Dietary supplements during the cold season increase rumen microbial abundance and improve rumen epithelium development in Tibetan sheep

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Livestock on the Qinghai-Tibetan Plateau are faced with extreme harsh winters and are often in negative energy balance during this period. Dietary supplementation can improve growth performance of Tibetan sheep and, consequently, we hypothesized that it would also increase microbial abundance and rumen epithelium development. To test this hypothesis, we examined the effect of feed supplementation during the cold season on rumen microbes, fermentation, epithelium development, and absorptive capability in Tibetan sheep. Eighteen 1-yr-old ewes (BW = 29.4 ± 1.79, kg) were offered oat hay ad libitum for 60 d and divided randomly into three groups: 1) no supplement; control group (**CON**); 2) urea-molasses lick block supplement (**BS**); and 3) concentrate feed supplement (**CS**). The ADG of CS ewes (143.3, g/d) was greater ($P < 0.05$) than BS ewes (87.9, g/d), which was greater ($P < 0.05$) than CON ewes (44.5, g/d). Serum concentrations of GH, IGF-1, and IGF-2 in the CS and BS groups were greater than in the CON group ($P < 0.05$). Greater relative abundance of protozoa, *Ruminococcus albus*, *Fibrobacter succinogenes*, *Streptococcus bovis*, and *Ruminobacter amylophilus* was observed in the CS and BS groups than in the CON group ($P < 0.05$), and relative abundances of rumen fungi, *Butyrivibrio fibrisolvens*, and *Prevotella ruminicola* in the CS group were greater than in the BS and CON groups ($P < 0.05$). Ruminal total VFA, ammonia, and microbial protein concentrations in the CS and BS groups were greater than in the CON group ($P < 0.05$), and in the CS group were greater than in the BS group ($P < 0.05$). Ruminal papillae width and surface area in the CS and BS groups were greater than in the CON group ($P < 0.05$), while in the CS group were greater than in the BS group ($P < 0.05$). The mRNA expressions of IGFBP5, NHE1 (sodium/hydrogen antiporter, isoform 1), DRA (downregulated in adenoma), and Na^+/K^+-ATPase (sodium/potassium ATPase pump) in ruminal epithelium were greater in the CS and BS groups than in the CON group ($P < 0.05$), and in the CS group was greater than in the BS group ($P < 0.05$), while NHE3 (sodium/hydrogen antiporter, isoform 3), MCT1 (monocarboxylate transporter 1), and MCT4 (monocarboxylate transporter 4) mRNA expressions in the CS group were greater than in the BS and CON groups ($P < 0.05$). It was concluded that supplementing Tibetan sheep during the cold season increases rumen microbial abundance and improves fermentation parameters, rumen epithelium development, and absorptive capability.

Key words: absorptive capability, rumen morphology, ruminal microbes, supplementation in cold season, Tibetan sheep

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INTRODUCTION

Tibetan sheep (*Ovies aries*), numbering approximately 50 million animals, are well-adapted to the conditions of the Qinghai-Tibetan Plateau (Zhou et al., 2015) and play a vital role in the livelihoods of Tibetan pastoralists (Li et al., 2015). Under traditional management, Tibetan sheep graze on rangeland all year-round without receiving feed supplements. The large increase in livestock numbers over the years has led to overgrazing of the pasture and degradation of the grasslands (Shang et al., 2014). During the long cold season, there generally is a severe shortage of herbage, and Tibetan sheep often lose BW (Dong et al., 2003; Xin et al., 2011; Sun et al., 2015). Severe snowfalls can prevent the livestock from grazing and can cause the death of large numbers of animals (Shang et al., 2012). Supplementary feed can improve the reproductive capability of Tibetan sheep (Jing et al., 2017) and can also improve their ADG or reduce their weight loss during the harsh winter (Liu et al., 2007; Feng et al., 2013; Jing et al., 2017). Dietary nutrients intakes not only affect animal performance, but also affect the rumen microbial community, epithelial development, and absorptive capabilities of the rumen in ruminants (Shen et al., 2004; Kamra, 2005; Pitta et al., 2010). However, little is known of how feed supplementation during the cold season affects the rumen microbial community and rumen epithelium development in Tibetan sheep. We hypothesize that feed supplementation to Tibetan sheep during the cold season would improve their rumen microbial community structure and rumen epithelial development, and, consequently, increase the ruminal fermentative and absorptive capabilities. To test this hypothesis, Tibetan sheep were offered oat hay with or without supplementary feed during the cold season, and BW change, the abundance of ruminal microbes, rumen fermentation, rumen epithelium morphology, and absorptive capability were examined.

MATERIALS AND METHODS

The experimental procedures in this research were approved by the Institutional Animal Care Unit of Sichuan Agricultural University.

Study Location

The experiment was conducted at the Haibei Demonstration Zone of the Plateau Modern Ecological Husbandry Science and Technology (6°44′–7°39′N, 100°23′–101°20′E; 3,200 m a.s.l.), Xihai Town, Haiyan County, Haibei Tibetan Autonomous Prefecture of Qinghai Province, China. Traditional husbandry is practiced in this area in which the livestock, mainly Tibetan sheep and yaks, only graze the grasslands. In this area, overgrazing over an extended period resulted in rangeland degradation, a process which has been increasing in recent years. The site is characterized by extreme harsh, cold conditions, with a semiarid plateau monsoon climate, having an average annual precipitation of about 400 mm and annual sunshine of about 2,980 h. During the experiment, the highest and lowest air temperatures in the sheep shelter were 12 °C and −25 °C, respectively.

Animals and Experimental Design

Eighteen healthy, 1-yr-old Tibetan ewes (29.4 ± 1.79, kg) were purchased from a Tibetan herder in the surrounding area of Haibei Demonstration Zone of Plateau Modern Ecological Husbandry Science and Technology. Before this study, the sheep grazed only natural pasture and were not offered supplements. The sheep were assigned randomly to one of three groups and were offered oat hay and water ad libitum throughout the experiment. The first group did not receive supplements and served as controls (**CON**); the second group was supplemented with a urea-molasses lick block (Table 1) ad libitum (**BS**); and the third group was supplemented with 200 g/d concentrate (Table 1) per sheep (**CS**). The composition of the oat hay, urea-molasses lick block, and concentrate is presented in Table 1. The experiment took place over 60 d (February 28, 2014 to April 28, 2014) after 15 d of adaptation to the experimental diets and conditions.

Animals Management

Each sheep was maintained in an individual pen (2 m × 4 m), equipped with a water tank and feed trough. The pens were under a three-sided roofed shelter, and thus the sheep were always exposed to outside temperatures. The sheep were fed twice daily, at 0800 and at 1600, and oat hay intake was recorded each day. The lick blocks were weighed every week to calculate the average daily intake. Concentrate feed (200, g/ewe) was offered the CS sheep at 0800 h each day. Sheep were weighed on d 0, 30, and 60 before feeding and ADG was calculated.

Hormones Determination

Ten milliliters of jugular blood were collected in glass tubes (Shanghai Kehua Bio-Engineering

Table 1. Composition of oat hay, urea-molasses lick block, and concentrate (DM basis)

Item	Lick block	Concentrate	Oat hay
Ingredient, % of DM			
Corn	15.7	55.6	-
Wheat bran	6.50	17.5	-
Rapeseed dregs	5.00	16.6	-
Soybean meal	0	5.20	-
Molasses	13.5	0	-
Urea	8.00	0	-
Rapeseed oil	0	1.00	-
Sodium chloride	17.0	1.00	-
Calcium carbonate	2.50	0.6	-
Calcium hydrophosphate	1.00	0.4	-
Sodium thiosulfate	1.00	0	-
Sodium bicarbonate	5.00	1.00	-
Compound vitamin	0.90		-
Monensin	0.50	0.1	-
Commercial premix[a]	1.00	1.00	-
Bentonite	8.40	0	-
Portland cement	14.0	0	-
Composition, % of DM			
DE[b], MJ/kg	7.24	14.05	10.4
CP, g/kg	325	171	100
ADF, g/kg	20.3	74.8	390
NDF, g/kg	46.7	167	630

[a]Commercial premix of lick block provided the following per kg of diets: Fe 524 mg; Cu 275 mg; Mn 1,288 mg; Zn 839 mg; I 10.3 mg; Se 1.10 mg; Co 2.20 mg; commercial premix of concentrate provided the following per kg of diets: Vitamin A 1700 IU; Vitamin D 190 IU; Vitamin E 18 IU; Fe 90 mg; Cu 20 mg; Mn 42 mg; Zn 48 mg; I 1.20 mg; Se 0.16 mg; Co 0.15 mg.

[b]DE = digestible energy, was a calculated value from the Feeding Standard of Meat-producing Sheep and Goats of China, NY/T 816-2004 (Ministry of Agriculture, MOA, PRC, 2004).

Co., Ltd, Shanghai, China) before feeding in the morning on d 0, 30, and 60. The tubes were maintained in a slanted position for 15 min, followed by centrifugation at 2,000 × *g* for 10 min at 4 °C. Serum samples were stored in 1.5 mL tubes (Eppendorf, GCS, New York, NY) at −80 °C till analysis. Concentrations of serum GH, IGF-1, and IGF-2 were determined by commercial ELISA kits (Shanghai Lengton Bioscience Co., Ltd, Shanghai, China).

Rumen Morphological Examination

At the end of the trial (on d 61), all sheep were slaughtered humanely 2 to 3 h after morning feeding (Metzler-Zebeli et al., 2013) and ruminal dorsal sac samples (2 × 2 cm) were collected immediately from each sheep. Samples were rinsed with physiological saline, then fixed in 4% (vol/vol) formalin solution, dehydrated with absolute ethanol (10%, 20%, 30%, 50%, 70%, 85%, 95%, and 100%), cleared with xylene (25%, 50%, 75%, and 100%, prepared with ethanol) twice, and saturated with and embedded in paraffin (Shen et al., 2004; Wang et al. 2009). The blocks were cut into 5-μm sections using a rotary microtome (RM2235, Leica, Germany) and the sections (four slices of each sample) were stained by hematoxylin and eosin (H&E). Ten images per slice in random fields were examined using a Nikon microscope (Eclipse E400, Tokyo, Japan). Average rumen papillae height, width, and area in each image were determined by Image-Pro Plus 6.0 software and the number of papillae in 1 cm^2 was counted, according to the method of Wang et al. (2009) and Melo et al. (2013).

Rumen Fermentation Parameters

Rumen fluids were collected immediately after slaughter and filtered through four layers of cheesecloth (Hristov et al., 2001; Bailey et al., 2012). The pH was measured immediately using a pH electrode (Model PHS-3C, Shanghai Precision & Scientific Instrument Co., Ltd, Shanghai, China). Rumen fluid samples of 10 mL were snap-frozen in liquid nitrogen and then stored at −80 °C for subsequent analysis. For analysis of VFA concentrations, ruminal fluid samples were thawed and centrifuged at 20,000 × *g* for 10 min (Hristov et al., 2001), and then analyzed using a Varian CP-3800 gas chromatograph

(Varian Inc., Palo Alto, CA). Samples were injected into a CP-FFAP capillary column (25 m × 0.32 mm i.d. × 0.3 μm film thickness, Varian Inc., Palo Alto, CA) and were run at a split vent flow of 40 mL/min, air flow of 450 mL/min, make-up gas flow of 35 mL/min, with a capillary column temperature of 100 °C for 1 min, and then increased to 190 °C at a rate of 20 °C/min and held for 3 min at 190 °C. The injection port temperature was 220 °C, and the flame ionization detector temperature was 250 °C. Crotonic acid was used as internal standard. Peak integration was determined using Galaxie Software (Varian Inc., Palo Alto, CA). The ammonia and microbial protein (**MCP**) content were quantified by commercial kits (Nanjing Jian Cheng Bioengineering Institute, China).

DNA Extraction and Abundance of Rumen Microbes

Rumen fluid samples were slow thawed at 4 °C, centrifuged at 500 × *g* for 15 min at 4 °C to remove feed particles while keeping bacterial cells, then the total genomic DNA was extracted using the TIANamp Bacterial DNA Kit (TIANGEN BIOTECH, Beijing, Co., Ltd, Beijing, China) according to the manufacturer's protocol. DNA concentration was estimated by spectrophotometry at a wavelength of 260 and 280 nm. The quality of RNA was also checked by 1.0% agarose gel electrophoresis. The DNA was amplified by real-time PCR using a SYBR Green real-time PCR master mix kit (Takara Biotechnology Co., Ltd, Dalian, China) with the BIO-RAD CFX96 (Hercules, CA) in a total volume of 12.5 μL. The PCR plate was incubated at 95 °C for 2 min, followed by 39 cycles of 95 °C for 10 s, annealing at a temperature of each primer for 30 s and 72 °C for 30 s. Total microbial DNA was diluted to 1:10 before use in real-time PCR assays. The primers of total bacterial, total fungi, total protozoa, *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Fibrobacter succinogenes*, *Butyrivibrio fibrisolvens*, *Streptococcus bovis*, *Prevotella ruminicola*, *Ruminobacter amylophilus* are shown in Table 2. The oligonucleotides were synthesized by Takara Biotechnology Co., Ltd, Dalian, China. Quantification for bacteria was expressed as a proportion of total rumen bacterial 16S rDNA according to the equation: relative quantification = $2^{-(Ct\ \text{target} - Ct\ \text{total bacterial})}$, where C_t represents threshold cycle (Chen et al., 2008).

RNA Extraction and mRNA Expression Determination

Immediately after slaughter, epithelium samples of the ruminal dorsal sacs were collected,

Table 2. Primer sequences and parameters

Target	Primer sequences (5′→3′)	Products size, bp	T_a, °C	Reference
Total bacterial	F[b]: CGGCAACGAGCGCAACCC R[c]: CCATTGTAGCACGTGTGTAGCC	130	60.0	Denman and Mcsweeney (2006)
Total fungi	F: GCACTTCATTGTGTGTACTG R: GGATGAAACTCGTTGACTTC	120	60.0	Denman and Mcsweeney (2006)
Total protozoa	F: GCTTTCGWTGGTAGTGTATT R: CTTGCCCTCYAATCGTWCT	223	55.0	Sylvester et al. (2004)
Ruminococcus albus	F: CCCTAAAAGCAGTCTTAGTTCG R: CCTCCTTGCGGTTAGAACA	176	55.0	Koike and Kobayashi (2001)
Ruminococcus flavefaciens	F: CGAACGGAGATAATTTGAGTTTACTTAGG R: CGGTCTCTGTATGTTATGAGGTATTACC	132	60.0	Denman and Mcsweeney (2006)
Fibrobacter succinogenes	F: GTTCGGAATTACTGGGCGTAAA R: CGCCTGCCCCTGAACTATC	121	60.0	Denman and Mcsweeney (2006)
Butyrivibrio fibrisolvens	F: TCTGGAAACGGATGGTA R: CCTTTAAGACAGGAGTTTACAA	295	55.0	Koike and Kobayashi (2001)
Streptococcus bovis	F: TTCCTAGAGATAGGAAGTTTCTTCGG R: ATGATGGCAACTAACAATAGGGGT	127	60.0	Stevenson and Weimer (2007)
Prevotella ruminicola	F: GGTTATCTTGAGTGAGTT R: CTGATGGCAACTAAAGAA	485	53.0	Tajima et al. (2001)
Ruminobacter amylophilus	F: CAACCAGTCGCATTCAGA R: CACTACTCATGGCAACAT	642	57.0	Tajima et al. (2001)

[a] T_a = optimal PCR annealing temperature.
[b] F = forward.
[c] R = reverse.

repeatedly rinsed with physiological saline, cut into small pieces, placed into 1.5 mL tubes (Eppendorf, GCS, New York, NY), snap-frozen in liquid nitrogen, and stored at −80 °C till RNA extraction. Total RNA was extracted using Trizol reagent (Takara Biotechnology Co., Ltd, Dalian, China) according to the manufacturer's protocol, and measured spectrophotometrically at 260 and 280 nm. The ratio of light absorbance at 260 nm to that at 280 nm was between 1.8 and 2.0, indicating that samples were pure and clean. The quality of RNA was also checked by 1.0% agarose gel electrophoresis. RNA was extracted for reverse transcription reaction by Prime Script RT Reagent Kit (Takara Biotechnology Co., Ltd, Dalian, China) according to the manufacturer's protocol. The resulting cDNA was diluted to 100 µL with RNase-free water and stored at −20 °C. The cDNA was amplified by real-time PCR using a SYBR Green real-time PCR master mix kit (Takara Biotechnology Co., Ltd, Dalian, China) with the BIO-RAD CFX96 (Hercules, CA) in a total volume of 12.5 µL. The PCR plate was incubated at 95 °C for 2 min, followed by 39 cycles of 95 °C for 10 s, annealing at a temperature of each primer for 30 s and 72 °C for 30 s. The IGFBP5, NHE1 (sodium/hydrogen antiporter, isoform 1), NHE3 (sodium/hydrogen antiporter, isoform 3), MCT1 (monocarboxylate transporter 1), MCT4 (monocarboxylate transporter 4), DRA (downregulated in adenoma), Na^+/K^+-ATPase (sodium/potassium ATPase pump), and β-actin primers were designed using Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, CA) (Table 3). The oligonucleotides were synthesized by Takara Biotechnology Co., Ltd, Dalian, China. The melting peaks of all samples were determined routinely by melting curve analysis to ascertain that only the expected products had been generated. Relative gene expression levels are presented as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

Statistical Analysis

Data were analyzed with the Statistical Package for Social Science (SPSS, version 22.0, SPSS Inc., Chicago, IL). Single factor ANOVA was used to analyze the data, and Duncan's multiple test was used to separate means where significant differences were found. A level of $P < 0.05$ was considered statistically significant.

RESULTS

Daily Intakes and BW Change

CP and DE intakes of the CS and BS groups were greater ($P < 0.05$) than the CON group, and the CS group was greater ($P < 0.05$) than the BS group (Table 4). The BW of the CS group was greater ($P < 0.05$) than both the CON and BS groups on d 60. The ADG of the CS group (143.3,

Table 3. Primers of fluorescent quantitation PCR

Target gene[a]	GenBank ID	Primer sequences (5′→3′)	Products size, bp	T_a[b], °C
IGFBP5	NM-001129733.1	F[c]: CTGTGACCGCAAAGGGTTCTA R[d]: AACATTGCTGCTGTCAAAGGTG	160	56.0
DRA	HM010763.1	F: TCGTTGCCATCGGATTTCTCTT R: CGCCACAATCTTCGTATTTCCAC	115	53.0
MCT1	AJ315929.1	F: TGGCTGTCATGTACGGTGGA R: GCACAGTGTTACAGAAGGAAGCAG	132	54.0
MCT4	XM-012186382.1	F: TGGTGTCTGCGTCCTTCTGTG R: GATGAGTGAGGGCTGGAAGTTG	101	56.0
NHE1	XM-004005085.2	F: GCCGTCACTGTGGTCCTGTATC R: ACACCACGAAGAAGCTCAGGAA	103	55.0
NHE3	XM-012097233.1	F: GAAACAGCAGCATTCCCAACG R: CCGCCACTCATCTCATCATCATA	130	55.0
Na^+/K^+-ATPase	NM-001009360.1	F: GAGAACGGCTTCCTCCCTAATC R: TGTTCATAGGTCCACTGCTGCC	107	56.0
β-actin	U39357.1	F: ATCGGCAATGAGCGGTTCC R: GCGTAGAGGTCTTTGCGGATGT	137	56.0

[a]DRA = downregulated in adenoma; MCT1 = monocarboxylate transporter 1; MCT4 = monocarboxylate transporter 4; NHE1 = sodium/hydrogen antiporter, isoform 1; NHE3 = sodium/hydrogen antiporter, isoform 3; Na^+/K^+-ATPase = sodium/potassium ATPase pump.

[b]T_a = optimal PCR annealing temperature.

[c]F = forward.

[d]R = reverse.

g/d) was greater ($P < 0.05$) than the BS group (87.9, g/d) which was greater ($P < 0.05$) than the CON group (44.5, g/d) (Table 5).

Serum Hormone Concentrations

The CS and BS groups had greater ($P < 0.05$) concentrations of GH, IGF-1, and IGF-2 than the CON group, on d 30 and d 60 (Table 6). In addition, the IGF-2 concentration in the CS group was greater ($P < 0.05$) than in the BS group on 60 d.

The Abundance of Ruminal Microbes

Greater relative abundances of protozoa, *R. albus*, *F. succinogenes*, *S. bovis*, and

Table 4. The average daily intakes of Tibetan sheep

	Treatments[a]				
Item	CON	BS	CS	SEM	*P*-value
Daily intake of diets[b]					
Oat hay, kg	1.09	1.12	0.98	0.039	0.169
Block, g		19.8			
Concentrate, g			185		
Daily intake of chemical composition[c]					
DM, kg/d	0.96	1.07	1.17	0.041	0.112
DE[d], MJ/d	9.94[c]	11.07[b]	12.89[a]	0.390	0.001
CP, kg/d	0.10[c]	0.12[b]	0.14[a]	0.011	0.007
ADF, kg/d	0.42	0.43	0.40	0.020	0.357
NDF, kg/d	0.68	0.70	0.65	0.021	0.453

[a,b,c]Means in same row with different superscripts are different from each other ($P < 0.05$).

[a]Treatments: CON = control (no supplements); BS = supplemented with urea-molasses lick block; CS = supplemented with concentrate.

[b]Daily intake of diets were expressed as DM basis.

[c]Daily intake of chemical composition of DE, CP, ADF, and NDF were expressed as DM basis.

[d]DE = digestible energy, was a calculated value from the Feeding Standard of Meat-producing Sheep and Goats of China, NY/T 816-2004 (Ministry of Agriculture, MOA, PRC, 2004).

Table 5. The growth performance of Tibetan sheep supplemented with urea-molasses lick block or concentrate in the cold season

	Treatments[a]				
Item	CON	BS	CS	SEM	*P*-value
Initial BW, kg	29.4	29.6	29.2	0.40	0.938
Final BW, kg	32.7[b]	34.7[b]	37.6[a]	0.55	0.001
ADG, g/d	44.5[c]	87.9[b]	143.3[a]	14.74	<0.0001

[a,b,c]Means in same row with different superscripts are different from each other ($P < 0.05$).

[a]Treatments: CON = control (no supplements); BS = supplemented with urea-molasses lick block; CS = supplemented with concentrate.

Table 6. The serum GH, IGF-1, and IGF-2 concentrations in Tibetan sheep supplemented with urea-molasses lick block or concentrate in the cold season

		Treatments[a]				
Item	Day	CON	BS	CS	SEM	*P*-value
GH, ng/L	0	316	312	315	3.42	0.916
	30	264[b]	337[a]	321[a]	11.63	0.001
	60	138[b]	368[a]	355[a]	37.38	<0.0001
IGF-1, ng/mL	0	125	121	126	2.27	0.680
	30	93[b]	162[a]	155[a]	11.28	<0.0001
	60	72[b]	168[a]	182[a]	17.46	<0.0001
IGF-2, ng/mL	0	65	64	65	0.89	0.984
	30	44[b]	74[a]	78[a]	5.44	<0.0001
	60	34[c]	78[b]	95[a]	9.13	<0.0001

[a,b,c]Means in same row with different superscripts are different from each other ($P < 0.05$).

[a]Treatments: CON = control (no supplements); BS = supplemented with urea-molasses lick block; CS = supplemented with concentrate.

R. amylophilus were observed in the CS and BS groups than in the CON group ($P < 0.05$); *R. albus*, *S. bovis*, and *R. amylophilus* in the CS group were greater ($P < 0.05$) than in the BS group, and protozoa in the BS group was greater ($P < 0.05$) than in the CS group (Table 7). Relative abundances of ruminal fungi, *B. fibrisolvens*, and *P. ruminicola* in the CS group were greater ($P < 0.05$) than in the BS and CON groups. The BS group had the greatest ($P < 0.05$) relative abundance of *R. flavefaciens* among groups.

Rumen Fermentation Parameters

Ruminal pH of the CS group was lowest ($P < 0.05$) among groups, but there was no difference between the BS and CON groups (Table 8). The CS and BS groups had greater ($P < 0.05$) concentrations of propionate, butyrate, total VFA, ammonia, and MCP than the CON group, and the CS group had greater ($P < 0.05$) concentrations than the BS group. The ratios of acetate/propionate in the CS and BS groups were lower ($P < 0.05$) than in the CON group, and the ratio in the CS group was lower ($P < 0.05$) than in the BS group.

Ruminal Morphology

Morphology analysis of ruminal tissue is summarized in Table 9. Width and surface area of ruminal papillae in the CS and BS groups were greater ($P < 0.05$) than in the CON group, and greater ($P < 0.05$) in the CS group than in the BS group. In addition, the height of ruminal papillae in the CS group was greater ($P < 0.05$) than in the CON group. Representative micrographs are presented in Figure 1.

The Gene Expression of Ruminal Epithelium

The mRNA relative expression levels of IGFBP5 were greater ($P < 0.05$) in the CS and BS groups than

Table 7. The relative abundance of ruminal microbes in Tibetan sheep supplemented with urea-molasses lick block or concentrate in cold season.

	Treatments[a]				
Item	CON	BS	CS	SEM	*P*-value
Rumen fungi	0.0301[b]	0.0257[b]	0.0529[a]	0.0045	<0.0001
Protozoa	0.7781[c]	1.7596[a]	1.4387[b]	0.1512	0.001
Ruminococcus albus	0.0041[c]	0.0054[b]	0.0069[a]	0.0004	0.001
Ruminococcus flavefaciens	0.0157[b]	0.0240[a]	0.0123[b]	0.0018	0.001
Fibrobacter succinogenes	0.0198[b]	0.2683[a]	0.3085[a]	0.0456	<0.0001
Butyrivibrio fibrisolvens	0.5698[b]	0.6282[b]	1.3083[a]	0.1233	<0.0001
Streptococcus bovis	0.1040[c]	0.5379[b]	1.5213[a]	0.2129	<0.0001
Prevotella ruminicola	0.0269[b]	0.0337[b]	2.2248[a]	0.3671	<0.0001
Ruminobacter amylophilus	0.5398[c]	1.8825[b]	2.3032[a]	0.2685	<0.0001

[a,b,c]Means in same row with different superscripts are different from each other ($P < 0.05$).

[a]Treatments: CON = control (no supplements); BS = supplemented with urea-molasses lick block; CS = supplemented with concentrate.

Table 8. The rumen fermentation parameters in Tibetan sheep supplemented with urea-molasses lick block or concentrate in the cold season

	Treatments[a]				
Item	CON	BS	CS	SEM	*P*-value
pH	6.94[a]	6.80[a]	6.55[b]	0.060	0.004
Acetate, mmol/L	58. 0	61.8	60.6	1.440	0.615
Propionate, mmol/L	12.4[c]	16.1[b]	27.6[a]	2.312	<0.0001
Butyrate, mmol/L	6.23[c]	8.59[b]	9.84[a]	0.551	<0.0001
Total VFA, mmol/L	76.7[c]	86.5[b]	98.0[a]	3.293	<0.0001
Acetate:propionate	4.72[a]	3.85[b]	2.20[c]	0.342	<0.0001
Ammonia, mmol/L	3.39[c]	5.29[b]	8.43[a]	0.761	<0.0001
MCP[b], mg/100 mL	116.3[c]	136.0[b]	163.5[a]	7.651	0.002

[a,b,c]Means in same row with different superscripts are different from each other ($P < 0.05$).

[a]Treatments: CON = control (no supplements); BS = supplemented with urea-molasses lick block; CS = supplemented with concentrate.

[b]MCP = microbial proteins.

Table 9. The rumen morphology in Tibetan sheep supplemented with urea-molasses lick block or concentrate in the cold season

	Treatments[a]				
Item	CON	BS	CS	SEM	*P*-value
Papillae density, n/cm^2	59.0	65.0	72.4	7.84	0.120
Papillae height, μm	1503.4^b	1587.3^b	1715.8^a	23.77	0.002
Papillae width, μm	338.9^c	376.1^b	432.8^a	9.65	<0.0001
Papillae surface, μm^2	455,780^c	547,542^b	636,063^a	22,643.8	<0.0001

[a,b,c]Means in same row with different superscripts are different from each other ($P < 0.05$).

[a]Treatments: CON = control (no supplements); BS = supplemented with urea-molasses lick block; CS = supplemented with concentrate.

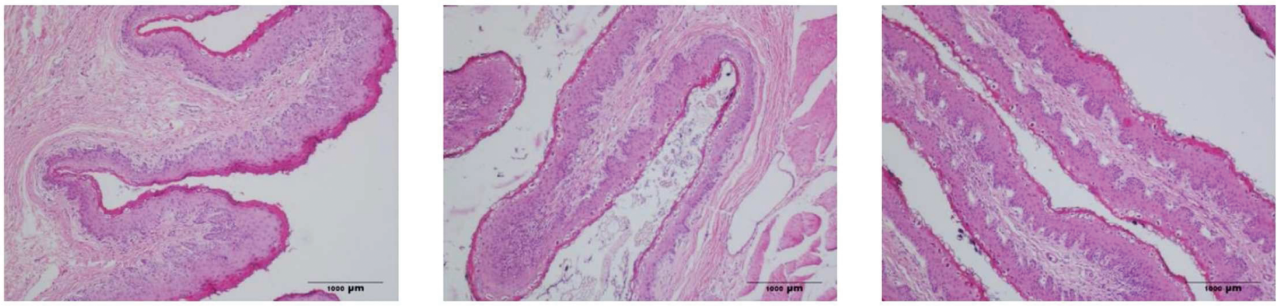

Figure 1. Representative micrographs of H&E staining in the ruminal papillae of Tibetan sheep (10×). CON = controls (no supplements); BS = supplemented with urea-molasses lick block; CS = supplemented with concentrate.

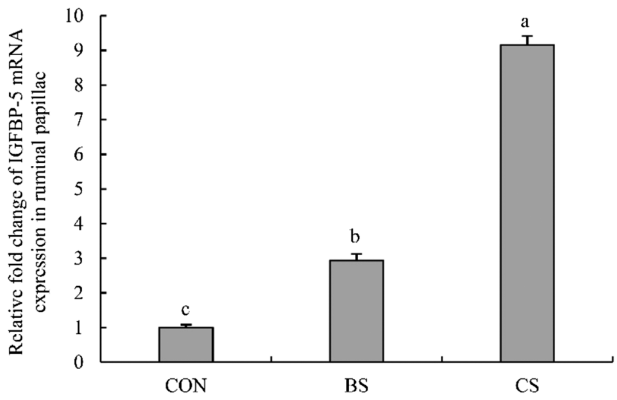

Figure 2. The relative expression of IGFBP5 mRNA in ruminal papillae of Tibetan sheep supplemented with urea-molasses lick block or concentrate in the cold season. CON = controls (no supplements); BS = supplemented with urea-molasses lick block; CS = supplemented with concentrate. Different letters indicate a statistically significant difference at $P < 0.05$.

in the CON group, and in the CS group ($P < 0.05$) than in the BS group (Figure 2). Similarly, the NHE1, DRA, and Na^+/K^+-ATPase mRNA expressions in ruminal epithelium were greater ($P < 0.05$) in the CS and BS groups than in the CON group, and in the CS group ($P < 0.05$) than in the BS group (Figure 3). The NHE3, MCT1, and MCT4 mRNA expressions in the CS group were greater ($P < 0.05$) than in the BS and CON groups, but no difference was found between the BS and CON groups.

DISCUSSION

Rumen Microbial Abundance and Rumen Fermentation

Rumen microbial community structure and diversity are highly responsive to dietary nutrients, and increase in dietary nonstructural carbohydrate content (Kocherginskaya et al., 2001; Tajima et al., 2001; Kamra, 2005; Pitta et al., 2010). Corn-fed animals displayed more diverse and rich bacterial populations than did hay-fed animals (Kocherginskaya et al., 2001), and forage-fed cattle, supplemented with sucrose, increased rumen microbial growth (Kennedy, 1980). In the present study, greater relative abundances of protozoa, *R. albus*, *F. succinogenes*, *S. bovis*, and *R. amylophilus* were observed in the CS and BS groups than in the CON group, and rumen fungi, *B. fibrisolvens* and *P. ruminicola* in the CS group than in the BS and CON groups. Supplementations in the CS and BS groups increased substrates for rumen microbial fermentation, which promoted growth and proliferation of rumen microbes. The concentrate supplementation contained more starch and real protein than the urea-molasses lick block, and provided more protein and energy. Accordingly, the CS group had a greater relative abundance of rumen fungi, *Butyrivibrio fibrisovens*, and *P. ruminicola*, the

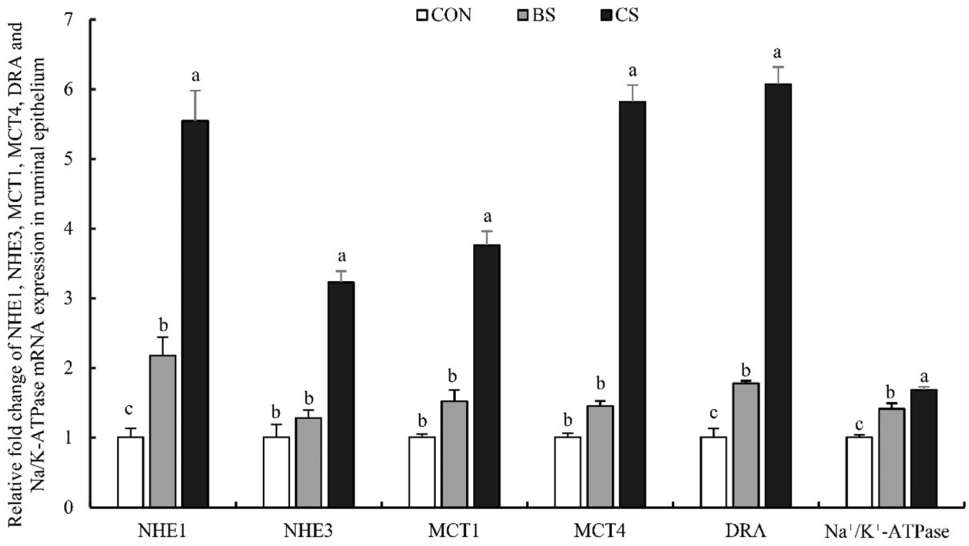

Figure 3. The relative expression of NHE1, NHE3, MCT1, MCT4, DRA, and Na^+/K^+-ATPase mRNA in ruminal papillae of Tibetan sheep supplemented with urea-molasses lick block or concentrate in cold season. CON = controls (no supplements); BS = supplemented with urea-molasses lick block; CS = supplemented with concentrate. DRA = downregulated in adenoma; MCT1 = monocarboxylate transporter 1; MCT4 = monocarboxylate transporter 4; NHE1 = sodium/hydrogen antiporter, isoform 1; NHE3 = sodium/hydrogen antiporter, isoform 3; Na^+/K^+-ATPase = sodium/potassium ATPase pump. Different letters indicate a statistically significant difference at $P < 0.05$.

main bacterial species for the catabolism of starch, protein, and peptides (Cotta, 1992; Attwood and Reilly, 1995). A greater relative abundance of protozoa was observed in the BS group than in the CS group. Protozoa can ferment soluble sugars very rapidly, as they possess a powerful glycolytic mechanism (Heald and Oxford, 1953; Denton et al., 2015; Teixeira et al., 2017). The urea-molasses lick block supplementation contained more molasses than the concentrate, and provided more soluble sugars to protozoa. It increased substrates for protozoa fermentation, which promoted their growth and multiplication.

VFA, consisting primarily of acetate, propionate, and butyrate are the most important end products of ruminal fermentation. Concentrate feed and urea-molasses lick block supplementation increased propionate, butyrate, and total VFA concentrations, and reduced acetate/propionate ratio, which is in agreement with previous studies (Getachew et al., 2004; Shen et al., 2004; Suárez et al., 2006) that reported diets rich in nonstructural carbohydrates increased propionate and butyrate production. *Streptococcus bovis* and *R. amylophilus* were found to be major nonstructural carbohydrate degradable bacterial species (Dehority, 1991; Cotta, 1992) and, accordingly, were greater in the supplemented groups than in the CON group. The greater VFA concentration in the CS group than in the other two groups resulted in the lowest pH in this group, as ruminal pH is maintained by a balance between acid production and its removal (Allen, 1997).

Ammonia, an important N source for MCP synthesis, is produced by rumen microbial fermentation and degradation of protein or urea in the rumen. In the present study, the supplemented groups consumed more CP than the CON group, which lead to greater ammonia concentrations in the supplemented groups. Ammonia and MCP increased with CP intakes, which was consistent with the finding of Bandyk et al. (2001).

Ruminal Morphology and Absorptive Capability

The rumen epithelium is essential for the absorption of fermentation end products as 50% to 85% of VFA is absorbed directly across the rumen epithelium. Absorption pathways by passive diffusion of undissociated VFA and by facilitated diffusion of dissociated VFA via carrier-mediated transport proteins across the rumen epithelium have been described (Kirat et al., 2006; Graham et al., 2007; Connor et al., 2010; Aschenbach et al., 2011). Consequently, the absorption rate of VFA by rumen epithelium is highly dependent on the papillae surface area and the availability of transport proteins (Bannink et al., 2008; Yang et al., 2012; Melo et al., 2013). In studies on goats and sheep, the width and surface area of ruminal papillae increased when energy-rich concentrate rations were offered (Shen et al., 2004; Odongo et al., 2006; Wang et al., 2009). In the present study, the ruminal papillae width and surface area were greater in the supplemented groups than in the CON group, which was consistent with their greater intakes

of energy and protein. In addition, the fermentation end products of VFA were greater in the supplemented groups. The larger surface area of the ruminal papillae in the supplemented groups when compared to controls increased the absorption capacity and, ultimately, allowed for a faster absorption rate of VFA in the supplemented groups than in the controls (Bannink et al., 2008; Melo et al., 2013).

The VFA, in particular butyrate, stimulate the growth and development of ruminal epithelium (Sander et al., 1959; Sakata and Tamate, 1978; Shen et al., 2005). Malhi et al. (2013) reported that transient increases in cyclin D1 transcription contribute to butyrate-induced papillae growth and subsequently to increased absorption of VFA by the ruminal epithelium and Mentschel et al. (2001) found that butyrate stimulated ruminal papillae development. The CS and BS groups had greater concentrations of butyrate than the CON group, which could explain the greater ruminal papillae growth and surface area in these groups when compared to controls. In addition, Shen et al. (2004) observed that an energy-rich diet altered rumen morphology and function, which was associated with changes in systemic IGF-1. In the present study, on d 30 and d 60, the CS and BS groups exhibited greater concentrations of serum GH, IGF-1, and IGF-2 than the CON group. Firth and Baxter (2002) reported that the cellular events of IGF-1 stimulated rumen morphology development, and that it was modulated by IGFBP. In the present study, the mRNA expression of IGFBP5 was upregulated in the ruminal papillae in the CS and BS group when compared with the CON group, as also occurred in cattle during the transition to a high-grain diet (Steele et al., 2011; Steele et al., 2012).

Monocarboxylate transporter 1 (MCT1) and monocarboxylate transporter 4 (MCT4) are transporters for dissociated VFA across the ruminal epithelium (Müller et al., 2002; Graham et al., 2007; Kirat et al., 2007). In addition, VFA^-/HCO_3^- exchange has been identified as a pathway for absorption of dissociated VFA (Bilk et al., 2005; Aschenbach et al., 2009). Moreover, downregulated in adenoma (DRA) participates in the transport of HCO_3^- and is responsible for movement of HCO_3^- from the rumen epithelium to the ruminal lumen for neutralizing acid and importing dissociated VFA (Bilk et al., 2005). Müller et al. (2002) reported that absorption of VFA across the ruminal epithelium is associated with a decreased intracellular pH; other studies demonstrated that exporting protons back into the lumen via Na^+/H^+ exchange (NHE) (Müller et al., 2000; Gäbel et al., 2002; Graham et al., 2007), energized by Na^+/K^+-ATPase (Aschenbach et al., 2011), plays a significant role in intracellular pH regulation. MCT, DRA, NHE, and Na^+/K^+-ATPase act in harmony to promote the absorption of VFA in the rumen epithelium. In the present study, relative expression levels of NHE1, DRA, and Na^+/K^+-ATPase mRNA of ruminal epithelium in the CS and BS groups were greater than in the CON group, and in the CS group were greater than in the BS group. Furthermore, the relative expression levels of NHE3, MCT1, and MCT4 mRNA in the CS group were greater than in the BS and CON groups. These results indicated that the supplements increased VFA absorption capacity of the ruminal epitheliums in the CS and BS groups, more so in the CS group than in the BS group. Yang et al. (2012) reported that NHE1 and NHE3 mRNA expression levels in the rumen epithelium were enhanced by a concentrate diet when compared with a forage diet, and observed positive correlations with these levels and VFA concentrations, but negative correlations with pH. Shen et al. (2004) observed similar results in that an energy-rich diet enhanced NHE activity in the rumen epithelium. Kuzinski et al. (2011) reported that the expression of Na^+/K^+-ATPase mRNA in rumen epithelium was upregulated in sheep fed a hay-concentrate diet when compared to hay-feeding alone, and, ruminants fed with highly fermentable diets demonstrated a potential increase in bicarbonate-dependent transport mediated via DRA (Penner et al., 2009; Connor et al., 2010; Aschenbach et al., 2011). In the present study, energy and the VFA concentrations in the CS and BS groups increased, which could explain the increase in the relative expression levels of NHE1, DRA, and Na^+/K^+-ATPase mRNA in the ruminal epithelium. In addition, Kiela et al. (2007) demonstrated that the expression of NHE3 mRNA increased with butyrate. The CS group had a greater concentration of butyrate than the BS and CS groups, and the relative expression of NHE3 mRNA in the CS group was also greater than in the BS and CON groups. It was reported that a high-grain diet upregulated MCT1 mRNA expression in ruminal epithelium (Metzler-Zebeli et al., 2013), and MCT4 was stably upregulated by ruminal butyrate infusion (Malhi et al., 2013). Consequently, the relative expression levels of MCT1 and MCT4 mRNA in ruminal epithelium were greater in the CS group than in the BS and CON groups.

Results suggested that Tibetan sheep with dietary supplements had a greater capacity for bicarbonate-dependent (VFA^-/HCO_3^- exchange)

absorption of VFA. Moreover, the CS group had a greater capacity for MCT-dependent absorption of VFA than the CON group. Supplementation with concentrate or urea-molasses lick block resulted in differences in ruminal papillae morphology and in concentration and composition of VFA and, ultimately, in the VFA absorption capacity between the two supplements.

CONCLUSIONS

Tibetan sheep, supplemented with energy and protein via concentrate feed or urea-molasses lick block in the cold season, increased ADG, rumen microbial abundance, and VFA production while improving fermentation parameters, rumen epithelium development, and absorptive capability. Consequently, nutrient supplementation to Tibetan sheep in the cold season could be an effective strategy to prevent BW loss and improve digestive processes. Adoption of this practice could decrease grazing pressures and promote a sustainable livestock production system on the grasslands, which is crucial for pastoralists to improve their livelihood on the Qinghai-Tibetan Plateau.

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