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Altered milk yield and rumen microbial abundance in response to concentrate supplementation during the cold season in Tibetan sheep

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ABSTRACT

Background: Weight loss and decline of milk yield in Tibetan sheep was a challenge for the dairy industry in Qinghai-Tibet Plateau, which were considered to be caused by underfeeding of the sheep during the harsh winter. The objective of this study was to assess the role of feed supplementation in the milk performance and rumen microbiome of ewes under forage-based diets. Based on parity, milking period, milk yield, and body weight, ten 1.5-yr-old ewes were allocated randomly into two groups. One group of ewes was fed no supplement Control group (CON) and the other group was fed with concentrate feed supplement (Treatment group, T). Individual milk yield was determined daily; both the milk composition and rumen bacterial characteristics were analyzed after the end of feeding trials.

Results: Results showed that lactose in the milk of the CON group was significantly lower ($P < 0.05$) than that of the T group at days 30 and 60. Milk yield in the T group was greater than in the CON group at day 30 ($P < 0.05$). Additionally, the dominant ruminal bacteria (phyla Bacteroidetes, Firmicutes, and Verrucomicrobia) were shared by both groups through 16S rRNA gene pyrosequencing. Greater relative abundance of Bacteroidales RF16 group in family level, Victivallales in order level, Lentisphaeria in class level, and Lachnospiraceae bacterium in species level were observed in the T group than in the CON group ($P < 0.05$).

Conclusions: These results demonstrated that supplementation of concentrate in the cold season improved milk lactose yield and milk production, and the rumen microbial abundance of Tibetan sheep.

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1. Introduction

Tibetan sheep (*Ovis aries*) is characterized by cold tolerance, hypoxia tolerance, stress resistance, and strong adaptability in the Qinghai-Tibet Plateau [1], providing an important economic resource for local Tibetans, such as pelage, meat, and milk production [2]. However, the long-lasting cold season and decline in nutritive value of pasture make it difficult to meet nutritional requirements for Tibetan sheep, resulting in growth retardation, hypoimmunity, and a higher mortality rate [3]. Previous researches demonstrated that concentrate supplements in diets contributed to nutrient requirements and improved the efficiency of feed usage in ruminants [4]. Therefore, a supplement of concentrate was crucial for superior availability of nutrients and economic benefits [5]. Distinct microorganisms (bacteria, archaea, fungi, and protozoa) lived in the rumen of ruminant herbivores in a symbiotic relationship [6]. These microbes would supply vitamins, proteins, and carbohydrates for the host by secreting lytic enzymes available for microbial fermentation [7]. Additionally, rumen microorganisms

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provided an improved host organic immunity and resistance to invading pathogens [8]. Furthermore, several bacteria remarkably detoxify harmful compounds (glycosides and sapongenins) in diets [9]. The compositions of microbial communities of the ruminants were highly responsive to change in diet type, physiological status, and management strategy [10]. Although several microbiome studies were performed on ovine rumen [8], the adaption of the rumen microbiome to changes in diets was rarely reported. It is speculated that feed supplementation to Tibetan sheep during the cold season would improve their milk production and rumen microbial community structure. Therefore, our study aimed to characterize and compare the composition and phylogenetic distributions of the ruminal microbiota at the diet-induced shifts, and assessed the role of concentrate supplementation in the milk performance of Tibetan sheep during the lactation period.

2. Material methods

2.1. Ethics statement

This animal experiment was approved by the Institutional Animal Care and Use Committee (State Key Laboratory of Plateau Ecology and Agriculture, Qinghai University) (Protocol 0115).

2.2. Experimental design and feeding

This experiment was conducted in Haiyan County, Qinghai province North, at latitude 15°30′N, longitude 32°33′E, and altitude 3000 m above sea level during the period from February 15, 2019, to April 30, 2019. Fifteen days were used to adapt to the diet changes and the experiment lasted for 60 d. Ten Tibetan sheep (aged 15 months and approx. 26 kg) of lactation were housed at the veterinary station, Haibei state, Qinghai province, China. Before this study, they grazed only natural pasture and were not offered supplements. Concentrate supplements were provided for the Tibetan sheep, which were grazed on the natural grassland until the experiment. All ewes were allotted randomly into two groups. One group of ewes was fed no supplement (Control group, CON) and the other group of ewes was fed concentrate feed supplement with 250 g/d concentrate per sheep (Treatment group, T), respectively (Table 1). According to NRC (2001), the chemical composition of concentrate supplementation was adjusted for T group requirements.

2.3. Milk production and composition

Individual milk yields were recorded daily throughout the experiment. Milk samples for analyzing the milk composition were collected at each milking for all ewes on days 0, 30, and 60. The fat, protein, and lactose of milk were determined using the infrared analysis (MilkoScan 4000; Foss, Hillerød, Denmark) [11].

2.4. Rumen bacterial characteristics

After slaughter, rumen fluid samples were filtered and collected by five layers of sterile gauze, and were stored at −80 °C. Total genomic DNA was extracted by the E.Z.N.A.® stool DNA kit (Omega Bio-tek, Norcross, GA, USA) [12]. Concentrations of genomic DNA from all samples were determined using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA).

The V3-V4 hypervariable region of the bacteria 16S rRNA gene was amplified by PCR according to primers forward (5′-CCTACGGGNGGCWGCAG) and reverse (5′-GGACTACHVGGGTATC TAAT). The cycling protocol was 95 °C for 2 min; 35 cycles at 95 °C for 2 min; 72 °C for 30 s; and 72 °C for 5 min. Purified amplifications were sequenced by GENE DENOVO (Guangzhou, China) on the Illumina HiSeq 2500 PE250 platform.

The 16S rRNA analysis was performed by R software (version 3.1.2), QIIME software (version 1.9.1) [13], and UPARSE software [14]. According to the UPARSE pipeline, multiplexed reads were clustered into operational taxonomic units (OTUs), based on 97% sequence identity. The 16S rRNA gene sequence was classified by the RDP classifier (version 2.2) [15]. Present figures were conducted using the R package [16].

2.5. Statistical analysis

SPSS software (version 25.0) was used for all the statistical analyses in this study. Differences in means between the relative abundance of bacteria were significant when *P*-values were <0.05 by paired T-test.

3. Results

3.1. Milk composition

The results of milk analyses are summarized in Fig. 1. Our results showed a decreasing trend in milk composition during the lactation period. The milk yield of the T group was greater than that of the CON group at day 30 ($P < 0.05$). Compared to the CON group, the increases in lactose of the T group were significant at days 30 and 60 ($P < 0.05$). No significant difference existed in the proportions of milk protein and fat at any time point after concentration supplementation ($P > 0.05$). After filtering out low-quality reads and chimeras of Illumina-sequenced reads, we obtained a total of 1,002,925 bacterial 16S rRNA effective sequences, with an average of 100,293 tags per sample. The number of sequences for each sample ranged from 87,134 to 113,715. The remaining high-quality sequences were clustered into OTUs according to a 97% similarity level by UPARSE software. All detected OTUs were 28,230 and the average value of OTUs for each sample was 2,823. We found 15 distinct phyla in all samples; the predominant abundant followed by Bacteroidetes (56.38%), Firmicutes (14.87%), and Verrucomicrobia (14.74%). The top ten phyla ranked by the abundant were prevalent in all samples (Table 2) and accounted higher than 98%. Compared with the CON group, the average value of relative abundance of Fibrobacteres for the T group was significantly higher ($P < 0.05$). The less abundant phyla included Planctomycetes (0.39%), SR1 (0.47%), Elusimicrobia (0.30%), Synergistetes (0.11%),

Table 1
Ingredients and chemical composition of the experimental diets.

Item	T group	CON group
Ingredient, % of Dry matter		
Corn	63.00	–
Rapeseed dregs	21.00	–
Cottonseed	6.00	–
Bean pulp	3.00	–
Glucose	3.00	–
Salts	1.00	–
Calcium hydrophosphate	0.50	–
Sodium bicarbonate	0.50	–
Mountain flour	1.00	–
Commercial premix[a]	1.00	–
Chemical analysis (%)		
Dry matter	87.00	88.00
Crude protein	15.30	6.50
Crude fat	2.60	2.30
Crude fibre	4.30	31.00
Crude ash	2.93	8.00

[a] Commercial premix following per Kg: Fe 9.0 g; Cu 1.8 g; Mn 6.0 g; Zn 10.0 g; I 100.0 mg; Se 25.0 mg; Co 25.0 mg.

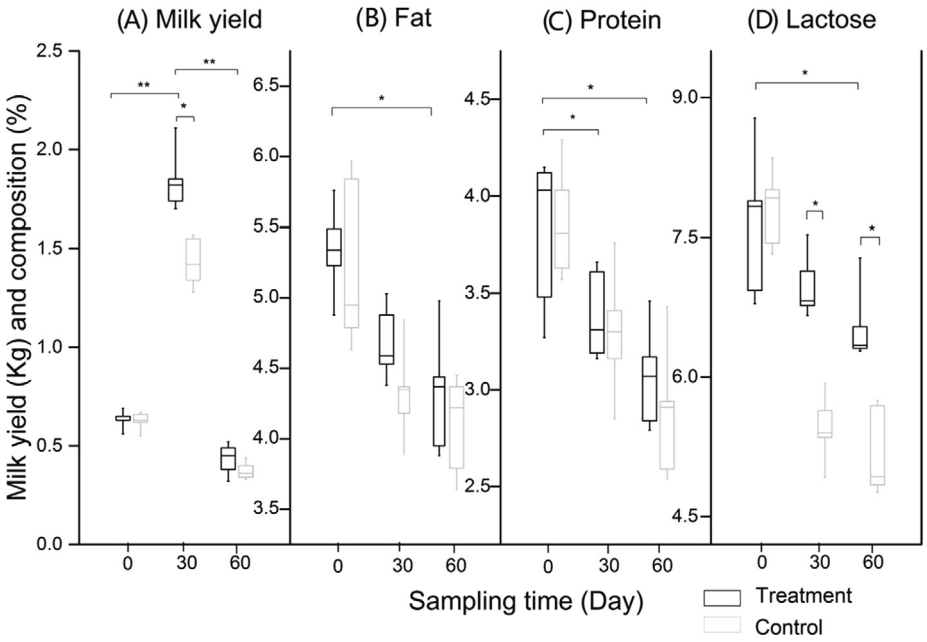

Fig. 1. The effect of concentrate supplementation on milk performance. $^{*}P < 0.05$. $^{**}P < 0.01$. $n = 5$.

Table 2
The relative abundance of main bacteria at the phylum in Tibetan sheep.

Bacteria	T group	CON group
Bacteroidetes	53.29 ± 6.72	59.46 ± 8.03
Firmicutes	14.59 ± 1.72	15.15 ± 1.18
Verrucomicrobia	16.23 ± 3.00	13.26 ± 3.74
Lentisphaerae	4.82 ± 0.96	3.48 ± 1.56
Fibrobacteres	3.62 ± 1.39[a]	1.09 ± 0.71[b]
Proteobacteria	1.49 ± 0.40	1.69 ± 0.41
Spirochaetae	1.61 ± 0.51	1.62 ± 0.50
Cyanobacteria	1.46 ± 0.43	1.61 ± 0.87
Tenericutes	1.25 ± 0.13	0.83 ± 0.41
Planctomycetes	0.30 ± 0.15	0.48 ± 0.19

Note: [a,b] Means with different superscripts are significantly different ($P < 0.05$). $n = 5$.

Saccharibacteria (0.12%), and Euryarchaeota (0.05%). Sixty-two diverse genera were detected and 57 genera existed for each individual. The top 20 genera were identified in all samples (Fig. 2), and the relative abundance of Prevotella 1 and Rikenellaceae RC9 gut group were significantly higher than others ($P < 0.05$).

3.2. Ruminal bacterial community

The observed species and Shannon index ranged from 2,704 to 2,976, and 8.82 to 9.20, respectively, suggesting no differences between the T and CON groups ($P > 0.05$). Of the 2253 OTUs that were shared for the two groups of ewes, 588 OTUs were unique to the CON group and 589 OTUs were unique to the T group (Fig. 3A). The PCoA analysis (Fig. 3B) revealed that the T and CON groups were separated; the first two axes of the PCoA1 explained 19.95% and 14.25% of the bacterial variation and the two principal components covered 34.19% of the variation.

The resolutions of principal component analysis (PCA) of the genus (Fig. 4A) and OTU (Fig. 4B) levels revealed distinct differences in the composition of CON and T groups. For the OTU level, the bacterial community of the CON group was separated from that of the T group by the second principal component (PC2), and the bacterial community of the CON group in the left side of the first principal component (PC1) tightness clustered into a major group. For the genus level, the bacterial community of the CON group was separated from that of the T group by the PC2.

3.3. Microbial community structure

We performed LEfSe analyses to identify the significance of different taxa (relative abundance > 1%) between the CON and T groups (Fig. 5A). LDA results from the LEfSe analyses are given in Fig. 5B. In the T group, we found that Lentisphaeria in class level, Victivallales in order level, Bacteroidales RF16 group, Clostridiaceae 1, Victivallaceae in family level, Senegalimassilia, Clostridium sensu stricto 1, Lachnoclostridium 12, Ruminobacter in genus level and bacterium MC2010, and Lachnospiraceae bacterium CG55 in species level, were significantly abundant taxa. In the CON group, we found that Deltaproteobacteria in class level, Rhizobiales in order level, Christensenellaceae, Comamonadaceae, and PL-11B10 in family level, and Christensenellaceae R-7 group, Lachnospiraceae NK3A20 group, and Lachnospiraceae UCG 009 in genus level, were significantly abundant taxa.

4. Discussion

The quantitative variation of concentrate supplementation in dietary and milk compositions in ruminants may fluctuate because of the change in nutritional intake [17]. Growing studies reviewed the effect of concentrate supplementation on the qualitative and quantitative composition of milk in ruminants [18]. Presently, concentrate supplementation in diet exhibited a prominent effect on the milk yield of Tibetan sheep, especially during the early stages of lactation, in agreement with Joy et al. [19], who concluded that concentrate supplementation of ewes improved milk yield under forage-based diets [19]. Similarly, in a study by Heublein et al. [20], when 6.0 Kg/day of concentrate supplementation was offered, supplemented cows produced more energy-corrected milk yield compared with non-supplemented cows [20]. The positive effect of concentrate supplementation on milk yield was probably caused by increased intake of energy as also stated by Munoz et al. [21].

The rumen microbes allowed ruminants to convert indigestible plant materials – through microbial fermentation – into a range of

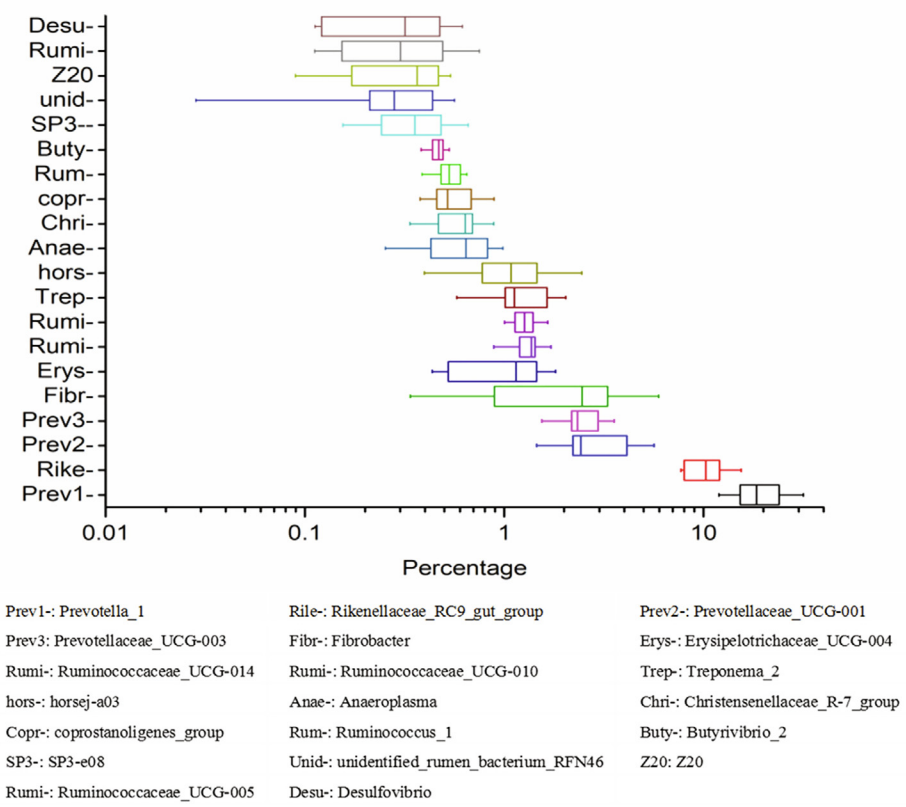

Fig. 2. The relative abundance of shared bacteria genera across ruminal samples are expressed using the box plot; the data are the relative abundance's Napierian logarithm of bacterial genera of all samples. n = 5.

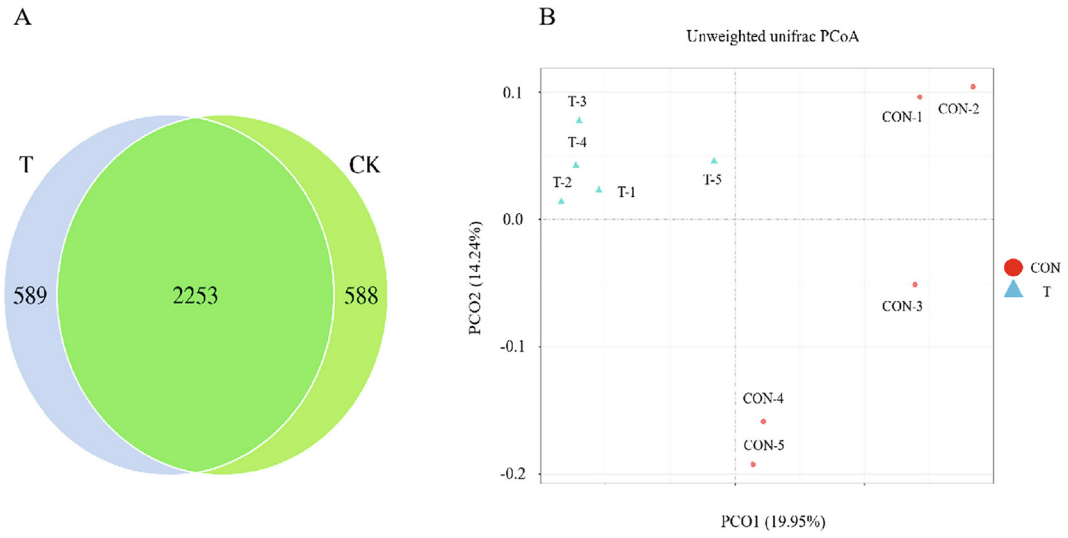

Fig. 3. The alpha diversity of bacteria community and OTUs between T and CON groups. A: Venn diagram test of bacteria. B: PCoA analysis.

volatile fatty acids [22] that constitute approximately 70% energy source for the host [23]. The ruminal microbial population is complex, diverse, and contains thousands of species of microbes, with only an estimated 10% of the bacterial species available in culture [24]. It was necessary to improve the representation of the community in the culture and to use culture-independent approaches. At present, the 16S rRNA amplicon sequencing is widely used to study microbial ecology, especially with regard to culture-independent methods and mechanism of metabolism in ruminants [25]. The ruminal microbial community structure exhibited dynamic balance and is especially susceptible to disruption by type and composition of dietary [10,26]. Considering the characteristics of the special environment and nutrient intake of the Tibet Plateau, we aimed to characterize the rumen microbiome and to ascertain the changes that occur when the supplement of concentrates was manipulated.

In the present study, 16S rRNA gene-based pyrosequencing data from the rumen of Tibetan sheep highlighted the predominance of

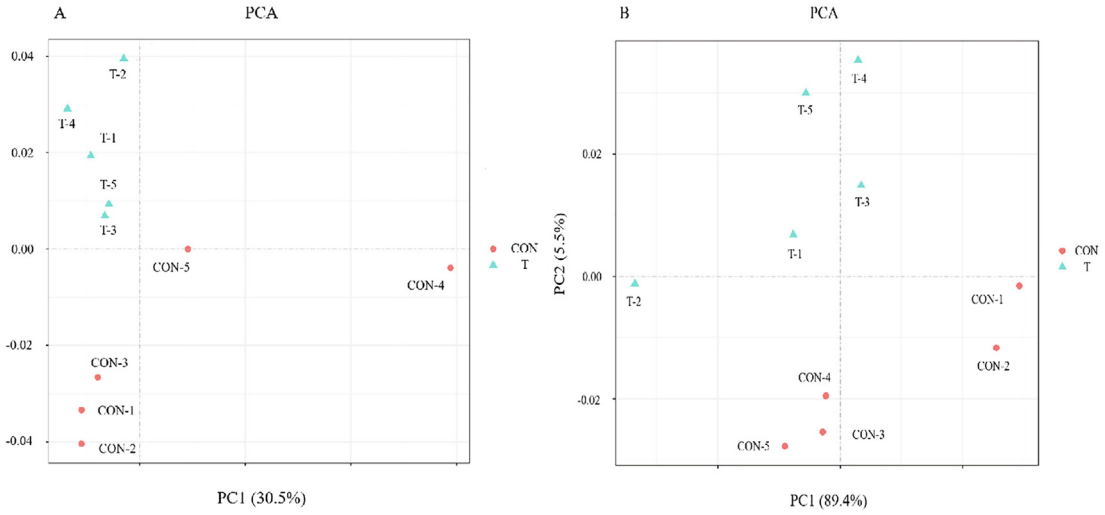

Fig. 4. Multivariate analysis based on the information at the genus level. A: PCA based on total OTU level information. B: PCA based on the genus level information.

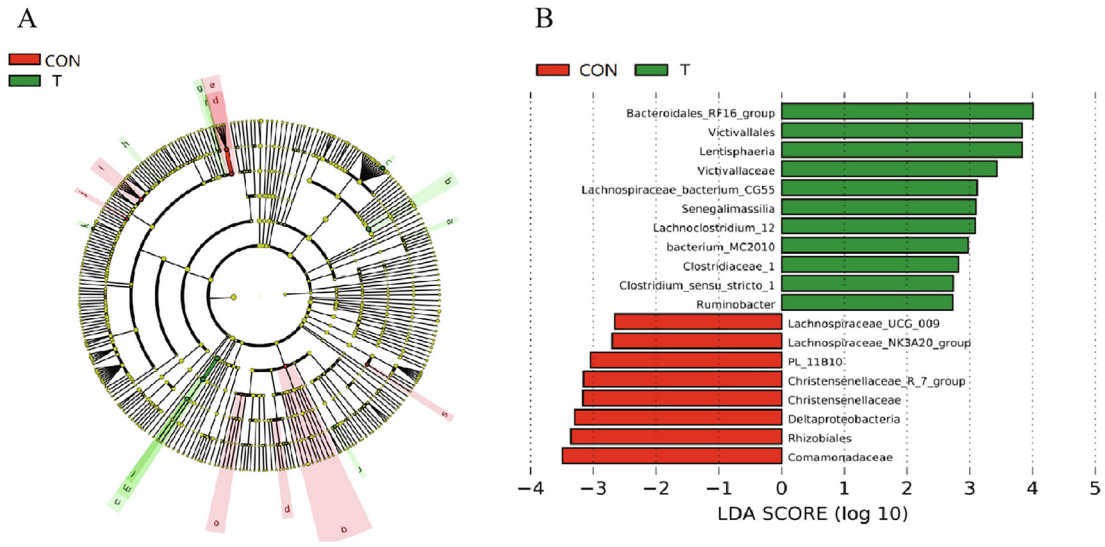

Fig. 5. A linear discriminant analysis effect size (LEfSe) method identifies the significantly different abundant taxa of bacteria. A. The taxa with significantly different abundances between the CON and T groups are represented by colored dots, yellow circles represent nonsignificant differences in abundance between the CON and T groups for that particular taxonomic group, and from the center to outward, they represent the kingdom, phylum, class, order, family, and genus levels. B: Only taxa meeting an LDA significance threshold of 2 are presented. a: Senegalimassilia, b: Bacteroidales RF16 group, c:Bacterium MC2010, d: Christensenellaceae R-7 group, e: Christensenellaceae, f: Clostridium sensu stricto 1, g: Clostridiaceae 1, h: Lachnoclostridium, i: Lachnospiraceae NK3A20 group, j: Lachnospiraceae UCG 009, k: Lachnospiraceae bacterium CG55, l: Victivallaceae, m: Victivallales, n: Lentisphaeria, o: Rhizobiales, p: Comamonadaceae, q: Deltaproteobacteria, r: Ruminobacter, s: PL-11B10. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

phyla gram-negative Bacteroidetes (56.38%) and gram-positive Firmicutes (14.87%), respectively. The results of bacterial composition were comparable to the previous rumen microbiome researches [27]. By targeting several polysaccharides, i.e., cellulose, pectin, and xylan, many of the Bacteroidetes have access to an amazing diversity of carbon sources from host-derived complex or plant glycans [28]. However, Bacteroidetes in the rumen were the preponderant microorganisms that caused carbohydrates degradation, thereby complementing host metabolism and developing the repertoire of enzymes [29]. Bacteroidetes colonized many different parts of the gastrointestinal tract and were more susceptible to the type and composition of feed [6]. Our present data reveal less dominance of Bacteroidetes with concentrate feed supplement in the dietary treatment compared to the CON group. These results were in agreement with Henderson et al. [30], in which a greater abundance of Bacteroidetes was observed in ruminants fed with a forage-based diet versus a concentrate diet [30]. Therefore, it might be reasonable speculation that the higher abundance of Bacteroidetes corresponding to the higher cellulosic plant constituent in the ratio probably came from the effect of substrate-induced diet.

Among the Firmicutes group, both Ruminococcaceae and Lachnospiraceae were dominant in our study similar to previous findings [31]. Although our data showed that diet had no effect on the family Firmicutes, it was noteworthy that the effect of dietary supplements was more pronounced on the abundance of Christensenellaceae (belonging to Firmicutes phylum). The Christensenellaceae family was one of the most inheritable taxa and initially described as bacteria that produce volatile fatty acids such as acetate and butyrate, and methanogenic archaea in ruminants [32]. Furthermore, the relationships with various bacteria promoted the function of the digestive system [33], as previously

demonstrated in humans [34] and pigs [35]. However, our results were in disagreement with Pitta et al., [28] in which a greater abundance of Christensenellaceae was observed in Kankrej cattle fed with a concentrate diet versus a forage-based diet [36]. Differences in the abundance of Christensenellaceae were largely explained as follows: (1) the analyzed breed had an insufficiently large population size, which might have negatively affected the statistical power. (2) interspecific difference (sheep vs cattle) and environmental deviation (temperature, altitude, and management style) could also be confounding factors in bacterial diversity determination.

5. Conclusions

This investigation confirmed that nutrient supplementation to Tibetan sheep effectively improved the yield and lactose of milk, and also increased the ruminal microbial abundance during the cold season. We propose that the nutrient supplementation of Tibetan sheep could be a beneficial strategy to offset the restriction of nutrition intake and to improve lamb survival in the harsh winter.

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Conflict of interest

The authors declare no conflict of interest.

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