metabolites between the two treatments. The fold change (FC) was used to evaluate the identification of significantly differential metabolites. In hierarchical clustering, hierarchical clustering analysis (HCA) for significantly different metabolites was used with Cluster 3.0 software. The online Human Metabolome Database (HMDB) (https://hmdb.ca), Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/) and Metabo Analyst 5.0 (http://www.metaboanalyst/) were conducted to map the enriched pathways of different metabolites.

Correlation analysis

The correlation between different rumen microbial genera ($P < 0.05$ and relative abundance > 0.1%), rumen fluid metabolites with VIP > 1.0, $P < 0.05$, rumen fermentation parameters and growth performance were assessed by Spearman's correlation analysis in R language package (Version 3.2.4, http://www.r-project.org). Spearman's rank correlation coefficient (r) was from − 1 to 1. The $|r|$ value indicated the degree of correlation between variables. These correlations with Spearman's correlation value and P value were visualized using the heat map drawn by Cytoscape. The connections with P-value < 0.05 and $|r| > 0.5$ were considered statistically significant.

Statistical Analysis

All data were evaluated using the Student's t-test of SPSS Statistics software (version 23.0, IBM, Chicago, USA). Statistical significance was declared at $P < 0.05$, and $0.05 < P < 0.10$ was considered to have a tendency.

Results

Growth performance and rumen fermentation characteristics

As shown in Table 2, compared with the CON group, final BW, ADFI, and ADG were significantly increased in the LBR group ($P < 0.01$). Meanwhile, the F/G was decreased in the LBR group ($P < 0.05$).

Table 2
Average daily feed intake, growth performance, and rumen fermentation characteristics for the dietary treatments.

Item	Treatments[1]		SEM[2]	*P*-value
	CON (*n* = 8)	LBR (*n* = 8)		
Growth performance				
Initial BW, kg	20.9	20.8	0.09	0.644
Final BW, kg	31.1[b]	33.0[a]	0.28	< 0.01
ADG, g/d	170.3[b]	203.4[a]	4.78	< 0.001
ADFI, g/d	1496.8[b]	1695.2[a]	25.71	< 0.001
F/G	8.82[a]	8.34[a]	0.116	0.034
Rumen fermentation parameters				
pH	6.56[a]	6.30[b]	0.015	0.040
NH_3-N, mg/dL	16.2[a]	10.6[b]	0.73	< 0.001
TVFAs, mmol/L	59.3[b]	67.9[a]	1.17	< 0.001
Acetate, mmol/L	38.7[b]	40.7[a]	0.32	< 0.001
Propionate, mmol/L	10.7[b]	13.7[a]	0.42	< 0.001
Butyrate, mmol/L	7.22[b]	10.2[a]	0.42	< 0.001
Isobutyric, mmol/L	0.91	0.97	0.022	0.207
Isovalerate, mmol/L	0.74[b]	1.08[a]	0.055	< 0.001
Valerate, mmol/L	1.01	1.11	0.031	0.099
A/P	3.62[a]	2.96[b]	0.094	< 0.001

[1] CON = control group; LBR = *Lycium barbarum* residue group.

[2] SEM = standard error of the mean.

BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; F/G = ADFI to ADG; TVFAs = total volatile fatty acids; NH_3-N = ammonia-nitrogen; A/P = acetate to propionate.

[a,b] Values within a row with different superscripts differ significantly at $P < 0.05$.

The ruminal pH differed ($P < 0.05$) between the LBR and CON groups. However, the NH_3-N concentration in the rumen decreased ($P < 0.01$) in the LBR group. The concentrations of TVFAs ($P < 0.01$), acetate ($P < 0.01$), propionate ($P < 0.01$), butyrate ($P < 0.01$) and isovalerate ($P < 0.01$) increased in the LBR group in comparison with the CON group. In addition, a significant decline in the acetate/propionate (A/P) ratio ($P < 0.01$) was noticed in the LBR group (Table 2).

Rumen microbe diversity and composition

1,277,062 raw reads were obtained from the Illumina MiSeq platform sequencing runs, averaging 63,853 sequences per sample. After quality control, 658,124 filtered sequences were retained, with a mean of 32,906 filtered sequences per

sample. The distributed range in length of all sequences was 51 to 439 base pairs. The rarefaction curve (Additional file 2: Fig. S1A) illustrated that the sequencing was sufficient to give the diversity of the rumen bacterial community. A Venn diagram demonstrated the shared and unique ASVs among the two treatment groups (Additional file 2: Fig. S1B). A total of 15,282 ASVs were identified in two groups, of which 2,330 ASVs were presented in all groups, accounting for 15.2% of the total number of ASVs. Alpha diversity indices analysis, including Chao1 index, Good's coverage, and Simpson and Shannon index, revealed that there were significant differences ($P < 0.01$) in all indices except Simpson between the LBR and CON groups (Fig. 2).

The taxonomical distributions of LBR and CON groups at phylum and genus levels for the rumen microbial samples are presented in Fig. 3. Ten bacterial phyla were identified in the rumen samples (Fig. 3A and Additional file 1: Table S1). Among these species, Bacteroidetes were the most dominant phylum (51.89% and 53.47%), followed by Firmicutes (35.08% and 42.05%) and Proteobacteria (10.16% and 3.0%) in the CON and LBR groups, respectively. The relative abundances of Bacteroidetes, Firmicutes, Tenericutes, Cyanobacteria, and TM7 were significantly higher ($P < 0.01$) in the LBR group than in the CON group. However, the relative abundances of Proteobacteria, Synergistetes, and Spirochaetes were significantly lower ($P < 0.01$) in the LBR group than in the CON group. 169 bacterial taxa were identified at the genus level, and 97 genera were observed in all samples. The top 10 genera presented in the samples were an indicator of the core microbiome in this study (Fig. 3B and Additional file 1: Table S2). Among these genera, *Prevotella* (41.79% and 43.74%), *Succiniclasticum* (3.82% and 9.14%), *Oscillospira* (5.72% and 5.28%), *Succinivibrio* (6.72% and 2.03%), *Ruminococcus* (2.29% and 2.74%) and *Coprococcus* (1.55% and 2.26%) were the dominant genera in the CON and LBR groups, respectively. Addition of LBR significantly increased the relative abundances of *Prevotella* ($P < 0.05$), *Succiniclasticum* ($P < 0.01$), *Ruminococcus* ($P < 0.01$), *Coprococcus* ($P < 0.01$), *Selenomonas* ($P < 0.01$) and *Butyrivibrio* ($P < 0.01$) in the treated group, whereas three genera (*Oscillospira*, *Succinivibrio*, and *Treponema*) were significantly higher ($P < 0.01$) in the CON group.

Alteration of ruminal microbiota

The PCoA and NMDS based on Bray-Curtis distance were used to identify beta diversity of the rumen microbial community across all the samples in CON and LBR groups (Fig. 4A and B). The rumen microbial communities of the LBR group and the CON group were separated from each other, demonstrating that LBR could affect the species and abundance of rumen microorganisms. The differences in rumen microorganisms between the LBR and CON groups were performed using LEfSe analysis and LDA (Fig. 5A and B). The relatively high abundance in the LBR group was mainly *Succiniclasticum*, *Selenomonas*, *Coprococcus*, *Ruminococcus*, *Butyrivibrio*, *Prevotella*, and *Anaerovibrio*. By comparison, *Succinivibrio*, *Treponema*, *Oscillospira*, *Alistipes*, *Pseudobutyrivibrio*, *Ruminobacter*, were more abundant in the CON group.

Prediction of rumen microbial function

Based on the 16S rRNA data, PICRUSt2 was conducted to predict the function of rumen microbiota in the LBR and CON groups. As shown in Additional file 2: Fig. S3A, biosynthesis, degradation/utilization/assimilation, detoxification, generation of precursor metabolite and energy, glycan pathways, macromolecule modification, and metabolic clusters were observed in the function of rumen microbe among the two groups at level 1. A total of 57 pathways were identified, and 21 were different among the LBR and CON groups at level 2. Compared with the CON group, supplemented with LBR was predicted to have a lower ($P < 0.05$) capability of controlling cell structure biosynthesis, secondary metabolite degradation, carbohydrate degradation, and metabolic regulator biosynthesis but a higher ($P < 0.05$) capability of influencing fatty acid and lipid biosynthesis, amino acid biosynthesis, nucleoside, and nucleotide biosynthesis, glycolysis, L-glutamate, and L-glutamine biosynthesis and nucleoside and nucleotide degradation (Additional file 2: Fig. S3A and B). Furthermore, flora correlated with D-arginine and D-ornithine metabolism, D-glutamine, and D-glutamate metabolism, fatty

acid biosynthesis, D-alanine metabolism, biotin metabolism, Vitamin B6 metabolism, pyrimidine metabolism, arginine, and proline metabolism ($P < 0.05$), were significantly higher in the LBR group at level 3 (Additional file 2: Fig. S3C).

Rumen fluid metabolome analysis

The untargeted LC-MS/MS approach was used to analyze the rumen fluid metabolome. The total ion chromatogram (TIC) curves of QC samples in positive and negative ion modes showed high stability, large peak identification capacity, and consistent retention time (Additional file 2: Fig. S4). The PCA revealed the overall difference and the degree of variation among rumen samples within the CON and LBR groups (Fig. 6A). All the pieces were within the 95% confidence interval (Hotelling's T-squared ellipse). The R^2X for PCA was 0.693, showing the reliability of the PCA model (Additional file 1: Table S3). The PCA indicated that the two groups were distributed in different areas. Furthermore, OPLS-DA score plots exhibited the differences in ruminal metabolites between the CON and LBR groups (Fig. 6B). As shown in Additional file 1: Table S3, the OPLS-DA model parameters R^2Y (cum) and Q^2 (cum) were above 0.90, explaining that the model remained stable and reliable. Furthermore, the value of Q^2 (-1.1 < 0) was negative in response to permutation testing, indicating that the models did not overfit (Fig. 6C).

Ruminal metabolites affected by LBR supplementation

In total, 431 differential metabolites were acquired with VIP > 1.0 and $P < 0.05$ from the rumen fluid samples between the CON and LBR groups. Among these metabolites, 287 were up-regulated by LBR (Fig. 7A), which included 94 organic acids and derivatives, 52 organoheterocyclic compounds, 51 lipids and lipid-like molecules, 24 nucleosides, nucleotides, and analogs, 20 organic oxygen compounds and 14 benzenoids. 144 metabolites were down-regulated in the LBR group (Fig. 7A), including 38 lipids and lipid-like molecules, 36 organic acids and derivatives, 26 organo-heterocyclic compounds, and 12 benzenoids. The HCA analysis was conducted on the top 100 different metabolites to visualize the differences in the rumen metabolome between the two groups (Fig. 7B). The differential metabolites were clustered separately between the CON and LBR groups. In addition, 12 significantly differential ruminal metabolites with VIP > 1.0 and $P < 0.01$ between CON and LBR were picked out. These metabolites were all elevated in the LBR group, except for 3-aminoisobutanoic acid (Table 3).

Table 3
HMDB compound classification of significantly different metabolites in Tan sheep among CON and LBR groups.

HMDB superclass[1]	HMDB class[1]	HMDB subclass[1]	Metabolites	VIP[2]	Log_2FC[3]	*P*-value
Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogs	L-Lysine	1.56	2.34	< 0.001
			L-Tyrosine	1.47	2.33	0.003
			L-Phenylalanine	1.40	1.43	0.002
			L-Proline	1.44	1.26	< 0.001
			D-Proline	1.22	0.59	< 0.001
			3-Aminoisobutanoic acid	1.43	-0.60	< 0.001
Organoheterocyclic compounds	Diazines	Pyrimidines and pyrimidine derivatives	Uracil	1.51	1.54	< 0.001
	Imidazopyrimidines	Purines and purine derivatives	Hypoxanthine	1.68	2.95	< 0.001
			Xanthine	1.11	1.20	< 0.001
Nucleosides, nucleotides, and analogues	Pyrimidine nucleosides	Pyrimidine 2'-deoxyribonucleosides	Thymidine	1.66	3.81	< 0.001
		_[4]	Uridine	1.59	1.74	< 0.001
Phenylpropanoids and polyketides	Phenylpropanoic acids	–	Hydrocinnamic acid	1.17	1.15	< 0.001

CON = control group; LBR = *Lycium barbarum* residue group.

[1] HMDB = Human metabolome database.

[2] VIP = Variable importance in the projection; VIP: the contribution value of metabolites to the difference between the two groups (VIP > 1).

[3] FC = Fold change; $log_2FC > 0$ represents the upregulated compounds, while $log_2FC < 0$ represents the downregulated compounds.

[4] –: no pathway information.

Metabolic pathway enrichment analysis of differential metabolites

The metabolic pathway enrichment analysis was performed based on the different metabolites identified between CON and LBR groups ($P < 0.05$). Given the high impact and *P*-value (Table 4), pyrimidine metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, arginine and proline metabolism, and phenylalanine metabolism were regarded as key metabolic pathways altered due to LBR supplementation (Additional file2: Fig. S5).

Table 4
Metabolic pathway enrichment analysis of significantly different metabolites among CON and LBR groups.

KEGG pathway	Metabolites	*P*-value
Pyrimidine metabolism	Uridine; Cytidine monophosphate; Cytidine; dCMP; Deoxyuridine; Thymidine; Dihydrothymine Ureidoisobutyric acid; Orotic acid Uracil; 3-Aminoisobutanoic acid	0.002
Phenylalanine, tyrosine, and tryptophan biosynthesis	Phenylpyruvic acid L-Phenylalanine; L-Tyrosine	0.006
Arginine and proline metabolism	Ornithine; Citrulline; L-Proline; L-Aspartic acid; L-Glutamic acid; N-Acetylornithine; D-Proline; 4-Aminobutyraldehyde N-Acetylputrescine; N4-Acetylaminobutanal 1-Pyrroline-2-carboxylic acid	0.009
Phenylalanine metabolism	L-Phenylalanine; 2-Phenylacetamide; L-Tyrosine; Phenylpyruvic acid	0.014

CON = control group; LBR = *Lycium barbarum* residue group.

Correlation between the differential rumen microbiome, metabolites, and rumen fermentation parameters

The Spearman correlation coefficient was used to conduct correlation analysis among several indicators (Fig. 8). As illustrated in Fig. 8A, the correlation between significantly differential bacteria and rumen fermentation parameters showed that the TVFAs, acetate, propionate, butyrate, isovalerate were most positively correlated to *Succiniclasticum*, *Coprococcus*, *Selenomonas*, *Ruminococcus*, *Butyrivibrio* and *YRC22* ($r > 0.7$, $P < 0.001$), but negatively correlated with *Succinivibrio* and *Treponema* ($r < -0.7$, $P < 0.001$). Nevertheless, NH_3-N concentration was most positively correlated to *Succinivibrio* and *Treponema* ($r > 0.9$, $P < 0.001$), but negatively correlated to *Succiniclasticum*, *Coprococcus*, *Selenomonas*, *Butyrivibrio* and *YRC22* ($r < -0.7$, $P < 0.001$). There was no correlation between pH, valerate, and isobutyric parameters with different bacteria.

Furthermore, the correlation analysis between significantly differential bacteria and metabolites manifested that *Succiniclasticum*, *Ruminococcus*, *YRC22*, *Selenomonas*, and *Coprococcus* were most positively correlated to L-proline, L-phenylalanine, L-lysine, L-tyrosine, D-proline, uracil, hypoxanthine, xanthine, thymidine, uridine and hydrocinnamic acid ($r > 0.7$, $P < 0.001$), but negatively correlated to 3-aminoisobutanoic acid ($r < -0.6$, $P < 0.01$). 3-aminoisobutanoic acid was most positively correlated to *Treponema* and *Succinivibrio* ($r > 0.7$, $P < 0.01$). *Prevotella* was positively associated with uracil, hypoxanthine, xanthine, thymidine, and uridine ($r > 0.5$, $P < 0.05$). Nevertheless, these metabolites were negatively related with *Oscillospira* ($r < -0.5$, $P < 0.05$). Additionally, *Butyrivibrio* was positively correlated with L-proline, L-lysine, D-proline, uracil, hypoxanthine, xanthine, thymidine, uridine and hydrocinnamic acid ($r > 0.5$, $P < 0.05$), but negatively correlated with 3-aminoisobutanoic acid ($r < -0.7$, $P < 0.001$) (Fig. 8B).

As shown in Fig. 8C, the relevance between significantly differential metabolites and rumen fermentation parameters revealed that TVFAs, propionate, and butyrate were positively correlated to L-proline, L-phenylalanine, L-lysine, L-tyrosine, D-proline, uracil, hypoxanthine, xanthine, thymidine, uridine and hydrocinnamic acid ($r > 0.6$, $P < 0.001$), but negatively correlated to 3-aminoisobutanoic acid ($r < -0.7$, $P < 0.001$). Nevertheless, NH_3-N was most positively correlated to 3-aminoisobutanoic acid ($P < 0.001$). The acetate and isovalerate were positively associated with L-proline, L-phenylalanine, L-lysine, L-tyrosine, uracil, hypoxanthine, xanthine, thymidine, uridine, and hydrocinnamic acid ($r > 0.6$, $P < 0.05$) but negatively correlated with 3-aminoisobutanoic acid ($r < -0.7$, $P < 0.001$). D-proline was negatively correlated with pH ($r < -0.6$, $P < 0.05$). Moreover, there was no correlation between valerate and isobutyric with different metabolites.

Discussion

Growth performance

The beneficial effects of *Lycium barbarum* supplementation have been demonstrated through human clinical trials and laboratory animal studies [29] and further studied in livestock animals [12, 16]. In the present study, dietary supplementation of LBR increased the final BW, ADG, and ADFI of Tan sheep but decreased the F/G, which could be due to the presence of LBPs, polyphenols, amino acids, and vitamins in LBR, which could improve the growth performance of Tan sheep. Previous studies have used polysaccharides from Chinese medicinal herbs, such as *Astragalus membranaceus* polysaccharide (AMP), *Aloe vera* polysaccharides, *Astragalus* polysaccharides (APS), *Ginseng* polysaccharide, and LBPs, to improve growth performance in piglets, chickens, and rabbits [11, 14, 30]. A diet with a mixture of *Lycium barbarum* and *Astragalus. membranaceus* increased the ADG and decreased the F/G of lambs [17]. Another study showed that diets with bee pollen polysaccharides improved the nutrient digestibility of calves [31]. Polyphenols are considered as the main bioactive components of *Lycium barbarum*, which positively regulates energy metabolism [32]. Consequently, the improvement of growth performance in the LBR group could be due to the elevated digestive enzymes, improved bioactive nutrient utilization, and enhanced antioxidant capacity of Tan sheep.

Rumen fermentation parameters

The ruminal pH can influence the rumen microbial community structure and change the production pathways of VFA [33]. The optimum pH value of a healthy rumen ranges between 5.9 and 7.2, in which the fibrinolytic microorganisms are most active [34]. In the present study, a lower ruminal pH was discovered in the LBR group, but the pH value was within the optimal pH, indicating that feeding LBR maintained a suitable acid-base status. In line with a previous study, the ruminal fluid with the lowest pH had the highest concentration of TVFAs, one of the main pH drivers [35]. Similar to the data reported by Ren et al. [36], the administration of LBPs could reduce the pH of rat caecal contents. The concentration of TVFAs in the LBR group was higher than that in the CON group, which could provide energy for ruminants. In addition, with supplementation of LBR, the concentration of acetate, propionate, and butyrate increased, whereas the A/P ratio decreased, as observed in this study. Acetate and butyrate are used to synthesize fatty precursors, and propionate is the major VFA used for gluconeogenesis in ruminants [37]. The concentration of NH_3-N was positively correlated with protein degradation.

Consequently, supplementation of LBR increased ADG and feed efficiency by offering more substrates for body protein and fatty acid syntheses in sheep. Furthermore, feeding LBR increased the fermentation of rumen microorganisms, increasing propionic production. Good fermentation characteristics facilitate the proliferation of major beneficial microorganisms in the rumen.

Rumen microbiome

Bacteria are the most abundant, diverse, and metabolically active microorganisms in ruminants, and were affected by diets, as reported previously [19]. There was a negative effect on Chao 1 value and Shannon index among LBR groups,

which was similar to previous studies [38]. These results could be attributed to the LBR having numerous potential health benefits, including antioxidative, antibacterial and antitumor activities [39], and immunoenhancing effects [40]. Also, LBR contains abundant polyphenols, stimulating the growth of beneficial bacteria and inhibiting pathogenic bacteria [41]. Therefore, LBR supplementation increased performance-enhancing microorganisms. In the present study, Bacteroidetes, Firmicutes, and Proteobacteria were the main microbes at the phylum level of Tan sheep, which was in accordance with the findings of a previous report [42]. Compared to the CON group, intake of LBR significantly increased the relative abundance of Bacteroidetes and Firmicutes in the treated group. Among these microorganisms, Bacteroidetes could assist the host in degrading a variety of complex polysaccharides, thereby improving nutrient absorption [43], promoting immune system development, and maintaining intestinal microecological balance [44], and the high LBPs content of the diet could explain the high abundance of Bacteroidetes. Firmicutes are important in the degradation of fibers and celluloses and are involved in many fiber-decomposing genera [45]. Enhancing cellulose degradation can improve animal feed utilization efficiency and promote animal production. The Firmicutes to Bacteroidetes ratio increased with supplementation of LBR. These findings are consistent with a report that obesity is positively associated with a high Firmicutes to Bacteroidetes ratio, which affects energy harvesting and fat deposition in the guts of mice [45]. In addition, the significantly decreased rumen microbes induced by LBR, including Proteobacteria and Spirochaetes, were mainly Gram-negative bacteria, including several potential pathogens.

In this study, *Prevotella*, *Succiniclasticum*, *Ruminococcus*, *Coprococcus*, *Selenomonas*, and *Butyrivibrio* were core microbiomes at the genus level, in line with previous reports [46, 47]. *Prevotella* plays a pivotal role in fiber degradation [48], which has the function of degrading non-fibrous polysaccharides and proteins, digesting and utilizing starch, xylan, and pectin, and producing short-chain fatty acids (SCFA), such as acetate, propionate, and succinate [49]. Hence, the increased VFA, acetate, and propionate concentrations in the LBR group could be attributable to the degradation of carbohydrates and fibers. Consistent with a previous study [50], *Ruminococcus* was abundant in the mammalian gut, which is beneficial for gut health, and its abundance was increased by LBR supplementation. *Ruminococcus* is rich in carbohydrate-active enzymes, which can degrade a wide variety of recalcitrant substrates in feed [51]. In the gut environment, the degradability of cellulose and hemicellulose components in plant materials enables *Ruminococcus* to break down these substrates, which are otherwise indigestible [52]. Furthermore, these substrates are fermented and produced short-chain VFA (acetate, propionate, and butyrate) that can be absorbed through the rumen epithelium and used as the primary carbon and energy sources supporting animal growth [53]. *Succiniclasticum* and *Selenomonas* specialized in fermenting succinate and converting it to propionate [54]. *Butyrivibrio*, a main butyrate-producing bacterium, also increased in the LBR group [55]. These findings showed that LBR significantly enhanced the growth of *Prevotella*, *Succiniclasticum*, *Ruminococcus*, and *Butyrivibrio*, leading to greater production of VFA and greater utilization of proteins in rumen fluid. Consistently, we found that numerous rumen microbes in the LBR were positively associated with TVFAs, acetate, propionate, and butyrate and negatively related to NH_3-N. Increased VFA production improved sheep's energy supply while inhibiting the proliferation of pathogens in the gastrointestinal tract [56]. Among these acids, propionate could improve colonization resistance of *Salmonella* by altering the pH, and butyrate could control the growth of pathogenic bacteria [57]. The rumen microbiota provides diverse metabolites required by the sheep.

In summary, LBR dietary supplement increased the abundance of SCFA-producing bacteria, increasing VFA and reducing pH, which would provide an acidic environment in the rumen and diminish pathogenic populations.

Rumen fluid metabolome

The ruminal microbial metabolites influence tissue and organ development, maintain the homeostasis of the gastrointestinal tract ecosystem and ensure the health of animals. In light of a previous report [58], the variations in the contents of various rumen metabolites could affect the rumen and host health. We used metabolomics analysis to uncover the differences in rumen metabolites between the LBR and CON groups. According to a previous study, the metabolomics data revealed that LBR supplementation significantly altered metabolites, including amino acids, peptides,

analogs, fatty acids and conjugates, and purines and purine derivatives [59]. Among the significantly altered metabolites, most of them were amino acids, which are from the degradation of proteins and microorganisms by rumen microbiota and acted as the key precursors for synthesizing proteins and polypeptides [60], such as 3-aminoisobutanoic acid, lysine and proline.

Meanwhile, an aromatic compound, hydrocinnamic acid [61], is a degradation product of amino acids. VFA and other substances are used as a carbon source, and nitrogen compounds, like ammonia, as a nitrogen source by the rumen microbe for *de novo* synthesis of amino acids [62]. In our study, higher concentrations of VFA and lower pH in the LBR group might promote amino acid synthesis in rumen.

Notably, most amino acids were positively related to SCFA-producing bacteria (*Succiniclasticum*, *Selenomonas*, *Butyrivibrio*) abundant in the LBR group. Therefore, LBR supplementation had positive effects on ruminal amino acid metabolism. Among these amino acids, L-proline, L-phenylalanine, L-lysine, L-tyrosine, and D-proline increased except for 3-aminoisobutanoic acid in the LBR group. In agreement, a previous study reported that 3-aminoisobutanoic acids and branched- chain fatty acids were competitive and negatively correlated with cellulolytic bacteria in the rumen [19]. Also, phenylalanine, tyrosine, and lysine could be used as precursors of acetate and butyrate [63]. Butyrate stems from lysine and glutamic acid, and proline is degraded to produce isoacids such as isobutyric acid, isovaleric acid, and valeric acid [64]. Thus, L-lysine, L-proline, L-phenylalanine, and L-tyrosine were positively correlated with SCFA-producing bacteria, TVFAs, propionic, butyric and isovaleric acid. L-proline is the main amino acid that maintains cell structure and function, and is an important regulator of cell metabolism and physiology [65]. Thus, LBR supplementation might promote gastrointestinal microbiota homeostasis and the synthesis of bacterial proteins.

In this study, we found that LBR supplementation affected pyrimidine and purine metabolism, characterized by elevated levels of uridine, thymidine, uracil, and xanthine hypoxanthine. Hypoxanthine is a purine derivative, while uracil, xanthine, and thymine are nucleic acid bases found in RNA and DNA, respectively [66]. A previous study showed that greater amounts of nucleic acids in the rumen could indicate greater availability of crude microbial protein (MCP) [67]. Among these, xanthine and hypoxanthine have been frequently used as biomarkers of microbial protein synthesis in the rumen [58]. The decomposition product of uracil is β-alanine, which can be further converted into propionic acid, explaining the positive correlation between uracil and *Succiniclasticum*. The above findings further suggested that supplementation with LBR might increase microbial protein synthesis in the rumen and improve health.

Conclusions

The integrative microbiome and metabolomics analysis investigated the effects of LBR on ruminal microbial communities, metabolites, and their associations with the growth performance of Tan sheep. The LBR increased the abundance of ruminal beneficial bacteria (such as *Prevotella*, *Succiniclasticum*, and *Ruminococcus*), and promoted amino acids synthesis and metabolism, pyrimidine metabolism, and organic acid production pathways in the rumen. These changed LBR associated rumen microbial communities and functions which in turn enhanced the growth performance and feed efficiency of Tan sheep.

Abbreviations

LBR: *Lycium barbarum* residue, LBPs: *Lycium barbarum* polysaccharides, AMP: *Astragalus membranaceus* polysaccharide, APS: *Astragalus* polysaccharides, ADG: Average daily gain, ADFI: Average daily feed intake, NDF: Neutral detergent fiber, ADF: Acid detergent fiber, TVFAs: Total volatile fatty acids, VFAs: Volatile fatty acids, NH_3-N: Ammonia nitrogen, BW: Body weight, A/P: Acetate to propionate ratio, DM: Dry matter, DE: Digestible energy, CP: Crude protein, EE: Ether extract, FCR: Feed conversion ratio, F/G: Gain feed ratio, ASVs: Amplicon sequence variants, PCoA: Principal coordinates analysis, NMDS: Nonmetric multidimensional scaling analysis, LEfSe: Linear discriminant analysis effect,

LDA: Linear discriminant analysis, FC: Fold change, HCA: Hierarchical clustering analysis, VIP: Variable importance in the projection, PCA: principal component analysis, HMDB: Human Metabolome Database, PICRUSt: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, OPLS-DA: Orthogonal partial least squares discriminant analysis, KEGG: Kyoto Encyclopedia of Genes and Genomes, RPT: Response permutation testing, MCP: Crude microbial protein, SCFA: Short-chain fatty acids, TIC: Total ion chromatogram, IDA: Information-dependent acquisition, QC: Quality control

Declarations

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Authors' contributions

YJZ: Performed the study, data collection and analysis, drafted the manuscript. BW, DWW and YSG: Assisted in data analyses and manuscript preparation. MD: Participated in data intepration and manuscript preparation. GJZ: Designed the study, data interpretation and finalized the manuscript.

Availability of data and materials

The data analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The experimental protocols and treatments were approved by the Institutional Animal Care and Use Committee of Ningxia University (NXUC20200618).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

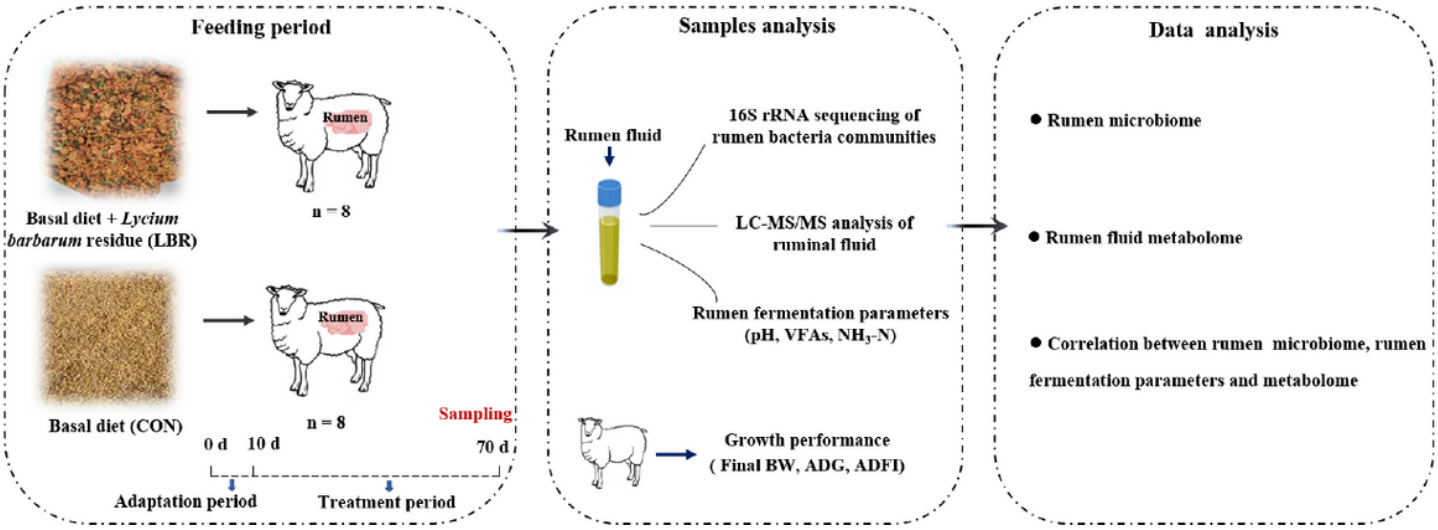

Figure 1

The experimental design and workflow.

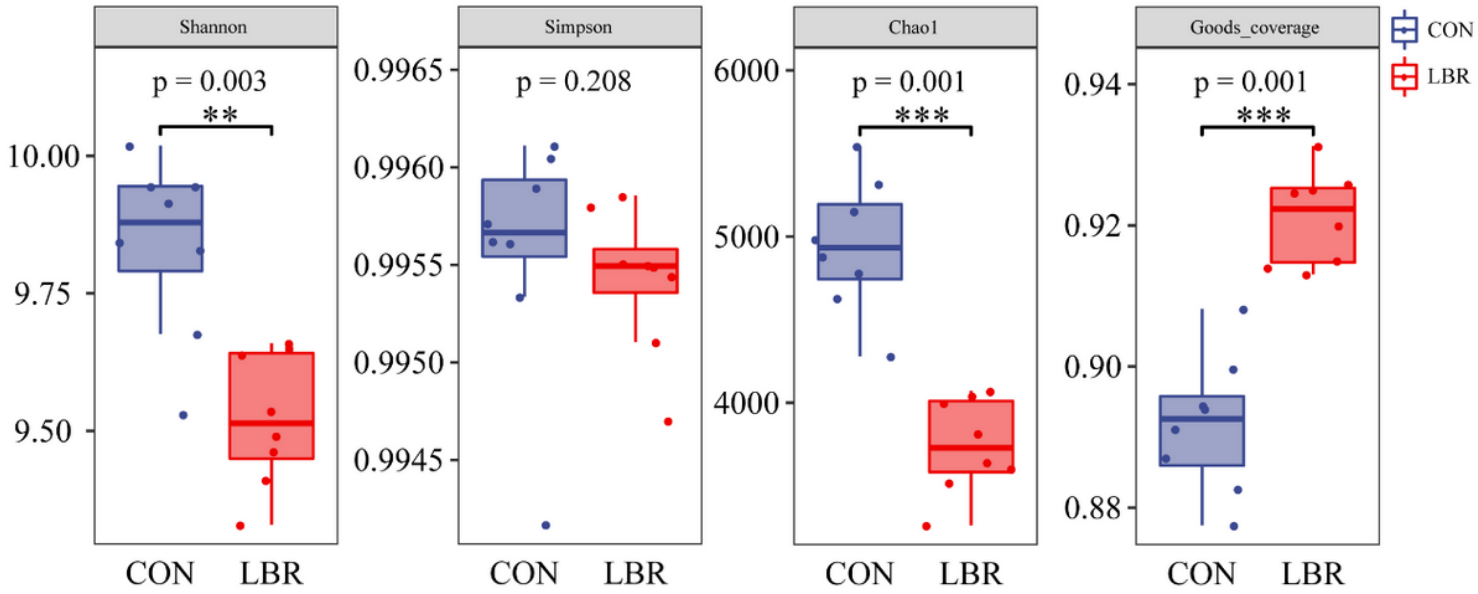

Figure 2

Alpha diversity index analysis of ruminal bacterial diversity and richness due to CON and LBR. CON, control group. LBR, *Lycium barbarum* residue group. The significant difference is indicated by $P < 0.05$.

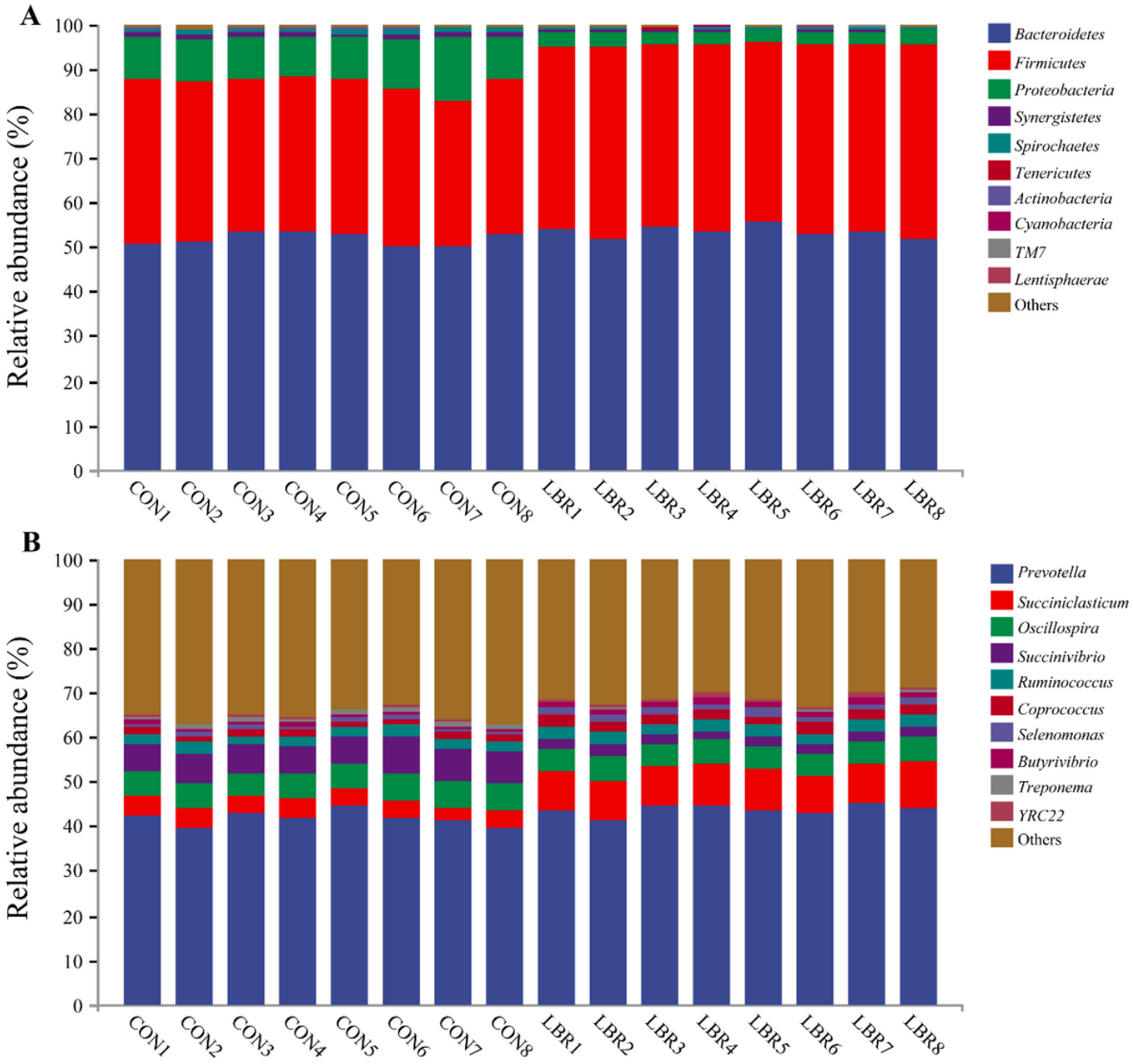

Figure 3

The ruminal bacterial community composition was distributed across the CON and LBR treatment groups under the (A) phylum and (B) genus levels. CON, control group. LBR, *Lycium barbarum* residue group. The different colors of the bars represent different species, and the length of the bars represents the relative abundance of the species.

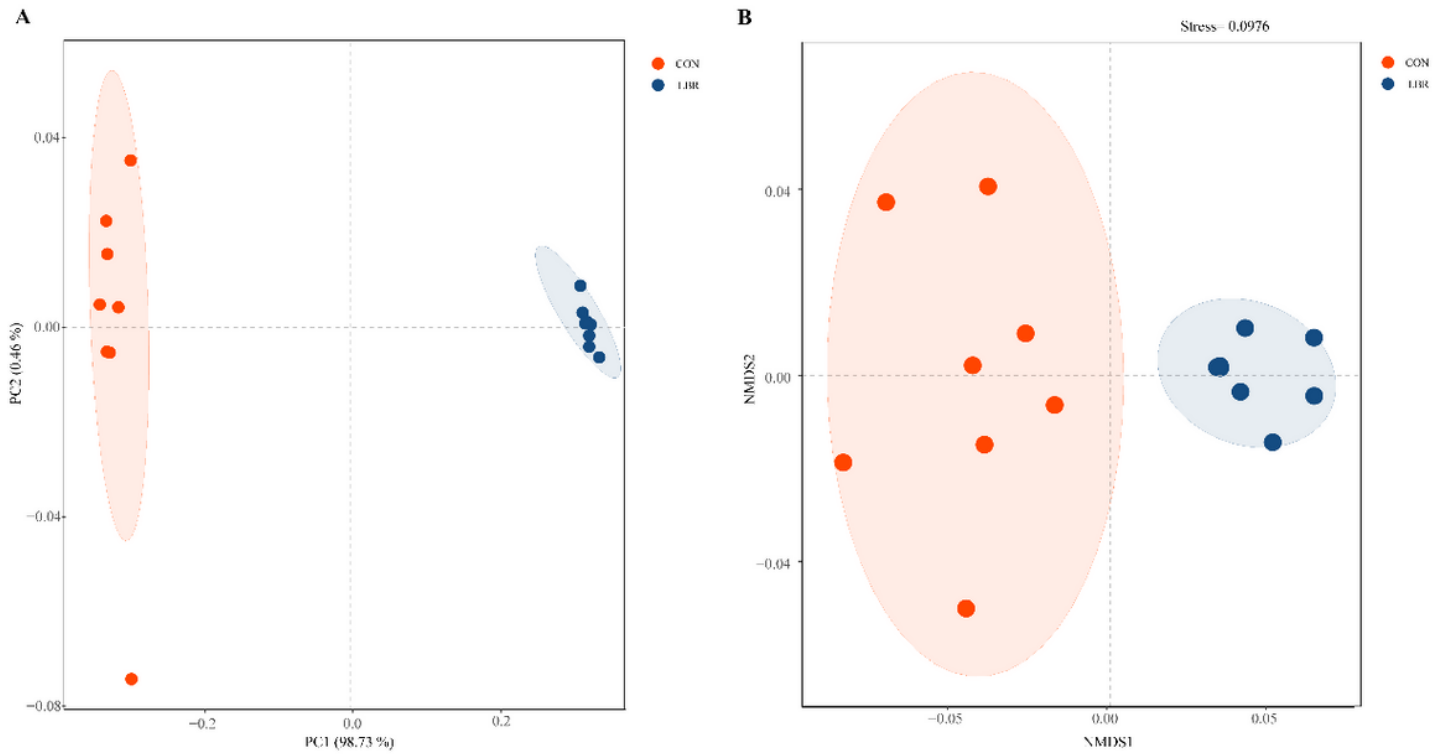

Figure 4

Beta diversity analysis of rumen microbial community through (A) principal coordinate analysis (PCoA) and (B) non-metric multidimensional scaling analysis (NMDS) among CON and LBR groups. CON, control group. LBR, *Lycium barbarum* residue group. PC = principal components.

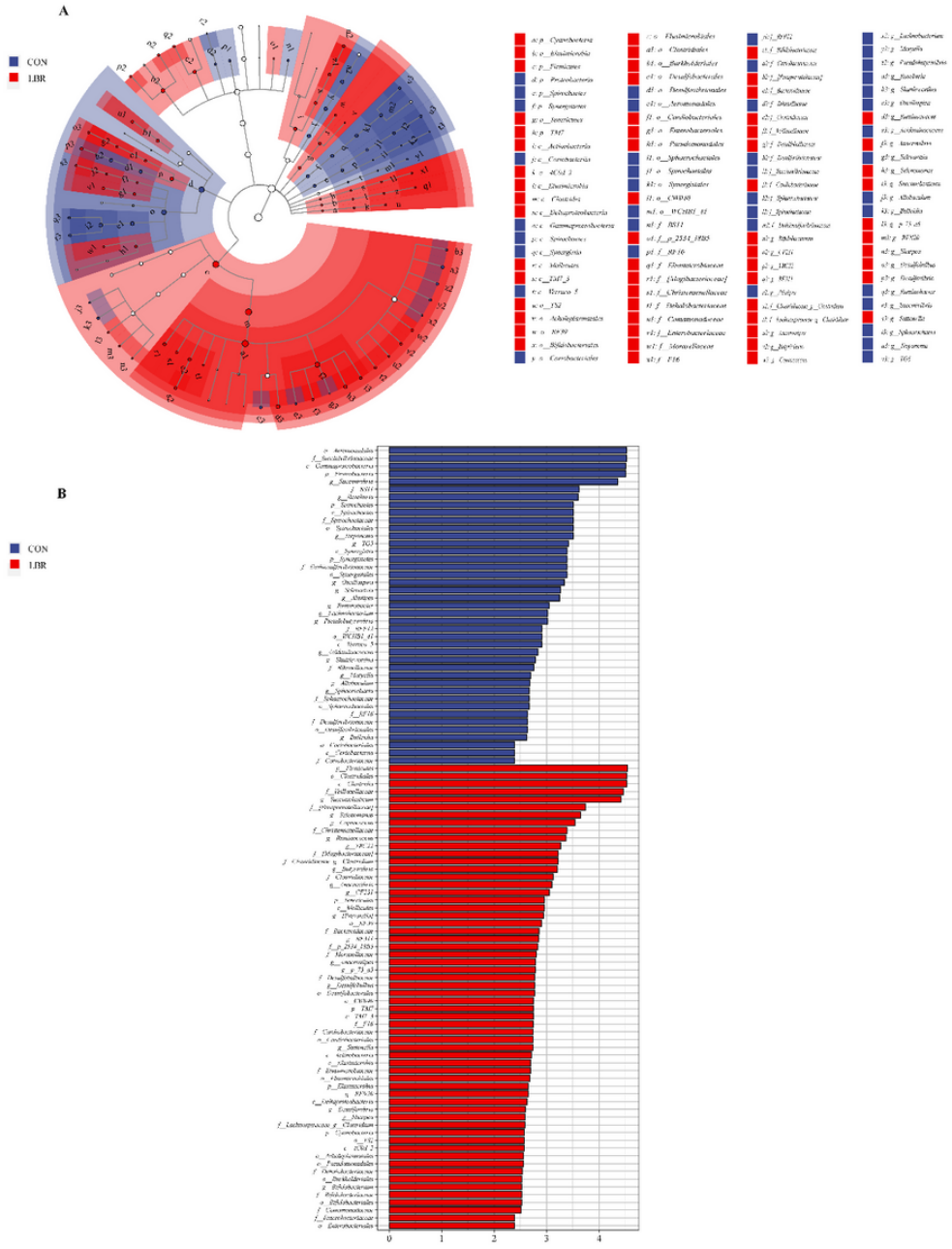

Figure 5

Linear discriminant analysis effect size (LEfse) analysis of differential rumen microorganisms between the LBR and CON groups. (A) Cladogram displayed the significantly different bacteria from phylum to genus level. (B) The linear discriminant analysis (LDA) bar displayed the effect of the abundance of each species on the difference between the 2 groups. CON, control group. LBR, *Lycium barbarum* residue group.

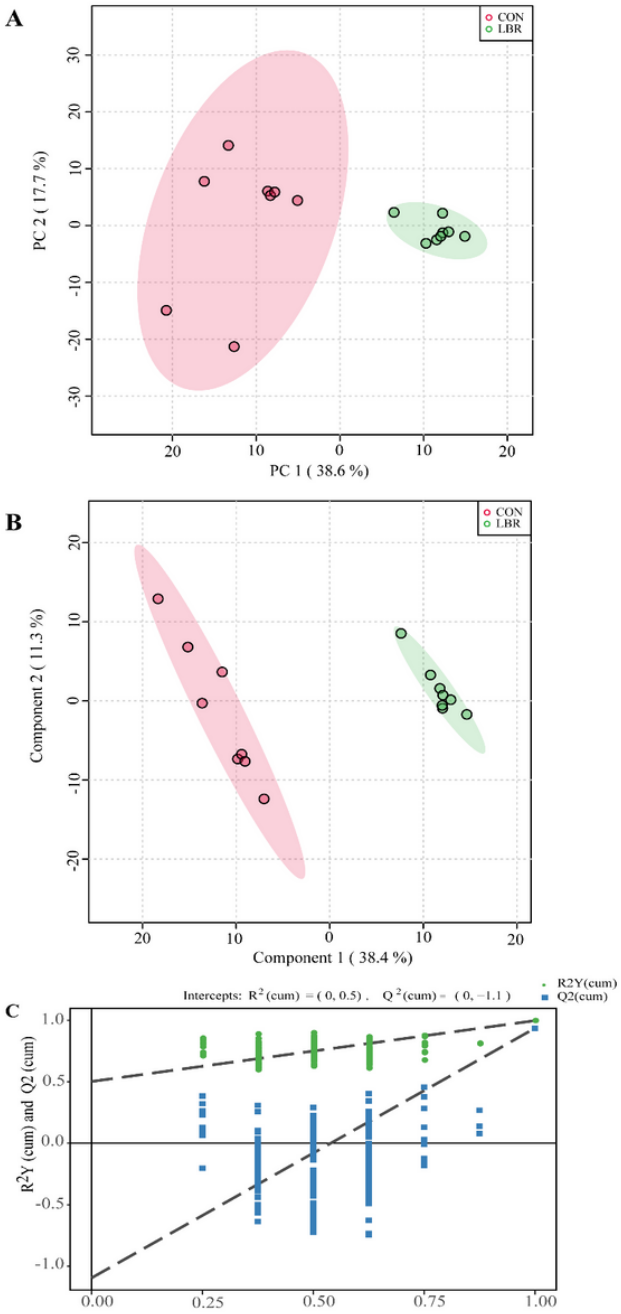

Figure 6

Ruminal fluid metabolomics analysis between the LBR and CON groups. (A) Score scatter plot of principal component analysis (PCA), Two regions within 95% confidence intervals were formed in the PCA plots, including CON and LBR. (B) Score plot of orthogonal partial least squares discriminant analysis (OPLS-DA) and (C) response permutation testing (RPT) of rumen samples for the CON and LBR group. R^2Y (cum) and Q^2 (cum) represent the interpretability and predictability of models, respectively. CON, control group. LBR, *Lycium barbarum* residue group.

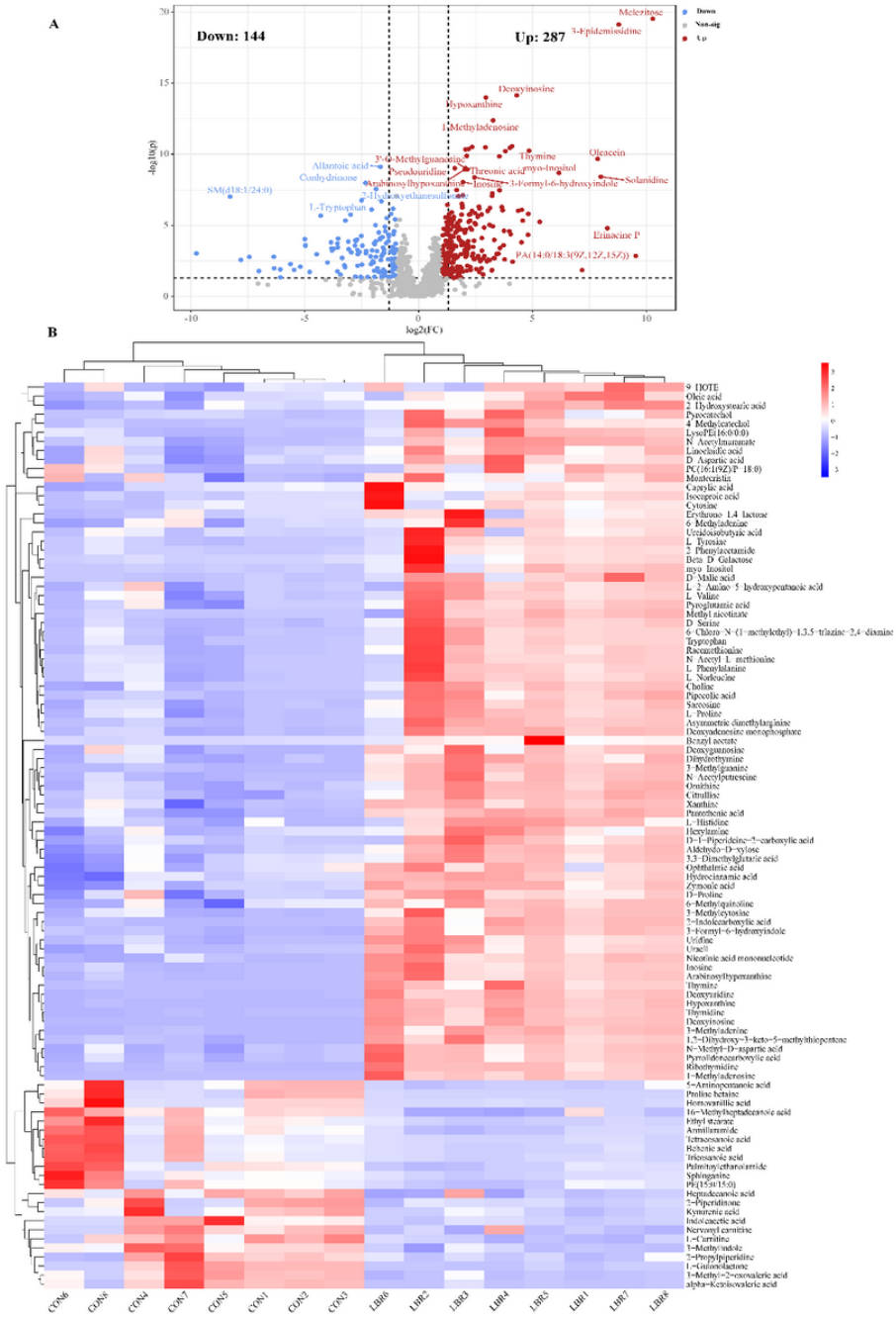

Figure 7

Significantly different ruminal metabolites between the LBR and CON groups. (A) Volcano plots showed the differentially metabolites in the CON and LBR groups. The upregulated and downregulated metabolites were shown in red and blue, respectively, whereas gray dots represented the metabolites without significance in each group. (B) Hierarchical clustering analysis (HCA) for the top 100 of significantly differential metabolites in the rumen by comparison of the LBR and CON groups. CON, control group. LBR, *Lycium barbarum* residue group.

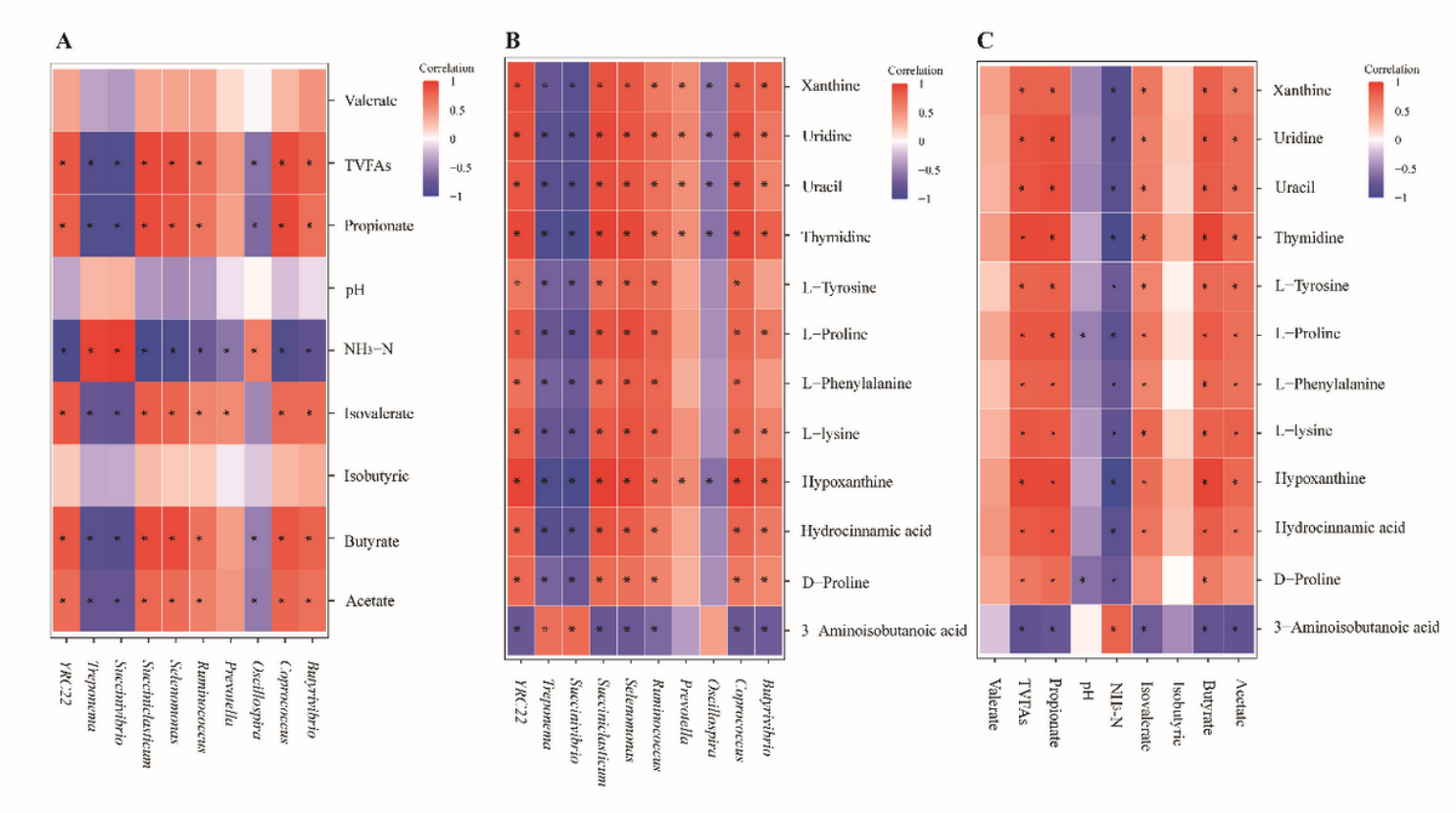

Figure 8

Correlation analysis between (A) significantly differential bacteria and rumen fermentation parameters, (B) differential bacteria and differential metabolites, and (C) differential metabolites and rumen fermentation parameters. TVFAs, total volatile fatty acids; NH_3-N, ammonia-nitrogen. Positive correlations are shown in red and negative correlations in blue. *means $P < 0.05$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.docx
- Additionalfile2.docx