

## Effects of Cold Season Supplementary Feeding on Digestive Tract Enzyme Activity and Apparent Digestibility in Tibetan Sheep (Postprint)

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### Abstract

This experiment aimed to investigate the effects of two nutritional supplementation methods, urea-molasses block or concentrate supplementation during the cold season, on growth performance, digestive enzyme activities in the digestive tract, and apparent digestibility in Tibetan sheep. Eighteen 1.5-year-old Tibetan ewe sheep were selected and randomly divided into 3 groups, with 6 sheep per group. The control (CON) group was fed oat hay ad libitum, the urea-molasses block supplementation (BS) group was allowed free access to urea-molasses blocks in addition to the CON group diet, and the concentrate supplementation (CS) group was supplemented with concentrate at 200 g/(head · d) in addition to the CON group diet. After the 60-day feeding trial, a 4-day digestion trial was conducted, followed by a slaughter trial. The results showed: 1) Compared with the CON group, the average daily intake of digestible energy in the CS and BS groups increased by 29.68% and 40.00%, respectively, and that of crude protein increased by 11.37% and 20.00%, respectively ( $P < 0.05$ ); 2) The average daily gain (ADG) in the CS and BS groups was 3.22 and 1.98 times that of the CON group, respectively ( $P < 0.05$ ); 3) Compared with the CON group, supplementation significantly increased the concentrations of serum glucose (GLU), total cholesterol (CHO), triglycerides (TG), total protein (TP), globulin (GLO), and albumin (ALB) ( $P < 0.05$ ); 4) Compared with the CON group, supplementation significantly increased the apparent digestibility of dry matter (DM), neutral detergent fiber (NDF), and acid detergent fiber (ADF) ( $P < 0.05$ ), and the CS group was higher than the BS group ( $P < 0.05$ ); 5) Compared with the CON group, both concentrate supplementation and urea-molasses block supplementation significantly increased the activities of xylanase, protease, and aminopeptidase in rumen fluid ( $P < 0.05$ ), and the CS group was significantly higher than the BS group ( $P < 0.05$ ); concentrate supplementation

also significantly increased the activities of  $\alpha$ -amylase and trypsin in the mucosa of various small intestine segments ( $P < 0.05$ ). The results suggest that during the cold season, supplementation with urea-molasses blocks or concentrate can significantly increase the intake of energy, protein, and trace elements in Tibetan sheep, significantly increase digestive enzyme activities in the digestive tract, thereby improving nutrient apparent digestibility and growth performance of Tibetan sheep; under the conditions of this experiment, the higher the supplemental intake level of energy and protein during the cold season, the higher the daily gain of Tibetan sheep.

## Full Text

### Effects of Supplementation on Digestive Enzyme Activities and Apparent Digestibility of Tibetan Sheep in Cold Season

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## Abstract

This study investigated the effects of two nutritional supplementation strategies—urea-molasses lick blocks and concentrate—on growth performance, digestive enzyme activities, and apparent nutrient digestibility in Tibetan sheep during the cold season. Eighteen 1.5-year-old Tibetan sheep ewes with similar body weight [(29.36 $\pm$ 1.79) kg] were randomly assigned to three groups (n=6 per group): a control group (CON) fed oat hay ad libitum, a lick block supplementation group (BS) receiving oat hay ad libitum plus free access to urea-molasses lick blocks, and a concentrate supplementation group (CS) receiving oat hay ad libitum plus concentrate at 200 g/(ewe · d). Following a 60-day feeding trial, a 4-day digestion trial was conducted, after which all sheep were slaughtered for sample collection. The results demonstrated that: (1) CS and BS groups consumed 29.68% and 11.37% more digestible energy, and 40.00% and 20.00% more crude protein than the CON group, respectively ( $P < 0.05$ ); (2) average daily gain (ADG) in the CS and BS groups was 3.22 and 1.98 times higher than in the CON group ( $P < 0.05$ ); (3) supplementation significantly elevated serum concentrations of glucose (GLU), total cholesterol (CHO), triglycerides (TG), total protein (TP), globulin (GLO), and albumin (ALB) compared to the CON group ( $P < 0.05$ ); (4) apparent digestibility of dry matter (DM), neutral detergent fiber (NDF), and acid detergent fiber (ADF) was significantly improved by supplementation ( $P < 0.05$ ), with the CS group showing higher values than the BS group ( $P < 0.05$ ); and (5) both supplementation strategies significantly increased

ruminal xylanase, proteinase, and aminopeptidase activities ( $P < 0.05$ ), with the CS group exhibiting significantly higher activities than the BS group ( $P < 0.05$ ). Additionally, concentrate supplementation specifically enhanced  $\alpha$ -amylase and trypsin activities in various small intestinal segments ( $P < 0.05$ ). These findings indicate that supplementation with either urea-molasses lick blocks or concentrate during the cold season significantly increases energy, protein, and trace mineral intake, enhances digestive enzyme activities throughout the gastrointestinal tract, and consequently improves nutrient apparent digestibility and growth performance in Tibetan sheep. Under the conditions of this study, higher supplemental intake levels of energy and protein corresponded to greater average daily gain.

**Keywords:** Tibetan sheep; nutritional supplementation strategy; digestive enzyme activity; apparent digestibility; growth performance

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## 1. Materials and Methods

**1.1 Experimental Period and Location** The experiment was conducted from February to April 2014 at the Haibei Plateau Modern Ecological Husbandry Science and Technology Demonstration Park in Xihaizhen, Haiyan County, Haibei Tibetan Autonomous Prefecture, Qinghai Province, China.

**1.2 Experimental Design and Diets** Eighteen healthy Tibetan sheep ewes aged 1.5 years with similar initial body weight [(29.36 $\pm$ \$1.79) kg] were randomly allocated to three groups (n=6 per group): control (CON), block supplementation (BS), and concentrate supplementation (CS). The basal diet consisted of oat hay. The CON group received oat hay ad libitum only, the BS group received oat hay ad libitum plus free access to urea-molasses lick blocks, and the CS group received oat hay ad libitum plus concentrate at 200 g/(ewe  $\cdot$  d). The concentrate formulation followed the Chinese Sheep Breeding Standard (NY/T 816-2004) and consisted primarily of corn, wheat bran, rapeseed meal, soybean meal, rapeseed oil, salt, and premix. The nutrient composition of oat hay, urea-molasses lick blocks, and concentrate is presented in Table 1. The concentrate supplementation level was determined based on previous research findings [14]. The experiment comprised three phases: a 14-day preliminary period, a 60-day formal feeding trial, a 4-day digestion trial (days 61-64), and subsequent slaughter.

**1.3 Animal Management** Prior to the experiment, all pens and feeding equipment were thoroughly cleaned and disinfected. All experimental sheep were treated with ivermectin for parasite control. Following the preliminary period, sheep were individually housed in separate pens and fed twice daily at 08:00 and 16:00. Feed offered and refusals were recorded daily. Urea-molasses lick blocks provided to the BS group were placed in feed troughs for free consumption, with periodic weighing to calculate intake. Concentrate for the CS

group was provided once daily at 08:00. Fresh water was available ad libitum throughout the experiment. Pens were cleaned daily and disinfected weekly.

**1.4 Sample Collection** **1.4.1 Blood Collection** Blood samples (10 mL) were collected via jugular venipuncture from all sheep on days 1 and 60 of the formal feeding period before morning feeding. Samples were allowed to clot at 4°C for 15 minutes, then centrifuged at 1,368×g for 15 minutes. Serum was harvested, aliquoted into 1.5 mL tubes, and stored at -20°C for subsequent biochemical analysis.

**1.4.2 Diet and Fecal Sample Collection** A total collection digestion trial was conducted on days 61-64. Daily feed intake was recorded and diet samples were collected and stored at -20°C. Following the method of Gorski et al. [16], custom fecal collection bags were attached to the sheep using a figure-eight wire frame and rubber straps to prevent spillage. Total feces were collected twice daily at 08:00 and 18:00, weighed, and thoroughly mixed. A 20% subsample was preserved by adding 10% hydrochloric acid (20 mL) for nitrogen fixation and stored at -20°C for analysis.

**1.4.3 Rumen Fluid and Intestinal Mucosa Collection** Immediately after slaughter, the rumen and small intestine (duodenum, jejunum, and ileum) were separated. Rumen digesta were sampled from the dorsal sac, ventral sac, dorsal caudal blind sac, and ventral caudal blind sac in equal proportions, filtered through four layers of cheesecloth, pooled, and aliquoted into 10 mL tubes. Samples were snap-frozen in liquid nitrogen and subsequently stored at -80°C. For intestinal samples, 5 cm segments from the middle of the duodenum, jejunum, and ileum were excised, flushed with saline to remove digesta, and mucosa was scraped using a glass slide. Mucosal samples were collected in 1.5 mL tubes, snap-frozen in liquid nitrogen, and stored at -80°C.

**1.5 Laboratory Analyses** **1.5.1 Growth Performance** Daily intake of oat hay, urea-molasses lick blocks, and concentrate was recorded to calculate average daily intake. Body weight was measured before morning feeding on days 1 and 60 to determine average daily gain (ADG).

**1.5.2 Blood Biochemical Parameters** Serum concentrations of glucose (GLU), triglycerides (TG), total cholesterol (CHO), total protein (TP), albumin (ALB), globulin (GLO), and urea nitrogen (UN) were determined using a Hitachi 7020 automatic biochemical analyzer (Japan) according to the manufacturer's instructions.

**1.5.3 Apparent Digestibility** Diet and fecal samples were analyzed for dry matter (DM), crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), calcium (Ca), and phosphorus (P) following standard methods [17]. Trace minerals (Fe, Cu, Mn, Zn, I, Co, Se) were determined by atomic absorption spectroscopy (CONTAA700, Jena, Germany) according to GB/T 13885-2003 [18]. Apparent digestibility of nutrients was calculated using the

formula:

Apparent digestibility (%) = [(Nutrient intake - Nutrient in feces) / Nutrient intake] × 100.

**1.5.4 Digestive Enzyme Activities** Activities of xylanase, endoglucanase,  $\alpha$ -amylase, proteinase, and aminopeptidase in rumen fluid, and  $\alpha$ -amylase, trypsin, chymotrypsin, and lipase in duodenal, jejunal, and ileal mucosa were determined using commercial ELISA kits (Shanghai Bangyi Biotechnology Co., Ltd.) according to the manufacturer' s instructions. Absorbance was measured using a Model 680 microplate reader (Bio-Rad, USA).

**1.6 Statistical Analysis** Experimental data were initially processed using Excel 2013 and subsequently analyzed using one-way ANOVA in SPSS 22.0. Differences among treatment means were evaluated using Duncan' s multiple range test. Statistical significance was declared at  $P < 0.05$ . Data are presented as means with standard error of the mean.

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## 2. Results

**2.1 Effects of Supplementation on Nutrient Intake** As shown in Table 2 , the CS group consumed 29.68% and 16.44% more digestible energy than the CON and BS groups, respectively ( $P < 0.05$ ), and 40.00% and 16.67% more crude protein than the CON and BS groups ( $P < 0.05$ ). The BS group consumed 11.37% more digestible energy and 20.00% more crude protein than the CON group ( $P < 0.05$ ). Total trace mineral intake in the BS group was 49.12% and 27.74% higher than in the CON and CS groups ( $P < 0.05$ ), while the CS group consumed 16.74% more trace minerals than the CON group ( $P < 0.05$ ). These results demonstrate that both urea-molasses lick block and concentrate supplementation significantly increased intake of digestible energy, crude protein, and total trace minerals in Tibetan sheep during the cold season.

**2.2 Effects of Supplementation on Growth Performance** Table 3 presents the effects of supplementation on growth performance. Final body weight in the CS group was significantly higher than in the BS and CON groups ( $P < 0.05$ ). Average daily gain in the CS group was 1.63 times higher than in the BS group and 3.22 times higher than in the CON group ( $P < 0.05$ ), while ADG in the BS group was 1.98 times higher than in the CON group ( $P < 0.05$ ). Both supplementation strategies significantly improved ADG in Tibetan sheep during the cold season.

**2.3 Effects of Supplementation on Blood Biochemical Parameters** As shown in Table 4 , serum concentrations of GLU, CHO, TG, TP, ALB, GLO, and UN did not differ among groups on day 1 ( $P > 0.05$ ). On day 60, serum GLU, TP, and GLO concentrations in both the CS and BS groups were significantly

higher than in the CON group ( $P < 0.05$ ), with no significant difference between the CS and BS groups ( $P > 0.05$ ). Serum CHO, TG, and ALB concentrations were highest in the CS group, intermediate in the BS group, and lowest in the CON group ( $P < 0.05$ ). Serum UN concentration was significantly higher in the CS group than in the BS and CON groups ( $P < 0.05$ ), while no difference was observed between the BS and CON groups ( $P > 0.05$ ).

#### **2.4 Effects of Supplementation on Nutrient Apparent Digestibility**

Table 5 shows that apparent digestibility of DM, NDF, and ADF was significantly higher in the CS group than in both the CON and BS groups ( $P < 0.05$ ), and higher in the BS group than in the CON group ( $P < 0.05$ ). Organic matter (OM) digestibility in the CS group was significantly higher than in the CON group ( $P < 0.05$ ). Crude protein digestibility was significantly higher in the CS group compared to both the CON and BS groups ( $P < 0.05$ ), with no significant difference between the BS and CON groups ( $P > 0.05$ ).

#### **2.5 Effects of Supplementation on Rumen Digestive Enzyme Activities**

As presented in Table 6, activities of xylanase, proteinase, and aminopeptidase in rumen fluid were highest in the CS group, intermediate in the BS group, and lowest in the CON group ( $P < 0.05$ ). Endoglucanase activity was significantly higher in the CS group than in the BS and CON groups ( $P < 0.05$ ), with no difference between the latter two groups ( $P > 0.05$ ).  $\alpha$ -Amylase activity was significantly higher in the CS group than in the CON group ( $P < 0.05$ ).

#### **2.6 Effects of Supplementation on Small Intestinal Mucosal Digestive Enzyme Activities**

Table 7 summarizes the effects on small intestinal enzyme activities. In duodenal mucosa,  $\alpha$ -amylase activity was significantly higher in the CS group than in the CON group ( $P < 0.05$ ). In jejunal mucosa,  $\alpha$ -amylase activity was significantly higher in the CS group than in both the BS and CON groups ( $P < 0.05$ ). In ileal mucosa, both the CS and BS groups showed significantly higher  $\alpha$ -amylase activity than the CON group ( $P < 0.05$ ). Trypsin activity in duodenal mucosa was significantly higher in the CS group than in the BS and CON groups ( $P < 0.05$ ), while in jejunal and ileal mucosa, trypsin activity was highest in the CS group, intermediate in the BS group, and lowest in the CON group ( $P < 0.05$ ). Chymotrypsin activity in duodenal and jejunal mucosa was significantly higher in both supplemented groups compared to the CON group ( $P < 0.05$ ). Lipase activity in duodenal mucosa was significantly higher in the CS and BS groups than in the CON group ( $P < 0.05$ ), whereas no significant differences were observed among groups in jejunal and ileal mucosa ( $P > 0.05$ ).

### 3. Discussion

#### 3.1 Effects of Supplementation on Serum Biochemical Parameters

Serum biochemical parameters reflect the metabolic status of nutrients in animals. Total protein concentration indicates protein intake and utilization efficiency. TP comprises albumin and globulin, where ALB is primarily involved in tissue synthesis and repair, while GLO is associated with humoral immunity. Nutritional deficiency typically leads to decreased TP, ALB, and GLO concentrations [19-20]. In this study, serum TP, ALB, and GLO concentrations decreased in the CON group but increased in both supplemented groups during the experimental period, with significantly higher values in the BS and CS groups than in the CON group on day 60. This pattern aligns with the increased protein intake observed in the supplemented groups. Serum UN concentration reflects protein or nitrogen metabolism and positively correlates with nitrogen input [21-22]. The significantly higher UN concentration in the CS group compared to the BS and CON groups is consistent with its higher CP intake. Although the BS group consumed significantly more CP than the CON group, their serum UN concentrations did not differ significantly, possibly because the soluble sugars, urea nitrogen, and trace minerals in the lick blocks were rapidly fermented and utilized by rumen microbes, promoting microbial protein synthesis and enhancing nitrogen utilization efficiency, thereby reducing urea nitrogen entry into the bloodstream.

Serum GLU in ruminants primarily originates from carbohydrate degradation to monosaccharides in the small intestine and hepatic gluconeogenesis. Previous studies have reported that increased energy intake significantly elevates serum GLU concentration [23-24]. In this experiment, both the CS and BS groups, which consumed significantly more digestible energy than the CON group, exhibited correspondingly higher serum GLU concentrations, consistent with previous findings. Serum TG and CHO concentrations are associated with lipid metabolism. On day 60, both the CS and BS groups showed significantly higher TG and CHO concentrations than the CON group, indicating enhanced lipid absorption and anabolic metabolism in the supplemented groups, whereas the CON group exhibited enhanced lipolysis. This pattern aligns with differences in digestible energy intake among groups and is consistent with the findings of Yang et al. [24].

#### 3.2 Effects of Supplementation on Digestive Enzyme Activities and Nutrient Apparent Digestibility

Digestive enzyme secretion and activity represent crucial indicators of digestive function, influencing nutrient absorption and subsequent growth, development, and production performance [25]. This study demonstrated that both concentrate and urea-molasses lick block supplementation enhanced activities of xylanase, endoglucanase,  $\alpha$ -amylase, proteinase, and aminopeptidase in rumen fluid, indicating improved ruminal fermentation and digestion of protein, cellulose, and starch. Rumen digestive enzymes are secreted by rumen microorganisms; therefore, their activities and types are

closely related to microbial populations [26], which are in turn influenced by diet composition and nutrient levels [27-28]. In this study, the CS and BS groups consumed more energy and protein, providing additional carbon and nitrogen sources for rumen microbial fermentation, thereby promoting microbial growth and metabolism and increasing the quantity and activity of secreted enzymes. Chen et al. [29] reported that nutrient restriction decreased cellulase activity in Small-tailed Han sheep, while nutritional compensation restored enzyme activity, findings that align with our results. Enhanced ruminal cellulolytic enzyme activity subsequently improved NDF and ADF apparent digestibility.

The small intestinal epithelium and pancreas secrete  $\alpha$ -amylase, trypsin, chymotrypsin, and lipase to further digest digesta entering the small intestine. Higher enzyme activity indicates stronger digestive capacity. Previous research has demonstrated that small intestinal enzyme activity is closely related to diet type and nutrient levels, with improved dietary nutrition significantly enhancing enzyme activities [30-33]. Liu et al. [34] reported that activities of amylase, trypsin, chymotrypsin, and lipase in the small intestine increased with dietary non-structural carbohydrate, protein, and fat content within certain ranges. In this study, supplementation with concentrate or urea-molasses lick blocks significantly increased digestible energy and CP intake in the CS and BS groups, likely increasing the flow of microbial protein, rumen-undegraded protein, and starch to the small intestine, thereby stimulating secretion and activity of small intestinal digestive enzymes. The increased activities of starch-hydrolyzing, protein-hydrolyzing, and lipid-hydrolyzing enzymes in the rumen and small intestine of the BS and CS groups, corresponding to increased nutrient intake, enhanced apparent digestibility of DM, OM, CP, NDF, and ADF, thereby promoting nutrient absorption and improving growth performance. These findings are consistent with Zhang [12], who reported that increasing concentrate supplementation levels improved nutrient apparent digestibility and growth performance in Tibetan sheep. Differences in enzyme activities between the CS and BS groups may be attributed to variations in supplemental nutrient levels and their degradation characteristics in the digestive tract, leading to differential effects on nutrient digestibility.

**3.3 Effects of Supplementation on Growth Performance** Average daily intake of protein, energy, and trace minerals determines normal physiological functions, health status, and growth performance [35]. Previous studies have demonstrated that energy or protein supplementation during the cold season improves livestock growth performance [36-37], with combined energy and protein supplementation being more effective than single-nutrient supplementation [38]. Traditional grazing management often results in mineral element deficiencies and imbalances that limit livestock productivity [39-42]. Zhang et al. [43] reported that supplementation with mineral lick blocks significantly increased trace mineral intake and average daily gain in grazing Alpine Merino sheep. In this study, both the CS and BS groups exhibited significantly higher ADG than the CON group, with the CS group outperforming the BS group, consis-

tent with the magnitude of increased energy, protein, and trace mineral intake and in agreement with previous research. Animal growth performance depends not only on nutrient intake but also on nutrient digestibility and absorption. This study demonstrated that increased nutrient intake enhanced digestive enzyme activities in both rumen and small intestine, improved apparent nutrient digestibility, and consequently increased growth performance in Tibetan sheep.

#### 4. Conclusions

Based on the findings of this study, two main conclusions can be drawn. First, supplementation with either urea-molasses lick blocks or concentrate during the cold season significantly increased intake of energy, protein, and trace minerals in Tibetan sheep, enhanced digestive enzyme activities throughout the gastrointestinal tract, and improved apparent digestibility of DM, OM, CP, NDF, and ADF, thereby promoting growth performance. Second, under the experimental conditions employed, higher supplemental intake levels of energy and protein corresponded to greater average daily gain in Tibetan sheep during the cold season.

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