



Ligularia virgaurea improved nutrient digestion, ruminal fermentation, and bacterial composition in Tibetan sheep grazing on the Qinghai–Tibetan plateau in winter

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ABSTRACT

Modulation of rumen microbiota is a prominent function of dietary supplements to improve the performance and health of grazing ruminants. This experiment evaluated the influence of the amount of *Ligularia virgaurea* (LV) supplementation on nutrient digestibility, ruminal fermentation, and bacterial composition in Tibetan sheep consuming low-quality, cold-season forage. Thirty-two intact male yearling Tibetan rams (30 ± 1.66 kg body weight, mean \pm SD) were used in a completely randomized block design, randomly assigned among four experimental groups, and fed for 35 days. Sheep were fed a basal diet (freshly native pasture) with either no LV (control), or with 100, 200, or 300 mg/kg BW of LV per animal/d, with prairie forage available ad libitum. Results showed that the dry matter intake and digestibility of nutrients differed significantly across treatments ($P < 0.05$). Ruminal fermentation parameters and the bacterial composition were affected by the dietary LV supplementation amount and there was a strong correlation between them. Bacteroidetes and Firmicutes were the dominant phyla in the ruminal bacterial community for all groups. *Prevotella* and *Rikenellaceae_RC9_gut* group were the dominant genera, whose relative abundances decreased and increased, respectively, with increasing LV supplementation. Overall, LV ingestion improved the rumen bacterial community abundance associated with cellulose and saccharide degradation, thus contributing to nutrient digestibility and energy supply in Tibetan sheep in the cold season. A comparison of metabolic functional prediction across the four treatment groups revealed an enrichment of metabolic pathways related to lipid metabolism, metabolism of terpenoids and polyketides, folding, sorting and degradation, and membrane transport in the LV supplementation groups. Genomics-based knowledge on the benefits of autochthonous functional plants to Tibetan sheep ruminal

Abbreviations: FNH, functional native herbage; LV, *Ligularia virgaurea*; QTP, Qinghai-Tibetan Plateau; BACs, biologically active compounds; BW, body weight; DM, dry matter; DMI, dry matter intake; OM, organic matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; EE, ether extract; DMD, apparent digestibility of dry matter; OMD, apparent digestibility of organic matter; NDFD, apparent digestibility of neutral detergent fiber; ADFD, apparent digestibility of acid detergent fiber; EED, apparent digestibility of ether extract; CPD, apparent digestibility of crude protein; NH₃-N, Ammonia nitrogen; TVFA, total volatile fatty acids; A/P, Acetate/Propionate ratio.

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microbiota functions and compositions will contribute to their selection as antibiotic alternatives for traditional pastureland.

1. Introduction

Tibetan sheep are one of the most important livestock types on the Qinghai–Tibetan Plateau (QTP) and are raised at an altitude of between 3000 and 5000 m above sea level. They are well adapted behaviorally, physiologically, and anatomically to the harsh conditions of the QTP, with over 50 million animals providing milk, meat, and income for most inhabitants of the plateau (Guo et al., 2020). The Tibetan sheep production system on the QTP is typical of alpine meadow grazing systems (Hou et al., 2021), as it relies on the use of rangelands of wide botanical diversity associated with a marked seasonality in plant resources. Most Tibetan sheep are still raised under traditional management; namely, they are grazed all year round on alpine meadows without supplements (Fan et al., 2021). On the QTP, the native herbage encompasses diverse annual and perennial browsed species, many of which are nutritionally valuable forage. Approximately 75 % of the plant species present in alpine rangeland contribute to grazing-livestock diets, while the biomass of other species not preferred by livestock ranges from 4.45 to 70.57 g/m² (Wang et al., 2022). However, due to changes in nutritional quality and palatability of forage, diets selected by grazing livestock depend largely on the season (Xiao et al., 2020). Owing to the harsh natural environment of the QTP, the grass-growing season is short, at only about 120 days per year. In the long cold season, the nutritional value and availability of forage can decline, especially its protein and energy content, and existing pastures are insufficient for grazing livestock in terms of ensuring their nutritional requirements during this season (Cui et al., 2019). In addition, most pastoral areas are overgrazed and herders do not have the practices or conditions to supplement the forage, so the nutrient requirements of Tibetan sheep are usually not satisfied (Cui et al., 2021). This is a yearly cycle resulting in low flock production with resultant food insecurity and economic challenges for producers.

Most of feed resources rich in biologically active compounds (BACs), such as saponins, tannins and flavonoids, are endowed with rumen-modulating properties. Plant parts or extracts rich in BACs have been used in medicine or dietary supplements/feed additives for thousands of years, and in many parts of the world such products are still used for this purpose (Durmic and Blache, 2012; Jia et al., 2022). Used as a traditional source of medicinal plants, grasslands offer a huge reservoir of BACs (Poutaraud et al., 2017). However, in natural grasslands, these plants are often not favored by herbivores because of their unique taste or poor palatability. Such undesirable plants are abundant all year round and thus can be used as non-conventional feed resources or “functional native herbage (FNH)” owing to their high nutritional value coupled with their biological and functional potential to alleviate the nutritional problems that livestock production faces. In recent decades, with increasing concerns and challenges surrounding the use of antimicrobials in livestock production, there has been growing interest in FNH rich in BACs, which is driving research towards plants or byproducts of plant origin as dietary additives in animal nutrition for their potential positive effects on livestock productivity and health (Halme-mies-Beauchet-Filleau et al., 2018), with fewer side-effects (Mesmar et al., 2022) and better cost-effectiveness compared to conventional feed resources (Debnath et al., 2022). Many FNH, with tonic effect or medicinal function, have been used as feed additives or antibiotic alternatives for livestock to reduce mortality and/or improve growth performance (Xie et al., 2022; Zhang et al., 2022). If used at an appropriate level, these lesser known feed resources can improve feed efficiency, solve the crisis of fodder/feed deficiency, and bring desirable changes in quality and quantity of livestock products.

Ligularia virgaurea (LV), a perennial herb belonging to the *Asteraceae* family, is distributed widely on the QTP at an altitude of 2600–4700 m. The whole plant of LV possesses anti-inflammatory, antioxidant, analgesic, and other physiologically beneficial properties due to the presence of active compounds such as flavonoids and a large family of terpenoids including monoterpenoids, diterpenoids, triterpenes and sesquiterpenes (Sun et al., 2007; Tori, 2016; Qi et al., 2017). In Chinese traditional medicine, the leaves of *Ligularia* species are used to help prevent or treat gastric ulcers, as well as treat dyspepsia and spleen disorders (Lee et al., 2010); specifically, *L. stenocephala* and *L. fischeri* have obvious therapeutic effects against ulcerative colitis (Fu et al., 2022). Meanwhile, LV has been used as a traditional medicine for the treatment of stomachache and nausea (Okamoto et al., 2010). However, due to its unique smell, it is generally not eaten by grazing livestock during the growth season. Similar to LV, *Coleus amboinicus* Lour is generally not used for animal feed because of its poor palatability related to its strong aroma; and the active compounds present in this species are diterpenes and polyphenolics. However, a recent report suggested that the addition of *C. amboinicus* to lamb diets can improve the rumen environment by regulating rumen bacteria involved in the fermentation and biohydrogenation of fatty acids, as well as the meat quality by increasing the deposition of n-3 polyunsaturated fatty acids (Yanza et al., 2022). On the basis of these findings, it is evident that unexploited FNHs have the potential for metabolic modulation in ruminants. It could therefore be advantageous to incorporate these plants at low levels in animal feed to reduce the feed cost as well as obtain desirable rumen fermentation properties for ensuring a healthy nutrient supply (Villalba et al., 2017). Recent studies have shown that the aboveground parts of LV are rich in crude protein, essential amino acids, and mineral elements, and low in tannin and alkaloids, which suggests value in being developed and utilized as feed (Fu, 2020). The stems and leaves of LV are available during most of the year and could be a good source of feed but mainly during the dry season as an energy and protein supplement for ruminant livestock. However, most herdsmen are lacking in understanding of the potential of LV use in animal husbandry and commonly employed management strategies. Past studies have mainly focused on the potential effects of LV on ecological aspects (Ade et al., 2021), with little attention having been paid to its effects on animals. Determination of the optimum proportion of LV in grazing-livestock diets is necessary.

The objective of the present study was to determine the effects of adding an FNH containing BACs as a natural dietary additive to cold-season and low-quality forage diets for Tibetan sheep on the digestibility of nutrients, rumen fermentation, and rumen bacterial

composition.

2. Materials and methods

2.1. Experimental site and animal ethics statement

This experiment was conducted from December 2019 to January 2020 at the Animal Husbandry Science and Technology Demonstration Park in Maqu County, Gannan Tibetan Autonomous Region, northwestern China (101° 52′ 11.24″ E, 33° 41′ 3.56″ N). All animals utilized were cared for in accordance with experimental procedures and acceptable practices reviewed and approved by the Lanzhou University Research Animal Resource Ethics Committee (file No: 2012–1 and 2012–2).

2.2. Experimental animal feeding and management

The experimental periods lasted 35 days, in which the first 14 days were used for dietary adaptation and the last 5 days for sampling. During the trial, fresh forage was cut and weighed from LV-free fenced alpine meadow native pasture as the base diet for feeding animals. In the full flowering stage (August), the vegetation was assessed with quadrats and by recording the proportion of pasture cover of each dominant family—namely, *Cyperaceae*, *Gramineae*, *Ranunculaceae*, *Compositae*, *Leguminosae*, *Liliaceae*, and others (Table 1). To the best of our knowledge, no study concerning the effects of LV on ruminants has been performed previously. Therefore, we used the study by Li et al. (2017) as a guideline for the dosage of LV addition. A total of 32 intact male Tibetan sheep (one year old, 30 ± 1.66 kg body weight) were randomly assigned to one of four treatments ($n = 8$): LV addition levels of 0 (LVC group), 100 (LVL group), 200 (LVM group), and 300 (LVH group) mg/kg BW (body weight) per day per sheep.

Sheep were fed individually and housed in individually raised mesh floor-type shaded pens (specifically designed to collect feces and urine), allowed ad libitum access to food and water, and were fed thrice daily at 08:00, 13:00 and 18:00, with food offered at 10 % more than their actual consumption. Heavily grazed degraded pasture adjacent to the LV-free fenced alpine meadow native pasture was the source of LV. The LV was dried at 50 °C in a forced-air oven (Kenton GX-130, Guangzhou Kenton Apparatus Co., Ltd., China) for 48 h, finely powdered in a Willey mill (1 mm) (FZ102, Beijing Zhongxing Weiye Instrument Co., Ltd., China), and stored in a closed container and used throughout the experimental period. Due to the hygroscopicity of the LV powder, it was weighed and rolled into pills with the same shape and size. The LV powder supplement was offered into two equal halves at 08:00 and 13:00, and a feeder was used to dose the pills via the mouth. The basal diet and LV ingredients and nutritional compositions are shown in Table 1.

2.3. Collection and processing of experimental samples

During the sample collection period, the feed offered to and refused by each sheep was recorded daily to calculate the dry matter (DM) and intake (DMI). Diet and ort sampling were carried out daily from day 31–35 and kept frozen. Samples were composited by the period. Also, the total feces from each sheep was collected and the weight was recorded daily. A total of 100 g of fecal grab samples were collected daily from all sheep, pre-feeding, at 08:00, as sub-samples for chemical assessments (mixed with 10 mL of 10% sulfuric acid) to adjust the pH and prevent nitrogen (N) loss. Samples were composited by the period, with single composites of feces prepared for each sheep. At the end of the five days of sample collection, the individual sheep diets, Orts, and feces were pooled across days and sub-sampled, and thereafter dried at 105 °C in the oven for 18 h for initial DM analysis. The samples of feces and feeds were dried at 65 °C for 48 h to a constant weight and then ground to pass through a sieve with a pore size of 1 mm, before being analyzed for chemical composition. Rumenal fluid samples were obtained via the esophagus of each sheep by using a transesophageal sampler 2 h after feeding in the morning on day 35. To avoid contamination from saliva, the first 50 mL of the sample from each sheep was always discarded, and then a sample of about 50 mL of rumen fluid was collected from each sheep and stored at – 80 °C until DNA extraction. The pH of the rumen fluid samples was immediately measured using a portable pH meter (model PB-21, Sartorius, Germany).

2.4. Samples analysis

The feces and feed samples were analyzed for organic matter (OM; method 942.05), ether extract (EE; method 920.39) and crude

Table 1

The pasture botanical composition and ingredients and nutritional composition of basal ration and LV powder.

Ingredients	Biomass (g/m ² DM)	Components ^a	Pasture	LV
Total biomass	856	DM (g/kg air-dried matter)	915	908
<i>Cyperaceae</i>	214	Chemical composition (g/kg DM)		
<i>Gramineae</i>	145	OM	940	924
<i>Ranunculaceae</i>	145	CP	56	65
<i>Compositae</i>	137	NDF	685	401
<i>Leguminosae</i>	43	ADF	391	306
<i>Liliaceae</i>	34	EE	19	36
Others	137	Energy (MJ/kg)	17.8	17.9

^a DM = dry matter; OM = organic matter; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; EE = ether extract.

protein (CP; method 990.03) according to the Association of Analytical Communities (AOAC, 2006). The acid detergent fiber (ADF; method 973.18) and neutral detergent fiber (NDF) of the diets and feces were analyzed using an Ankom A200i Fiber Analyzer (Ankom Technology Corp., Macedon, New York, USA; aNDF analyzed with heat-stable amylase), as described by standard methods (Van Soest et al., 1991). The formulae used to calculate the digestive feed intake, nutrient digestion, and apparent digestibility of nutrients were:

Feed intake (kg/d) = feed amount (kg/d) – residual feed (kg/d);

DM digestion (g/d) = DM intake (g/d) – fecal amount (g/d);

Apparent digestibility of nutrients (%) = (nutrient intake (g/d) – the nutrient in the feces (g/d)/nutrient intake (g/d) × 100%.

The rumen fluid samples were analyzed for rumen fermentation products, including total volatile fatty acids (TVFA) (gas chromatography, Focus GC AI 3000 Thermo Finnigan analyzer, USA) and ammonia-N (NH₃-N) (colorimetric method, Agilent Cary 60 UV-Vis Spectrophotometer, USA). All analysis procedures were as described by Cui et al. (2021).

2.5. 16S rRNA gene sequencing and bioinformatic analysis

Total DNA extraction from rumen fluid samples and PCR amplification were performed following the procedure described in our previous study (Cui et al., 2021). Briefly, the total DNA of each sample was extracted using the cetyltrimethylammonium bromide (CTAB) DNA extraction method. The purity and concentration of DNA were checked with 1 % agarose gel electrophoresis and atomic spectrophotometry. The universal prokaryote primers 341F and 806R were used to amplify the V3–V4 variable region of the bacterial 16S rRNA gene. The amplicons were sequenced on a Miseq PE300 platform (Illumina, USA). Raw 16S rRNA gene sequencing reads were subjected to quality filtration according to Flash, version 1.20, and QIIME (Quantitative Insight into Microbial Ecology), version 1.8.0 (Caporaso et al., 2010). These sequences were grouped into operational taxonomic units (OTUs) based on a 97% sequence identity clustered by Vsearch, version 2.7.1. The assignments of taxonomic OTUs were conducted via the SILVA database to further annotate taxonomic information. In order to identify the taxonomic information and determine the phylogenetic relationships between OTUs, each sample was normalized using MUSCLE, version 3.8.31 (Edgar, 2004). The functional gene content of microorganisms was predicted using a bioinformatics tool (PICRUSt2) based on 16S ribosomal DNA sequence data.

Bioinformatic analysis was conducted using R software. The alpha- and beta-diversity parameters were calculated with QIIME, version 1.8.0, based on normalized data. The relationships between the abundance of bacteria genera and the DMI and digestibility of nutrients were explored by using constrained correspondence analysis (CCA) in the vegan package using R software, version 2.15.3. Analysis of similarity was used to test whether a significant difference existed.

2.6. Statistical analyses

Data for DMI, digestibility of nutrients, ruminal fermentation parameters, and pH values were analysed by analysis of variance, using the one-way ANOVA, Univariate Model procedure of SPSS 25.0 (IBM Corp., Armonk, NY, USA). Orthogonal polynomial contrasts were used to determine linear and quadratic responses of the treatments. Polynomials were considered where overall significant effects of dietary LV were recorded. The Kruskal–Wallis rank sum test was used to compare the bacterial relative abundances and alpha-diversity indices (SPSS, version 25.0). Differences between mean values were considered significant at $p < 0.05$, and a tendency was declared at $0.05 < P < 0.10$.

Table 2

The effects of supplementary LV on feed intake and nutrient digestibility in Tibetan sheep.

Items ^a	Groups ^b				SEM	P-value		
	LVC	LVL	LVM	LVH		Treatment	Linear	Quadratic
DMI (g/day)	683 ^b	710 ^{ab}	730 ^a	647 ^c	7.5	< 0.01	0.19	< 0.01
Apparent digestibility (g/kg)								
DM	593 ^a	649 ^b	672 ^b	633 ^{ab}	7.9	< 0.01	0.04	< 0.01
OM	633 ^a	677 ^b	691 ^b	670 ^b	6.1	< 0.01	0.02	< 0.01
NDF	537	572	582	544	6.6	0.04	0.63	0.02
ADF	549 ^a	595 ^{bc}	609 ^c	560 ^{ab}	7.4	< 0.01	0.48	< 0.01
EE	499 ^a	545 ^a	572 ^b	512 ^a	9.1	0.01	0.44	< 0.01
CP	426 ^a	471 ^{ab}	486 ^b	469 ^{ab}	8.2	0.05	0.05	0.02

^{a,b,c} Within rows, means without a common superscript differ ($P \leq 0.05$).

^a DM = dry matter; DMI = dry matter intake; OM = organic matter; NDF = neutral detergent fiber; ADF = acid detergent fiber; EE = ether extract; CP = crude protein.

^b Groups: LVC = 0 mg LV/kg BW; LVL = 100 mg LV/kg BW; LVM = 200 mg LV/kg BW; LVH = 300 mg LV/kg BW.

3. Results

3.1. DM intake and digestibility of nutrients

As shown in Table 2, the average DMI of the four treatment groups were 683, 710, 730 and 647 g, respectively. Increasing the LV levels in rations affected the DMI of Tibetan sheep ($P < 0.01$). Compared with the LVC group, the DMI was higher in the LVL and LVM groups. The apparent digestibility of OM, NDF, ADF, EE, and CP was significantly affected by the dietary treatment ($P < 0.05$), which increased significantly in the LV supplementation groups in comparison with the control group.

3.2. Rumen fermentation characteristics

There were significant changes in concentrations of $\text{NH}_3\text{-N}$ and TVFAs, and in the proportions of some individual VFAs among the samples in the four groups ($P < 0.05$), except for butyrate and isovalerate, but there was no effect on the ruminal pH ($P > 0.05$) (Table 3). The concentrations of ruminal TVFAs, propionate, and $\text{NH}_3\text{-N}$ increased quadratically ($P < 0.01$) with an increase in LV, and the highest values were found in the LVM group. The concentrations of valerate in the LVH group were significantly higher compared with the other groups ($P < 0.01$). Among the four groups, the acetate and acetate/propionate ratio (A/P) decreased quadratically as dietary LV increased from 0 to 300 mg/kg DM, and the lowest values were found in the LVM group (200 mg/kg).

3.3. Microbial diversity

A total of 2938,718 qualified sequences were obtained from the rumen fluid samples with an average of $86,899 \pm 31,597$ (means \pm standard deviation) per sample, and 64,788 OTUs with 2025 (standard deviation: 162) OTUs per sample were detected based on 97% similarity. The flattened rarefaction implied that our data provided sufficient sequencing depth to present the rumen bacterial communities for the samples examined (Fig. S1). Differences ($P < 0.05$) in alpha-diversity metrics, including Chao1 index, observed OTUs, PD_whole_tree and Shannon index, were detected among the four groups, demonstrating greater diversity and richness in the LVH group (Fig. 1).

3.4. Divergence of the rumen bacterial communities

A comparison of the effects of LV supplementation on the rumen bacterial composition of Tibetan sheep was performed through taxonomic analysis. A total of 22 bacterial phyla were obtained from the 32 rumen samples. The most predominant phyla ($> 90\%$) among the four groups were Bacteroidetes and Firmicutes (Fig. 2A). Other phyla, including Patescibacteria, Verrucomicrobiota, Spirochaetota, Cyanobacteria, and Synergistota, were present with a lower abundance. However, in the LV supplementation groups, the abundance of Verrucomicrobia, Cyanobacteria and Synergistota was significantly increased compared with the LVC group (Table S1, $P < 0.001$). At the genus level, 234 genera belonging to 22 phyla were identified in the samples. *Prevotella* (LVC = 27.5%, LVL = 22.8%, LVM = 13.7%, and LVH = 21.4%) was the most dominant genus in the rumen, followed by *Rikenellaceae_RC9_gut* group (LVC = 18.5%, LVL = 18.0%, LVM = 23.4%, and LVH = 20.8%, respectively), and a difference was found in the relative abundance among the four groups (Fig. 2B). There were 17 most abundant shared genera with a relative abundance $> 0.5\%$ in all the samples of the different treatments (Table S2). Compared with the LVC group, the relative abundances of uncultured_bacterium, *Prevotellaceae_UCG-003*, and *Ruminococcus*, were significantly higher ($P < 0.05$), while those of *Prevotella*, uncultured_rumen_bacterium,

Table 3
Effects of supplementary LV on ruminal fermentation in Tibetan sheep.

Items	Groups ^a				SEM	P-value		
	LVC	LVL	LVM	LVH		Treatment	Linear	Quadratic
pH	6.64	6.37	6.42	6.58	0.05	0.21	0.77	0.11
Ammonia-N ^b (mmol/L)	16.3 ^a	17.4 ^{ab}	18.6 ^b	17.6 ^{ab}	0.24	0.01	0.02	< 0.01
TVFA ^c (mmol/L)	32.6 ^a	34.4 ^{ab}	37.4 ^b	35.2 ^{ab}	0.53	0.07	0.02	< 0.01
Molar proportions (%)								
Acetate	72.1b ^c	70.2 ^{ab}	68.4 ^a	72.8 ^c	0.44	< 0.01	0.92	< 0.01
Propionate	12.8 ^a	16.8 ^b	17.2 ^b	13.6 ^a	0.44	< 0.01	0.48	< 0.01
Butyrate	6.22	4.72	5.63	5.05	0.21	0.06	0.17	0.23
Iso-butyrate	3.28 ^b	2.82 ^{ab}	3.43 ^b	2.07 ^a	0.18	0.03	0.06	0.07
Valerate	3.15 ^a	3.28 ^a	2.95 ^a	4.46 ^b	0.18	0.01	0.02	0.01
Iso-valerate	1.48	1.57	1.77	1.37	0.13	0.77	0.92	0.66
A/P ^d	5.70 ^b	4.26 ^a	4.02 ^a	5.38 ^b	0.15	< 0.01	0.40	< 0.01

^{a,b,c} Within rows, means without a common superscript differ ($P \leq 0.05$).

^a Groups: LVC = 0 mg LV/kg BW; LVL = 100 mg LV/kg BW; LVM = 200 mg LV/kg BW; LVH = 300 mg LV/kg BW.

^b Ammonia-N = ammonia nitrogen.

^c TVFA = total volatile fatty acids.

^d A/P = Acetate/Propionate ratio.

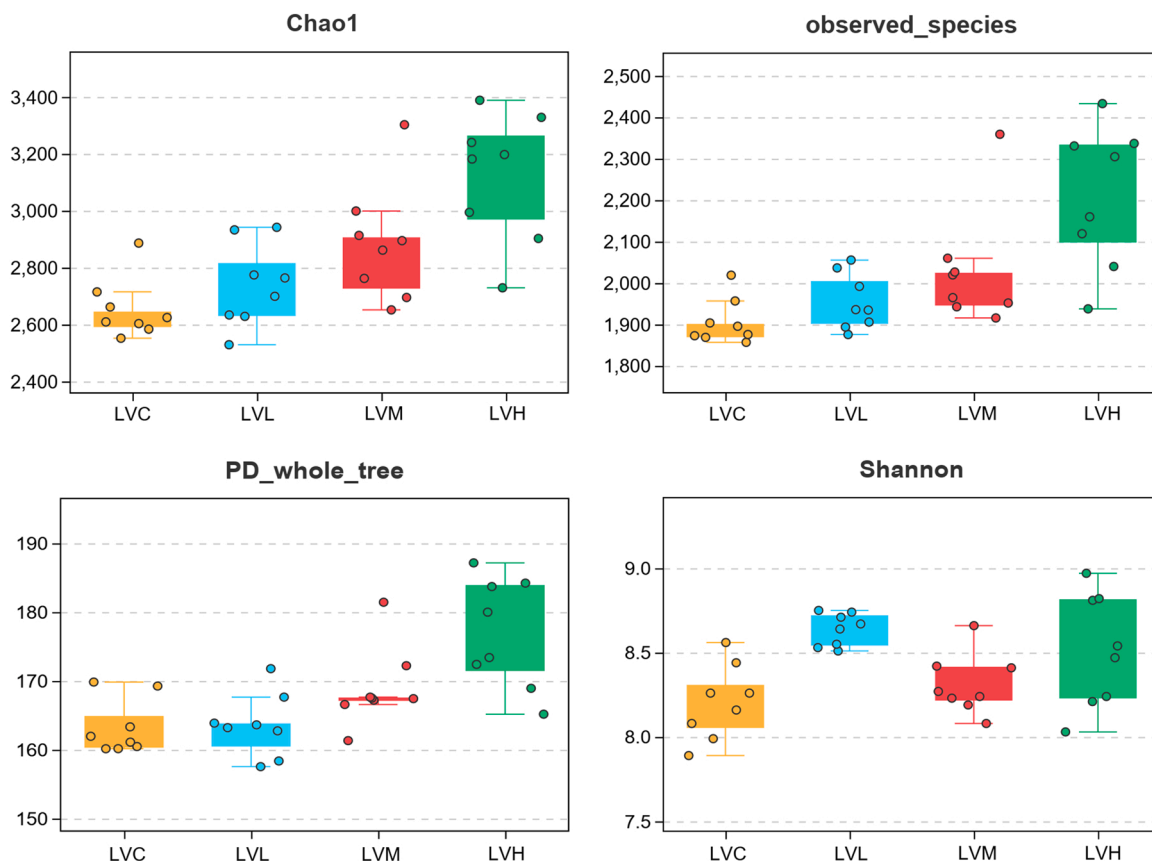


Fig. 1. Alpha diversity metrics of the bacterial composition in the rumen of Tibetan sheep.

Prevotellaceae_NK3B31_group, *Christensenellaceae_R-7_group*, *NK4A214_group*, and *Candidatus_Saccharimonas* were significantly lower ($P < 0.05$) in the LV supplementation groups. Furthermore, we performed a biomarker analysis using Linear discriminant analysis (LDA) effect Size (LEfSe) (Fig. 3A), and a cladogram (Fig. 3B) was generated from the LEfSe analysis of the ruminal bacteria community. The LEfSe analysis indicated that the composition of the bacterial communities differed significantly among the four groups with a linear discriminate analysis threshold of 4.0. The genera *Prevotellaceae_NK3B31_group* and *Prevotella* were significantly enriched in the LVC group; the phyla Synergistota and the genus *Fretibacterium* were enriched in the LVL group; the genus *Prevotellaceae_UCG_003* was enriched in the LVM group; while the genera *Rikenellaceae_RC9_gut_group* and *Ruminococcus* were enriched in the LVH group.

CCA was used to identify the associations between the treatment groups (Fig. 4). Seven physiological index factors (i.e., DMI, DMD, OMD, NDFD, ADFD, EED and CPD) were found to be correlated with the bacterial communities at the genus level. CCA showed that the rumen bacterial communities in the four treatments clustered according to their ration treatment. Coloring the samples by their different diet groups showed that the centroids of the four groups' clouds were well separated, which confirmed the close correlation between the physiological indices of the sheep and their rumen bacterial community. More information was uncovered by comparing the angles between the arrows. There is a clear separation of the LVM and LVH samples on the ordination plot with respect to those from LVC and LVL (horizontal axis). Moreover, several variables were correlated, or tended to be correlated, with the following sample distribution: DMI ($R^2 = 0.45$, $P < 0.01$); DMD ($R^2 = 0.32$, $P = 0.01$); OMD ($R^2 = 0.29$, $P = 0.01$); ADFD ($R^2 = 0.25$, $P = 0.02$); EE ($R^2 = 0.25$, $P = 0.02$). Furthermore, NDFD ($R^2 = 0.17$, $P = 0.07$) and CPD ($R^2 = 0.16$, $P = 0.09$) were positively correlated with the structure of the bacterial community of LVM samples, whereas they were negatively correlated to LVC samples.

3.5. Relationship between the bacterial community and phenotypic variables and VFAs

Spearman's rank correlation analysis showed that the relative abundances of *Prevotellaceae_UCG-003* taxa were positively correlated with DMD and EED; the relative abundances of uncultured_bacterium taxa were positively correlated with DMD, OMD, NDFD, ADFD, EED and CPD; *Ruminococcus* was positively correlated with OMD; the relative abundances of *Christensenellaceae_R-7_group* taxa were negatively correlated with EED; an uncultured_rumen_bacterium taxon was negatively correlated with OMD; *Prevotellaceae_UCG-001* was negatively correlated with DMD, NDFD and ADFD; *Candidatus_Saccharimonas* was negatively correlated with DMD, ADFD and EED; and the relative abundances of *Prevotella* were positively correlated with DMD, OMD, NDFD, and ADFD ($P < 0.05$, Fig. 5A).

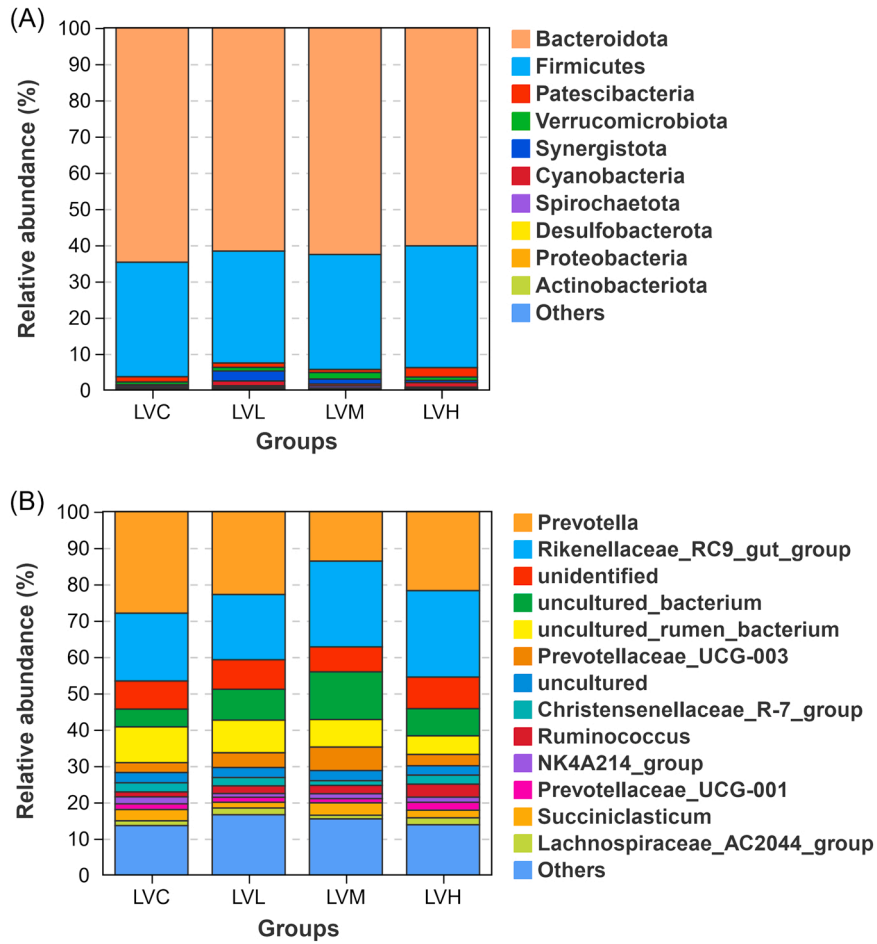


Fig. 2. Bacterial community composition at different taxonomic levels in the rumen of Tibetan sheep in all the four groups.

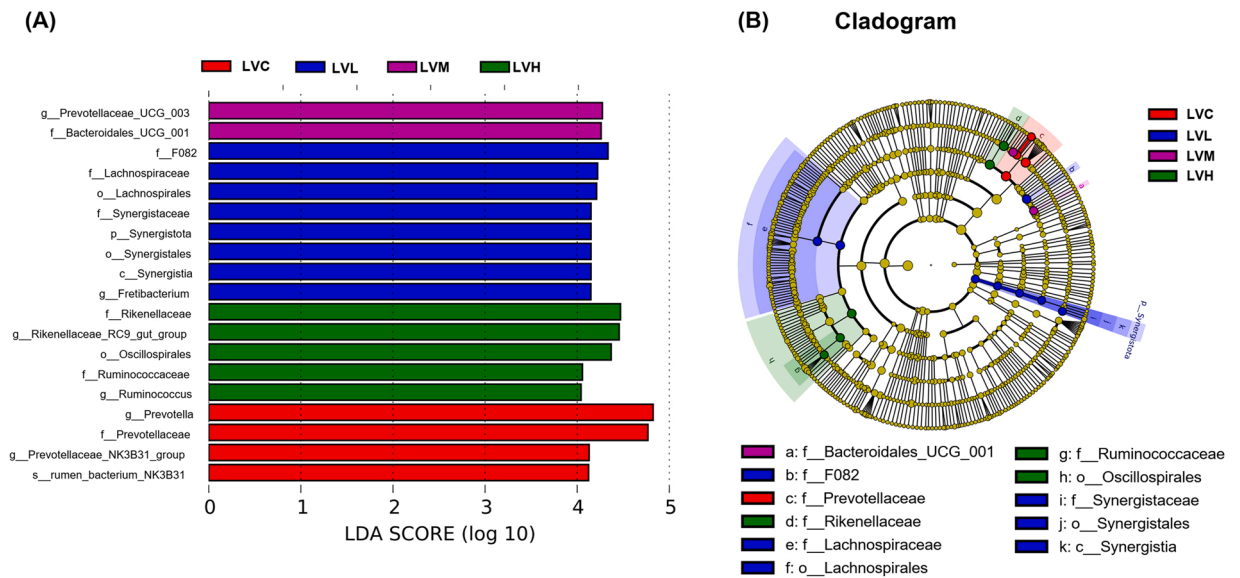


Fig. 3. Effect of LV on the composition of the rumen microbial communities in Tibetan sheep at different taxonomic levels.

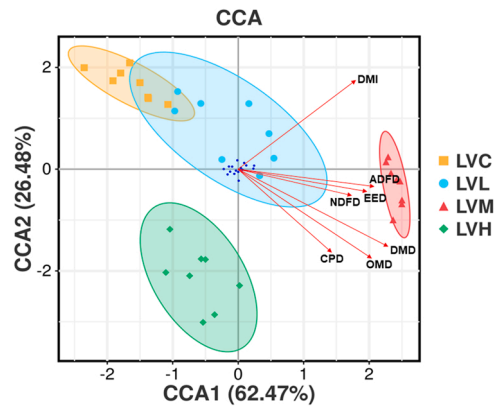


Fig. 4. Bi-plot from the constrained correspondence analysis (CCA) that shows the relationships between the bacterial community composition at the genus level and the environmental factors for four treatments.

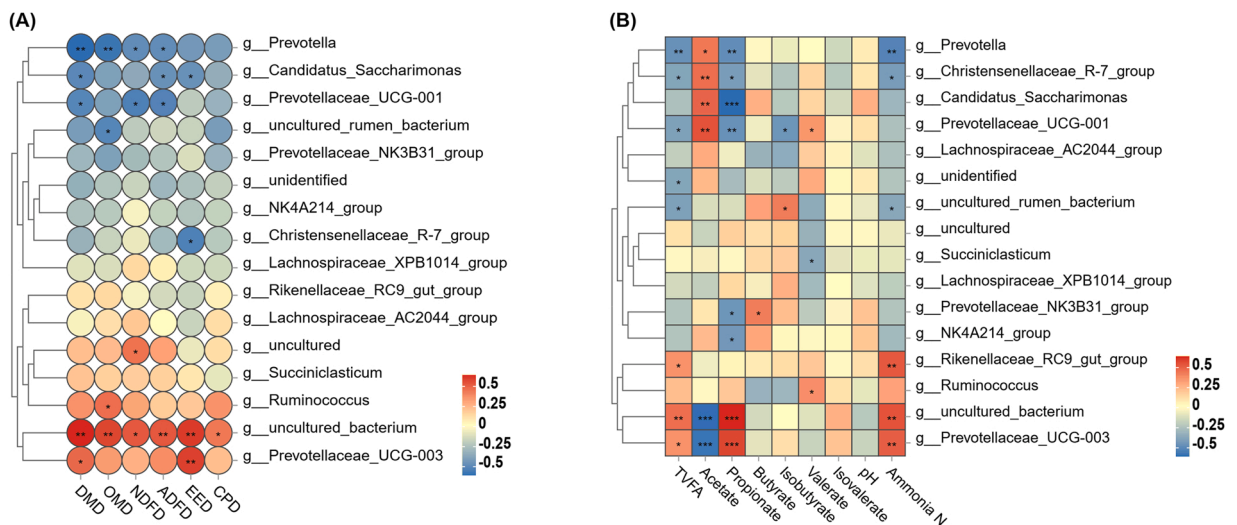


Fig. 5. Spearman's correlation showing that rumen bacterial communities (genus level) features associated with phenotypic variables (A) and ruminal fermentation (B).

Correlation analysis revealed significant relationships ($P < 0.05$) between the rumen microbial community and VFAs. Most microbes contributed strongly to the variation in TVFA, acetate, propionate, butyrate, isobutyrate, valerate, and $\text{NH}_3\text{-N}$, but did not relate to isovalerate or pH (Fig. 5B). Two clusters of microbes showed the strongest correlations with $\text{NH}_3\text{-N}$. The first cluster included *Prevotellaceae_UCG-003*, *uncultured_bacterium*, and *Rikenellaceae_RC9_gut_group* ($P < 0.01$), while the second one comprised *Candidatus_Saccharimonas*, *Prevotellaceae_UCG-001*, *Christensenellaceae_R-7_group*, and *Prevotella*. Meanwhile, rumen bacteria were also correlated with TVFA and major rumen metabolic measures, especially the concentrations of acetate and propionate (Fig. 5B). For example, *Prevotellaceae_UCG-003*, and *uncultured_bacterium* were positively correlated with propionate and negatively correlated with acetate concentrations, while *Prevotella*, *Christensenellaceae_R-7_group*, *Candidatus_Saccharimonas*, and *Prevotellaceae_UCG-001* were positively and negatively correlated with acetate and propionate concentrations, respectively ($P < 0.05$).

3.6. Metabolic pathway enrichment analysis of differentially abundant metabolites

The functional contributions of the bacteria in the samples were predicted based on OTUs using the PICRUSt2 package in R. In total, 32 functional pathways were successfully assigned to KEGG orthologs (KO) and their corresponding pathways (at level 2). Fig. 6 shows the 20 most important pathways based on PICRUSt2 functional predictions. These functions were found to be dominated by the following KEGG pathway-related processes: cellular processes (cellular community prokaryotes, transport and catabolism, cell growth and death, and cell motility); environmental information processing (membrane transport); genetic information processing (replication and repair, transcription, folding, sorting and degradation, and translation); and metabolism (carbohydrate metabolism, lipid metabolism, metabolism of cofactors and vitamins, energy metabolism, nucleotide metabolism, amino acid metabolism, metabolism of

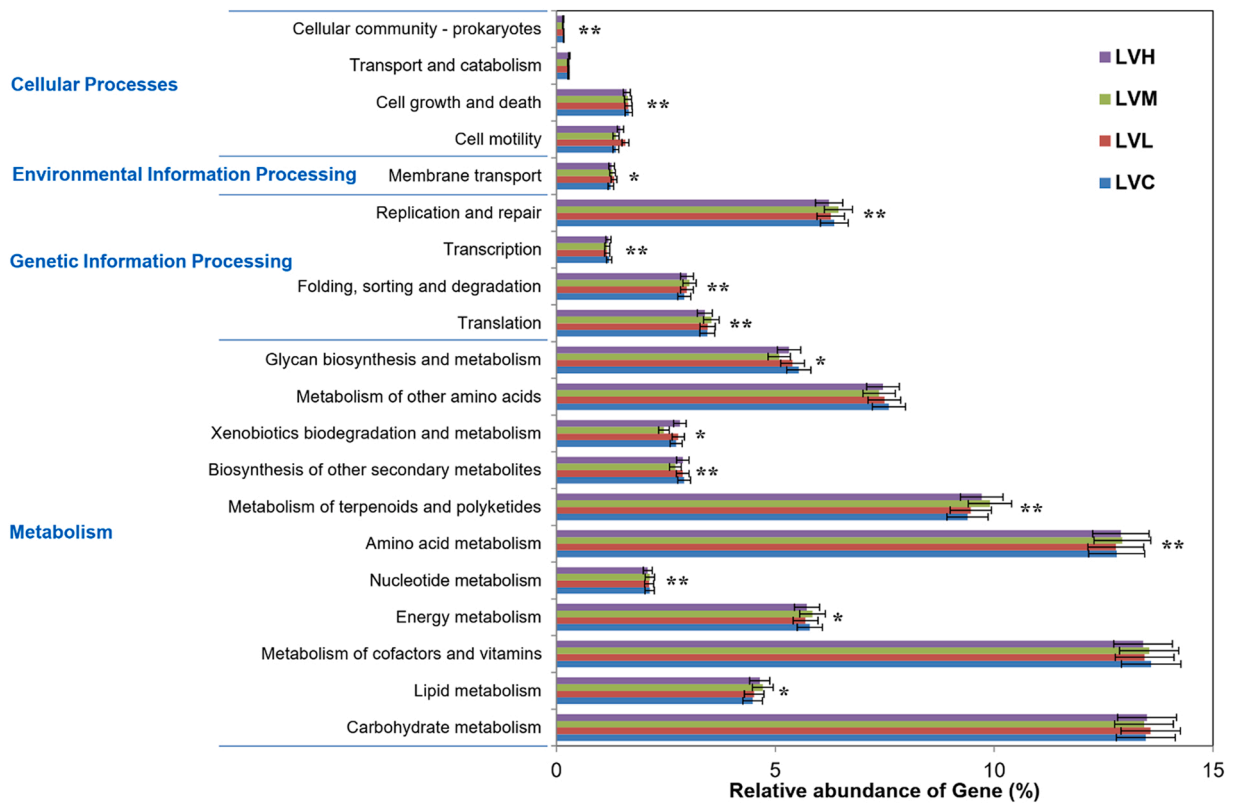


Fig. 6. Predictive functional analysis using PICRUST2 tool, showing relative abundance of assigned KEGG identity to the pathways of metabolisms at subsystem levels (at level 2 KOs).

terpenoids and polyketides, biosynthesis of other secondary metabolites, xenobiotics biodegradation and metabolism, metabolism of other amino acids, and glycan biosynthesis and metabolism). We found that most predicted functional categories changed significantly in the LV supplementation groups in comparison with the control group (level 2 KOs, Fig. 6).

4. Discussion

DM intake is an important indicator for measuring the health and production efficiency of animals, and the digestibility of nutrients can reflect the digestive characteristics of an animal's diet (Davison et al., 2021). Different macronutrients and bioactive compounds in forage can influence food preferences, which alter forage choices and impact dietary intake and nutrient digestion, leading to different nutritional statuses (Cui et al., 2019). Utilization of low-input food resources rich in plant bioactive compounds is a promising strategy for modulating the nutritional status and ruminal microbial populations of ruminants (Patra and Saxena, 2009). During long-term co-evolution, Tibetan sheep have developed a habit of eating a variety of foods, including FNHs containing bioactive compounds (Xiao et al., 2020). LV contains bioactive compounds, which can influence metabolism, nutrient digestion and ruminal microbial populations in herbivores. In the present study, increasing the proportion of dietary LV resulted in a tendency for a quadratic change in DMI, which was highest for Tibetan sheep in the LVM group and lowest in the LVH group. Therefore, it is possible that LV at a high level impairs DM intake. However, we did not observe an analogous decrease in nutrient digestion. Although the reasons for this are not clear, the potential decrease in DMI might not have been substantial enough to compromise the digestibility of nutrients. Other researchers have similarly reported that the inclusion of plant secondary metabolites in the diet of ruminants at high levels can generate an inverse effect, reducing the level of voluntary feed intake owing to the dynamic interaction between food flavor and post-ingestive feedback (Villalba et al., 2006; Jason and Villalba, 2006). The DMI and nutrient digestion of ruminants may be associated with the level of secondary metabolites in the diet. Overall, additional phytonutrients through LV supplementation may significantly improve the feed utilization efficiency of Tibetan sheep in cold conditions.

Rumen fermentation characteristics of ruminants varies with host, dietary composition, season, and other experimental treatments (Patra, 2010; Arnold, 2020). Tibetan sheep are the dominant livestock breed of the QTP, which are well adapted to the high altitude, strong ultraviolet, hypoxia and cold environment (Cui et al., 2019). The adaption of Tibetan sheep includes physiological adaptations such as a well-adapted microbiome and molecular regulation mechanisms, such as the regulation of metabolites and nutrient absorption by host genes and rumen microbiota (Fan et al., 2021). Due to changes in forage nutritional quality and palatability during different phenological stages, the rumen microbiota and ruminal fermentation characteristics will make physiological adjustment strategies to

improve plant biomass deconstruction and energy maintenance in response to seasonal and environmental factors (Cui et al., 2023). The convergent evolution of the rumen microbial community upregulates genes related to VFAs transport and absorption that helps Tibetan sheep to survive at high altitude (Zhang et al., 2016). As thus, Tibetan sheep may have completely different adaptive mechanisms and rumen fermentation patterns than ruminants living on the plains, and the development and application of FNH in Tibetan sheep production systems may even provide a unique opportunity to manipulate complex microbial ecosystems to improve the health and productivity of Tibetan sheep during the cold season.

There are in excess of 1000 BACs occurring naturally in FNH (Hart et al., 2008). Structurally, BACs can be classified as alcohol, esters, or aldehyde derivatives of phenylpropanoids and terpenoids (Greatehead, 2003). There are reports that the effects of plant-derived additives on rumen bacterial communities were dependent on the chemical nature of the additive provided as a supplement (Patra and Saxena, 2009). Indeed, the type of plant-derived additive used, the composition of their active components, as well as their dosage, may affect their ability to modulate the rumen environment. While the specific modes of action of natural plant or crude extracts as feed additives still remain to be determined, they are thought to be more effective in the direct addition of crude FNH compared to the isolated constituents it contains, as different types of compounds in plants may be more likely to affect microbial growth or survival through distinct mechanisms (Rasoanaivo et al., 2011). There is increasing evidence to suggest that many FNH rich in BACs have an effect on ruminal microbiota in vivo and can selectively alter specific rumen bacteria and consequently the metabolic processes of VFAs in the rumen (Fagundes et al., 2020). The concentrations of VFAs and $\text{NH}_3\text{-N}$ and the value of the pH are the main internal environmental indicators of rumen fermentation. $\text{NH}_3\text{-N}$ is the most important N source for microbial protein synthesis in the rumen (Calsamiglia et al., 2010). In the present study, increases in the LV level significantly increased the ruminal $\text{NH}_3\text{-N}$ and TVFA concentrations, which might have been due to the ability of the rumen system to adapt to different dietary LV supplementation levels through the self-adjustment of rumen microorganisms. An increase in the production of VFAs and $\text{NH}_3\text{-N}$ in the rumen often results in improved productivity, metabolic activity, and N efficiency in ruminants (Ogunade et al., 2020; Hartinger et al., 2018). Dietary supplementation of LV increased the VFA concentration, possibly due to increased substrate availability, because the DM intake was different among the treatments. Furthermore, LV supplementation significantly induced propionate concentrations but decreased the proportions of acetate, thereby resulting in a significant reduction in the acetate/propionate ratio. Providing an LV-supplemented diet shifted the fermentation pattern from acetate to propionate. Propionate is thought to be a major volatile fatty acid in ruminants that contributes to the gluconeogenesis pathway for net synthesis of glucose (Allen, 2014). Thus, the increase in propionate in the rumen was a positive sign for improving energy utilization as well as easing cold stress. A high propionate/acetate ratio can help hosts maintain their metabolic balance in the cold season. The molar proportions of butyrate, isobutyrate, valerate and isovalerate did not differ among the four treatments. One possible reason for this is that these short-chain fatty acids account for a relatively smaller part of the TVFA, and thus its change may be masked by the changes in propionate or acetate. It is worth noting that, although LV altered the bacterial community, similar pH values were achieved.

Ruminal microbiota plays a vital role in forage digestion by converting it to nutrients that can be used directly by the ruminants (Morgavi et al., 2013; Mizrahi et al., 2021). Rumen microbial diversity is highly responsive to the nutritional composition and dietary phytochemical content of forage (McAllister, 2019; Cui et al., 2021). High richness and diversity in bacterial microbiota are considered beneficial, which can increase the stability of the rumen (Weimer, 2015; Newbold and Ramos-Morales, 2020). A higher diversity and richness indicate an increase in the number of species stimulated by LV, and the dominance of certain species is enhanced. In the present study, comparison of the bacterial community in the rumen between the LVC and LV supplementation groups revealed that some bacterial taxa may play a role in improved digestibility.

Bacteroidetes and Firmicutes were the dominant bacteria in the rumen of Tibetan sheep in the cold season, which is consistent with findings in yak, cattle, and other ruminants (Yamano et al., 2019; Yang et al., 2020; Islam et al., 2021). After dietary intervention of LV, the abundances of Verrucomicrobiota, Cyanobacteria and Synergistota were relatively higher in the LV supplementation groups. It was previously reported that some members of Verrucomicrobia were found to help hosts improve the efficiency of energy utilization under cold stress (Chevalier et al., 2015). Synergistota have been found in a wide range of anaerobic habitats and some members have been implicated in amino acid transport (Hugenholtz et al., 2009; Oren and Garrity, 2021). Cyanobacteria have been observed to increase with a higher plant-based diet in humans (Di Rienzi et al., 2013), while in ruminants, the Cyanobacteria group are a common ruminal bacterial phylum and play an important role in hemicellulose and pectin degradation, as well as the reduction of methane production (Martinez-Fernandez et al., 2020; Prasanna et al., 2002). The variation in the relative abundance of this phyla is likely being driven by the change in the quality of the diet and their ability to degrade the hemicellulose and pectin content of the plant. These results suggest positive effects of LV supplementation on bacterial change and the possible improvement of energy utilization and amino acid fermentation in a low-temperature environment, as well as the potential to reduce methane emissions. Moreover, we observed that the abundances of Patescibacteria and Spirochaetota were significantly changed under the effect of LV in winter. It was reported that Spirochaetota abundance in the rumen is associated with pectin degradation, and some members are known as major hemicellulose degraders (Gharechahi et al., 2021). The superphylum Patescibacteria is commonly detected in anoxic environments and potentially involves anaerobic fermentative metabolisms, and the abundance of this superphylum in rumen could be influenced by the ambient temperature (Herrmann et al., 2019; Qiu et al., 2020).

We found that the relative abundance of the genera uncultured_bacterium, *Prevotellaceae*_UCG-003, and *Ruminococcus* were higher, while *Lachnospiraceae*_NK3A20, *Prevotella*, uncultured_rumen_bacterium, *Prevotellaceae*_NK3B31_group, *Christensenellaceae*_R-7_group, *Ruminococcaceae*_NK4A214_group, and *Candidatus_Saccharimonas* were lower, in the LV-treated groups than in the untreated groups. This could be a sign of improved cellulose and hemicellulose degradation ability due to LV feeding, as *Ruminococcus* have a substantial set of hemicellulase and oligosaccharidedegrading enzymes. For ruminants, *Ruminococcus* plays an important role in short-chain fatty acid production (La Reau and Suen, 2018). The increase in abundance of these microorganisms might be one reason for the increase in

TVFA generation. A study found results similar to this study insofar as it observed an increase in populations of *Ruminococcus* species in goats when fed *Andrographis paniculata* as a feed additive, and the animals were able to tolerate the level of polyphenols present in *A. paniculata* (Yusuf et al., 2017). The family Prevotellaceae is a genetically and metabolically diverse microbial population in the rumen that is widely considered to have the ability to efficiently degrade lignocellulosic feedstock, protein and pectin (Cui et al., 2021). Studies have shown that silage plantago species rich in glycosides, flavonoids, and effective mucilage polysaccharides and mono-terpenoids could significantly enhance the Prevotellaceae community in the rumen (Kara et al., 2022). Interestingly, in the present study, the abundance of Prevotellaceae_UCG-003, Prevotella and Prevotellaceae_NK3B31_group showed clearly distinct responses to dietary LV supplementation, possibly indicating that the various members of the Prevotellaceae family have different sensitivities for plant-derived additives. Like in this study, a previous report showed that some members of the Prevotellaceae family exhibited markedly distinct sensitivities when supplemented with daidzein (a natural isoflavone widely found in legumes) in lactating-cow diets (Zhao et al., 2017). We speculate that some members of these microorganisms might prefer metabolic pathways that are also highly adaptable to high BACs-containing conditions. As a common and abundant microbiome occurring in a variety of natural habitats, the genus Prevotella has increasingly come under the spotlight owing to conflicting reports about whether its effect on host health is positive or detrimental (Tett et al., 2021). A decrease in the number of members of the genus Prevotella was observed in the rumen of sheep fed a diet of *Rosmarinus officinalis* leaves (Cobellis et al., 2016). In addition, a recent study showed that having a Prevotella-rich gut microbiome decreased cholesterol levels, potentiated weight loss, and limited the bifidogenic effect in individuals consuming a fiber-rich diet (Chung et al., 2020). The present study indicates that Prevotella may be downregulated by feeding LV to Tibetan sheep, which motivates us to try to improve the production performance of grazing livestock by manipulating the abundance of Prevotella in the rumen. Candidatus_Saccharimonas belongs to the superphylum Patescibacteria, which is an important member of the oral microbiome, and is also found throughout the skin and gastrointestinal tract. The latest evidence shows that this microorganism can translocate into the intestine and alter its microbiota and possibly the immune system; plus, the abundance of this genus may be associated with inflammatory mucosal diseases such as intestinal inflammation (Cruz et al., 2020). Therefore, it is worth noting that supplementation with LV could reduce the abundance of Candidatus_Saccharimonas by as much as 65.3 %, suggesting the potential benefit of LV in terms of intestinal diseases. Ruminococcaceae_NK4A214_group is an important intestinal microorganism that is positively associated with diarrhea (Liang et al., 2021). Based on comprehensive analysis, we speculate that LV could enhance intestinal homeostasis by decreasing the abundance of Candidatus_Saccharimonas and Ruminococcaceae_NK4A214_group. The genus Christensenellaceae_R-7_group has been identified as a key component of the epithelial microbiota of ruminants (Pacífico et al., 2021). Christensenellaceae harbors several enzymes that can degrade sugary compounds, including α -arabinosidase, β -galactosidase, and β -glucosidase (Perea et al., 2017). A study found that a high relative abundance was associated with a lean host phenotype (Goodrich et al., 2014). Results from the present study showed that the decrease in Christensenellaceae_R-7_group in the LV addition groups may be beneficial in promoting growth and development of Tibetan sheep in winter, and this was most noticeable in the LVM group. Moreover, five other genera showed significant differences as well: Rikenellaceae_RC9_gut_group, Succiniclaticum, Lachnospiraceae_AC2044_group, and Lachnospiraceae_XPB1014_group, and these microorganisms had limited nutrient degrading capacities, yet they may play a complementary or accessory role in BACs metabolism and degradation in the rumen. Some members of Succiniclaticum can ferment succinate quantitatively to propionate (van Gylswyk, 1995). Rikenellaceae_RC9_gut_group can produce propionate and succinate as fermentation end products (Graf, 2014). This further explains why supplemental LV modulated the rumen fermentation towards increased concentrations of ruminal propionate precursors for net synthesis of glucose. Greater relative abundances of these genera possibly explain the increased propionate concentrations in sheep fed supplemental LV. Apart from the above classified genera, a large number of unclassified genera were identified, and these might play a crucial role in the rumen's metabolism, acidogenesis, and hydrolysis of BACs (Fink-Gremmels, 2010).

Functionally, microbiota showed significant alterations in predicted functional profiles in response to dietary treatments. The LV supplementation groups featured enrichment of taxa that may enhance folding, sorting and degradation, membrane transport, cell motility, metabolism of terpenoids and polyketides, and lipid metabolism. Specifically, the LVM diet obviously enhanced energy and amino acid metabolism, translation, folding, sorting and degradation, as well as replication and repair in the rumen. Ruminants can develop adaptation mechanisms through rumen microorganisms to neutralize the effects of BACs in diets (Fink-Gremmels, 2010; Loh et al., 2020). The enriched metabolism of terpenoids and polyketides in response to the LV diets was possibly related to the content of BACs in different treatments. The increased abundance of lipid metabolism genes can be explained as an increase in the regulation of energy homeostasis by the LV diets and was consistent with the increase in the production of VFAs in the sheep fed the LV diet (Conte et al., 2022). The increased abundance of genes related to cellular processes and amino acid metabolism can be explained as an increase in the growth of the taxonomic drivers by the LV diets and may indicate the potential of LV as a metabolic regulator in the cold season (Park et al., 2020).

5. Conclusion

Dietary inclusion of an FNH (LV) in the diet affected the metabolic parameters, ruminal fermentation, and bacterial composition linked to nutrient supply in Tibetan sheep in the cold season. Our results suggest that rumen bacteria tend to show a unique response to different LV supplementation levels. The bacterial composition improved under the 100 and 200 mg/kg treatments, but not under 300 mg/kg. This is because there are indications that dietary inclusion of LV at a high level could limit the efficacy of BACs from LV in modulating ruminal metabolism.

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CRediT authorship contribution statement

Xiongxiang Cui: Conceptualization, Software, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Zhaofeng Wang:** Conceptualization, Data curation, Formal analysis. **Qingshan Fan:** Formal analysis, Investigation. **Shenghua Chang:** Resources, Data curation, Formal analysis. **Tianhai Yan:** Methodology. **Fujiang Hou:** Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing. Thank Mr. Tsedan Guru for the help of sample collection.

Conflict of interests

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

Data availability

The datasets generated for this study are included in the article/[Supplementary Material](#), further inquiries can be directed to the corresponding author.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.anifeedsci.2023.115628](https://doi.org/10.1016/j.anifeedsci.2023.115628).

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