Effects of Dietary Copper Content on In Vitro Rumen Fermentation in Yaks (Postprint)

Authors: Xue Yanfeng, Hao Lizhuang, Liu Shujie, Chai Shatuo, Zhang Xiaowei, Zhao Suonan

Date: 2017-10-10T00:00:00+00:00

Abstract

To investigate the appropriate content of the trace element copper in yak diets, the present experiment conducted in vitro rumen fermentation using yak diet as substrate with copper glycinate as the additive form. The substrate copper contents were 5.0, 10.0, 15.0, 20.0, and 25.0 mg/kg, and fermentation lasted for 42 h. Gas production, rumen fermentation parameters, and digestive enzyme activities were measured after fermentation. The results showed that when substrate copper content was 15.0 mg/kg, dry matter digestibility (DMD), microbial crude protein (MCP), propionate, isobutyrate, butyrate, isovalerate, valerate, total volatile fatty acid concentrations, and the activities of lipase (LPS), trypsin (TYS), and cellulase (CLS) all reached maximum values of 63.858%, 4.289 g/L, 24.475 mmol/L, 0.470 mmol/L, 8.977 mmol/L, 1.159 mmol/L, 1.607 mmol/L, 81.583 mmol/L, 0.504 U/mL, 84.167 U/mL, and 79.956 U/mL, respectively, while the acetate/propionate ratio was lowest at 2.045. When copper content was 10.0 mg/kg, MCP and acetate concentrations reached maximum values of 4.289 g/L and 51.075 mmol/L, respectively, and other parameters were also at relatively high levels. Based on these results, under in vitro conditions, the recommended copper content in yak diets is 10.0–15.0 mg/kg.

Full Text

Effects of Dietary Copper Content on Rumen Fermentation of Yaks *in Vitro*

XUE Yanfeng[1], HAO Lizhuang[1], *LIU Shujie*[1], CHAI Shatuo[1], ZHANG Xiaowei[1], ZHAO Suonan[2]

[1]State Key Laboratory of Plateau Ecology and Agriculture, Key Laboratory of Plateau Grazing Animal Nutrition and Feed Science of Qinghai Province,

Qinghai Plateau Yak Research Center, Qinghai Academy of Animal Science and Veterinary Medicine, Qinghai University, Xining 810016, China
[2]Academy of Animal Science and Veterinary Medicine of Haibei Prefecture, Haiyan 810200, China

Abstract: To investigate the optimal copper content in yak diets, this experiment used cupric glycinate as an additive and yak diet as substrate for *in vitro* rumen fermentation. The substrate copper contents were designed as 5.0, 10.0, 15.0, 20.0, and 25.0 mg/kg, respectively, with a fermentation period of 42 h. Gas production, rumen fermentation parameters, and digestive enzyme activities were measured after fermentation. The results showed that when substrate copper content was 15.0 mg/kg, dry matter digestibility (DMD), concentrations of microbial protein (MCP), propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, and total volatile fatty acids, as well as activities of lipase (LPS), trypsin (TYS), and cellulase (CLS) all reached their maximum values, being 63.858%, 4.289 g/L, 24.475 mmol/L, 0.470 mmol/L, 8.977 mmol/L, 1.159 mmol/L, 1.607 mmol/L, 81.583 mmol/L, 0.504 U/mL, 84.167 U/mL, and 79.956 U/mL, respectively; the acetate/propionate ratio was lowest at 2.045. When copper content was 10.0 mg/kg, MCP and acetate concentrations reached their maximum values of 4.289 g/L and 51.075 mmol/L, respectively, and other indices also remained at relatively high levels. In conclusion, under *in vitro* conditions, the recommended copper content in yak diets is 10.0–15.0 mg/kg.

Key words: yak; cupric glycinate; *in vitro* gas production technique; digestive enzyme activity; volatile fatty acid

Introduction

The yak is the dominant livestock species in the Qinghai-Tibet Plateau region, where environmental conditions are extremely harsh (cold, dry, hypoxic, and high radiation), serving as the material foundation and economic pillar for local herders [1-2]. However, yaks face cold winters and long withered grass periods, experiencing not only severe weight loss in winter but also frequent starvation and freezing deaths, which seriously constrain the development of the yak industry. Therefore, in-depth research on yak nutrition, formulation of feeding standards, and rational supplementation are imperative. While certain research foundations have been established regarding energy [3] and protein [4] nutrition in yaks, studies on trace element nutrition in yaks are essentially in a completely blank state.

Copper is one of the essential trace elements for animals [5], participating in hematopoiesis and myelin protein synthesis [6-7], promoting bone and collagen formation [8], involved in hair pigment deposition, enhancing immunity [9-10], and promoting livestock growth [11-12]. Copper in basal diets is often deficient, but excessive supplementation can affect yak digestion and metabolism, growth, reproduction, and production performance, and even impact the health status of yaks.

Yang Feng [6] pointed out that trace elements including copper, manganese, iodine, iron, and zinc in animal diets are in a state of marginal deficiency. In actual production, most farmers add excessive amounts of trace element complexes to animal diets in the form of premixes to meet animal requirements. While this compensates for the deficiency of trace elements in animal diets and plays a certain positive role, it fails to maximize animal growth performance.

Yang Hongjian [13] recommended a copper supplementation level of 10 mg/kg in beef cattle diets. Perry et al. [14] suggested that dairy cows require 10–20 mg/kg of copper under low molybdenum levels. Ma Changxing [15] reported that the copper requirement for young pregnant cows (300–650 kg body weight, 250 days of gestation) is 12–16 mg/kg. Therefore, this experiment used cupric glycinate as the form of trace element copper supplementation and employed the *in vitro* gas production technique to study the effects of copper contents ranging from 5.0 to 25.0 mg/kg on artificial rumen fermentation, aiming to screen the optimal copper content in yak diets and provide references for improving yak feeding standards and scientific supplementation.

Materials and Methods

Experimental Animals and Management

Three healthy adult castrated yaks with similar body condition and permanent rumen fistulas were selected as rumen fluid donors for this experiment. The experimental diet consisted of concentrate and roughage (oat hay) at a concentrate-to-roughage ratio of 6:4, fed individually twice daily (08:00 and 18:00) with free access to water. Rumen fluid was collected in the morning after overnight fasting following a 15-day feeding period.

In Vitro Gas Production Method

Rumen fluid was collected from the three yaks in the morning after overnight fasting, mixed, and filtered through four layers of gauze. Artificial rumen fermentation medium was prepared following the method of Menke et al. [16], and CO_2 was infused to achieve anaerobic conditions. The medium was dispensed into culture tubes containing 200 mg of substrate using an automatic dispenser (30 mL per tube) and incubated in an artificial rumen incubator at (39.0$±0.5)°C.Attimepointsof2, 4, 6, 8, 12, 16, 20, 24, 30, 36, and48h, culturetubeswereremovedandthepistonsc
waterbathtoterminatefermentation.Blanktubeswithoutsubstratewerealsoprepared.ThepHandammonianitr
N) concentration of fermentation fluid were measured on-site. Fermentation fluid was also stored frozen for determination of digestive enzyme activities, microbial protein (MCP), and volatile fatty acid (VFA) concentrations. Fermentation residues were collected, dried at 105 °C for 12–24 h, and dry matter digestibility (DMD) was determined.

Referring to the Beef Cattle Feeding Standard (NY/T 815-2004) and the *Yak Nutrition Research Papers* [2], the concentrate formula of the basal yak diet was designed for yaks weighing 150 kg with a daily gain of 500 g. Oat hay was used as roughage to prepare the fermentation substrate at a concentrate-to-roughage ratio of 6:4. This experiment adopted a single-factor design with five treatments and three replicates per treatment. The copper content in the fermentation substrate was first determined to be 4.395 mg/kg by atomic absorption spectrophotometry. Cupric glycinate (containing 20% copper) was then added to the fermentation substrates of the five treatments at levels of 0.605, 5.605, 10.605, 15.605, and 20.605 mg/kg, respectively, so that the total copper contents in the five treatments reached 5.0, 10.0, 15.0, 20.0, and 25.0 mg/kg.

Copper Content Determination

Copper content was determined by flame atomic absorption spectrophotometry using a Beijing Puxi TAS-990 AFG atomic absorption spectrophotometer according to GB/T 13885-2003 [17]. The standard curve fitting formula for copper content in this experiment was:

$$Abs = 0.10030Conc + 0.00046667 \ (r = 0.9999,\ n = 4)$$

where *Conc* is copper content (g/mL), *Abs* is absorbance value, and wavelength was 324.8 nm.

Gas Production, Gas Production Rate, and DMD

Total gas production, methane production, and gas production rate were calculated based on gas production recorded at each time point. Total gas production (mL) = total gas production in test tubes - total gas production in blank tubes; Methane production (mL) = total gas production × percentage of methane; Gas production rate (mL/h) = gas production at stage / time interval.

$$\text{DMD (\%)} = \left\{\frac{[\text{sample DM weight} - \text{residue DM weight} + \text{blank tube DM weight}]}{\text{sample DM weight}}\right\}\times 100$$

pH and NH_3-N, MCP Concentration

pH was measured using a HANNA HI221 benchtop pH meter.

NH_3-N concentration was determined by the modified colorimetric method of Feng Zongci et al. [18]. The standard curve fitting formula for NH_3-N concentration in this experiment was:

$$Y = 0.0431X + 0.0241 \ (r = 0.995,\ n = 5)$$

where Y is NH_3-N concentration (mg/dL), X is absorbance value, and wavelength was 625.0 nm.

MCP concentration was determined using a kit provided by Nanjing Jiancheng Bioengineering Institute. The principle is based on the biuret reaction where peptide bonds (–CONH–) in protein molecules react with alkaline copper solution to form purple complexes. The absorbance value at 540 nm is measured to calculate protein concentration. The biuret reagent was prepared by mixing diluted reagent 1 and reagent 2 at a 1:2 ratio. Fermentation fluid (0.20 mL) was mixed with physiological saline (0.80 mL), homogenized in an ice-water bath, centrifuged at 2,500 r/min for 10 min, and the supernatant (50 L) was used for determination. Detailed steps are shown in Table 1 . Absorbance was measured at 540 nm using a TU-1810 UV-Vis spectrophotometer (preheated for 30 min) with a 1 cm cuvette path length, zeroed with ultrapure water.

$$MCP\ (g/L) = \frac{(Am - AK)}{(As - Ak)} \times Ck \times w$$

where *Am* is the absorbance of the determination tube; *AK* is the absorbance of the blank tube; *As* is the absorbance of the standard tube; *Ck* is the concentration of the protein standard (56.3 g/L); and *w* is the dilution factor.

VFA Concentration Determination

VFA concentration determination followed the methods described in relevant literature [19-20] for sample pretreatment. Fermented rumen fluid was filtered through four layers of gauze, and 5 mL was placed in a clean centrifuge tube, centrifuged at 3,000 r/min for 10 min. Then 2 mL of supernatant was transferred to a Tube, and 0.2 mL of 25% metaphosphoric acid solution was accurately added. After mixing and standing for 10 min to allow complete reaction, the mixture was centrifuged at 12,000 r/min and 4 °C for 10 min. The supernatant was transferred to a new Tube and stored at -80 °C for later use.

VFA determination was performed using a Shimadzu 2014 gas chromatograph with FID detector and a capillary column (30 m × 0.32 mm × 0.5 m). The column temperature program was: initial 60 °C, increased to 120 °C at 10 °C/min (held for 2 min), then increased to 180 °C at 15 °C/min (held for 5 min). Vaporization and detection temperatures were 250 °C. Injection volume was 1 L with high-purity nitrogen (99.99%) as carrier gas at 0.7 MPa. Standard curves are shown in Table 2 .

Digestive Enzyme Activity Determination

Activities of amylase (AMS), lipase (LPS), trypsin (TYS), and cellulase (CLS) were determined using kits provided by Nanjing Jiancheng Bioengineering Institute. Activity unit definitions: One unit of AMS activity is defined as the amount of enzyme solution that hydrolyzes 10 mg of starch in 30 min at 37 °C per milliliter; one unit of LPS activity is defined as the consumption of 1 mol of substrate per minute at 37 °C per milliliter of enzyme solution; one unit of TYS activity is defined as a 0.003 change in absorbance value (wavelength 253 nm) of the reaction system per minute at 37 °C and pH 8.0 per milliliter of enzyme solution; one unit of CLS activity is defined as the production of 1 g of glucose per minute per milliliter of enzyme solution.

Statistical Analysis

Experimental data were analyzed using the ANOVA procedure in SAS 9.0 software for one-way analysis of variance, with Duncan' s method for multiple comparisons. $P<0.05$ was considered statistically significant, and data for each group were expressed as "mean ± standard deviation" .

Results

Gas Production, Gas Production Rate, and DMD

As shown in Table 3 , when cupric glycinate was used as an additive, total gas production from rumen fermentation showed a trend of initial increase followed by stabilization with increasing copper content. Total gas production remained stable at approximately 75.0 mL when copper content was 10.0–20.0 mg/kg. Only when copper content reached 25.0 mg/kg was total gas production significantly higher than at 5.0 mg/kg ($P<0.05$), with no significant differences among other groups ($P>0.05$). Methane production showed a trend of initial increase followed by decrease with increasing copper content, reaching its maximum value of 10.54 mL at 10.0 mg/kg copper, which was significantly higher than at 5.0, 20.0, and 25.0 mg/kg ($P<0.05$), but not significantly different from the methane production of 10.42 mL at 15.0 mg/kg ($P>0.05$). DMD showed a trend of initial increase followed by decrease with increasing copper content, reaching its maximum value of 63.858% at 15.0 mg/kg copper, which was significantly higher than at 5.0 and 25.0 mg/kg ($P<0.05$).

As shown in Figure 1 [Figure 1: see original paper], with prolonged fermentation time, the gas production rate in all five groups showed a trend of initial increase followed by decrease, eventually approaching zero. The maximum gas production rate was reached between 5-9 h, presenting a single-peak shape.

pH and NH_3-N, MCP Concentration

As shown in Table 4 , when cupric glycinate was used as an additive, pH after *in vitro* fermentation showed a trend of initial increase followed by decrease with increasing copper content. pH reached a stable trend at copper contents of 10.0–15.0 mg/kg, which was significantly higher than at 5.0 mg/kg ($P<0.05$). The pH values after *in vitro* fermentation in all groups ranged from 6.71 to 7.16, all within the normal range of yak rumen fluid pH. NH_3-N concentration after *in vitro* fermentation showed a trend of initial stability followed by decrease with increasing copper content. When copper content was 5.0-15.0 mg/kg, NH_3-N concentration remained stably high, all above 10.00 mg/dL. When copper content was 20.0–25.0 mg/kg, NH_3-N concentration was slightly lower, below 10.00 mg/dL. NH_3-N concentration at 5.0 mg/kg copper was significantly higher than at 20.0 and 25.0 mg/kg (P<0.05). MCP concentration after *in vitro* fermentation showed a trend of initial increase followed by decrease with increasing copper content, reaching a stable state at copper contents of 10.0–15.0 mg/kg, both at 4.289 g/L, which was significantly higher than the MCP concentration at 5.0 mg/kg copper (P<0.05).

VFA Concentration

As shown in Table 5 , when cupric glycinate was used as an additive, concentrations of acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, and total volatile fatty acids (TVFA) after fermentation all showed a trend of initial increase followed by decrease with increasing copper content. Acetate concentration reached its maximum value of 51.075 mmol/L at 10.0 mg/kg copper. Concentrations of propionate, isobutyrate, butyrate, isovalerate, valerate, and TVFA all reached their maximum values at 15.0 mg/kg copper, being 24.475, 0.470, 8.977, 1.159, 1.607, and 81.583 mmol/L, respectively. The acetate/propionate ratio showed a trend of initial decrease followed by increase, reaching its minimum value of 2.045 at 15.0 mg/kg copper. When copper content was 10.0 mg/kg, acetate concentration after *in vitro* fermentation was significantly higher than at 5.0 and 25.0 mg/kg (P<0.05). When copper content was 15.0 mg/kg, propionate concentration after *in vitro* fermentation was significantly higher than in all other groups (P<0.05), while at 10.0 mg/kg copper, propionate concentration was significantly higher than at 5.0 and 25.0 mg/kg (P<0.05). When copper content was 10.0 and 15.0 mg/kg, isobutyrate concentrations after *in vitro* fermentation were not significantly different (P>0.05) but were significantly higher than in other groups (P<0.05). When copper content was 10.0 and 15.0 mg/kg, butyrate concentrations were both significantly higher than in other groups (P<0.05), with significant differences between these two groups (P<0.05). When copper content was 10.0 and 15.0 mg/kg, isovalerate concentrations were both significantly higher than in other groups (P<0.05), with no significant difference between these two groups (P>0.05). When copper content was 10.0 and 15.0 mg/kg, valerate concentrations were both significantly higher than in other groups (P<0.05), with significant differences between these two groups (P<0.05). When copper content was 10.0 and 15.0 mg/kg, TVFA concentrations were both significantly higher than at 5.0 and 25.0 mg/kg (P<0.05), with no significant difference between these two groups (P>0.05). When copper content was 15.0 mg/kg, the acetate/propionate ratio after *in vitro* fermentation was significantly lower than at 5.0, 10.0, and 20.0 mg/kg (P<0.05).

Digestive Enzyme Activities

As shown in Table 6 , with increasing copper content, AMS activity after *in vitro* fermentation showed a trend of initially remaining at high levels, then decreasing, and increasing again, while LPS, TYS, and CLS activities all showed a trend of initial increase followed by decrease. AMS activity reached its maximum value of 0.531 U/mL at 5.0 mg/kg copper, but was not significantly different from that at 10.0 and 25.0 mg/kg (P>0.05), with these three groups being significantly higher than the other two groups (P<0.05). When copper content was 15.0 mg/kg, LPS activity reached its maximum value of 0.504 U/mL, significantly higher than all other groups (P<0.05). When copper content was 15.0 mg/kg, TYS activity reached its maximum value of 84.167 U/mL, significantly higher than at 5.0, 20.0, and 25.0 mg/kg (P<0.05), but not significantly different from that at 10.0 mg/kg (P>0.05). When copper content was 15.0 mg/kg, CLS activity reached its maximum value of 79.956 U/mL, not significantly different from that at 25.0 mg/kg, with both groups being significantly higher than at 5.0 mg/kg (P<0.05).

Discussion

Rumen gaseous fermentation products mainly include carbon dioxide, methane, hydrogen, VFAs, etc. [21], derived from the degradation of organic matter in the diet. The amount of total gas production reflects the degree of diet degradation [22]; higher gas production indicates more complete diet fermentation, providing more energy for the body and being more conducive to animal growth. In this experiment, total gas production and methane production increased with increasing substrate copper content, indicating that cupric glycinate supplementation is beneficial for rumen fermentation. The gas production rate showed a single-peak trend of initial increase followed by decrease. This is because at the initial fermentation stage, soluble sugars and other components in the fermentation substrate are easily utilized by microorganisms, rapidly producing gas. As fermentation time extends, fermentable components become increasingly scarce, and the gas production rate gradually decreases, eventually approaching zero. pH is a comprehensive reflection of rumen fermentation, influenced by various factors such as fermentation substrate type and organic acid precipitation [23]. Only when pH is within the normal range can rumen fermentation and feed degradation proceed normally. The pH values after *in vitro* fermentation under different copper contents in this experiment were all within the normal range of 5.6–7.5 for ruminant rumen fluid.

DMD directly reflects the degree of diet degradation in the rumen. In this experiment, DMD increased initially and then decreased with increasing copper content, reaching its maximum value of 63.858% at 15.0 mg/kg copper, indicating that 15.0 mg/kg copper is most conducive to feed degradation. This basically aligns with the results of digestive enzyme activity measurements in the rumen-gastrointestinal tract, where activities of LPS, TYS, and CLS all reached their maximum values at 15.0 mg/kg copper. Only AMS activity showed a trend of initial decrease followed by increase, and DMD was also at a relatively high level at 10.0 mg/kg copper. Therefore, from the perspective of rumen digestive enzyme activities and DMD, copper content of 10.0-15.0 mg/kg is most conducive to diet degradation. The digestive enzyme activities measured in yak rumen in this experiment were basically at the same level as those reported by Zhang Haitao et al. [24] for AMS activity in calf rumen (0.29-1.74 U/mL), Liu Caijuan et al. [25] for CLS activity in dairy cow rumen (71.43-99.05 U/mL), and Moharrery et al. [26] for AMS activity (0.70-31.99 U/mL) and LPS activity (0.15-0.81 U/mL) in dairy cow duodenum. Only TYS activity was much higher than the 5.55-11.42 U/mL reported by Moharrery et al. [26] in dairy cow duodenum and the 18.09-24.56 U/mL reported by Wang Jie [27] in sheep rumen, reaching 32.713-84.167 U/mL. Goodrich et al. [28] demonstrated in an *in vitro* culture experiment with different copper levels added to rumen fluid that copper greatly promotes cellulose degradation. Saxena et al. [29] also showed that dietary copper supplementation can promote cellulose degradation by rumen microorganisms, and in this experiment, copper addition significantly increased CLS activity after yak rumen *in vitro* fermentation, which will play a positive role in rumen microbial cellulose degradation.

NH_3-N is derived from protein degradation in the diet and is mainly used for microbial synthesis of MCP [30], basically maintaining dynamic equilibrium in the rumen. In this experiment, NH_3-N concentrations in all five groups were within the normal range of 0.35-29.0 mg/dL [31-32], and showed a decreasing trend with increasing copper content. However, when copper content was 10.0-15.0 mg/kg, NH_3-N concentration remained at an overall high level. MCP provides 40%-60% of the protein requirement for ruminants. With increasing copper content, MCP concentration showed a trend of initial increase followed by decrease, reaching its maximum at copper contents of 10.0-15.0 mg/kg. This indicates that copper content of 10.0-15.0 mg/kg is most conducive to NH_3-N and MCP formation. When copper content is low, the weak ability of microorganisms to synthesize MCP may result in low MCP concentration, and also inhibit the conversion of NH_3-N to MCP, leading to high NH_3-N concentration.

VFAs are important energy substances for ruminants, providing 60%-80% of digestible energy [33-34]. In this experiment, concentrations of propionate, isobutyrate, butyrate, isovalerate, valerate, and TVFA all reached their maximum values at 15.0 mg/kg copper, while acetate concentration reached its maximum at 10.0 mg/kg copper. Meanwhile, for ruminants, the energy required by the animal body mainly comes from glucose produced by hepatic gluconeogenesis, and propionate is an important precursor for gluconeogenesis and an efficient acid. A lower acetate/propionate ratio indicates a greater proportion of propionate, which is more conducive to ruminant growth. In this experiment, the acetate/propionate ratio reached its minimum value at 15.0 mg/kg copper. This demonstrates that copper content of 10.0-15.0 mg/kg is most conducive to energy substance production and yak growth.

In summary, under *in vitro* conditions, for growing yaks, when cupric glycinate is used as the form of copper supplementation, a copper content of 10.0-15.0 mg/kg in yak diets is beneficial for rumen fermentation and diet degradation. However, the basal content of trace element copper in the yak diet used in this experiment was only 4.395 mg/kg, far below the yak’ s copper requirement of 10.0-15.0 mg/kg, indicating an extremely deficient state. Therefore, additional supplementation of trace element copper is necessary to maximally improve yak rumen fermentation and growth performance.

References

[1] WIENER G, HAN J L, LONG R J. The yak[M]. 3rd ed. Bangkok: RAP Publication, 2003: 1-18.
[2] HU Linghao. Yak Nutrition Research Papers Collection[M]. Xining: Qinghai People’ s Publishing House, 1997.
[3] HAN Xingtai, HU Linghao, XIE Aoyun, et al. Estimation of energy metabolism in growing yaks under rough feeding conditions[J]. Qinghai Animal Husbandry and Veterinary Medicine Magazine, 1992, 22(2): 21-22.
[4] XUE Bai, CHAI Shatuo, LIU Shujie. Study on protein requirements of growing yaks[J]. Qinghai Animal Husbandry and Veterinary Medicine Magazine, 1994, 24(4): 1-4.
[5] LIU Zongping. Modern Animal Nutrition and Metabolic Diseases[M]. Beijing: Chemical Industry Press, 2003: 132-142.
[6] YANG Feng. Animal Nutrition[M]. 2nd ed. Beijing: China Agriculture Press, 2003: 116-117.
[7] YUAN Shibin, HE Ping, CHEN Daiwen. Nutritional physiological function and growth-promoting mechanism of trace element copper[J]. Feed Industry, 2004, 25(7): 23-26.
[8] GUO Baolin. Study on Effects of Different Dietary Copper Sources on Copper Metabolism and Cellulose Degradation in Sheep[D]. PhD Dissertation. Beijing: China Agricultural University, 2004.
[9] YANG Wenzheng, ZHANG Shiyuan, LIN Zaiguang, et al. Animal Mineral Nutrition[M]. Beijing: China Agriculture Press, 1996: 6-9.
[10] WU Jianshe, GUO Yuming. Research progress on nutrition and immunity of trace element copper[J]. Foreign Animal Husbandry Science and Technology, 1999, 26(1): 5-9.
[11] LI Wenli, HUANG Xincheng. Test report on effects of trace element additives on cattle fattening[J]. Journal of Laiyang Agricultural College, 1994, 11(4): 313-315.
[12] YANG Zhengde. Effects of different copper supplementation levels on copper metabolism and fattening performance of beef cattle[J]. Chinese Journal of Animal Science, 2000, 36(3): 14-16.
[13] YANG Hongjian. Copper nutrition in beef cattle[J]. Feed Research, 1999(8): 1-3.
[14] PERRY J M, ZHAO Y, MARLETTA M A, et al. Cu^{2+} and Zn^{2+} inhibit nitric-oxide synthase through domain interaction reductase[J]. The Journal of Biological Chemistry, 2000, 275(19): 14070-14076.

[15] MA Changxing. Study on nutritional requirement of trace element copper for dairy cows[J]. Animal Husbandry and Veterinary Science and Technology Information, 2010(11): 43.
[16] MENKE K H, RAAB L, SALEWSKI A, et al. The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor *in vitro*[J]. Journal of Agricultural Science, 1979, 93(1): 217-222.
[17] General Administration of Quality Supervision, Inspection and Quarantine of the People' s Republic of China. GB/T 13885-2003 Determination of calcium, copper, iron, magnesium, manganese, potassium, sodium and zinc contents in animal feed—Atomic absorption spectrometry method[S]. Beijing: China Standards Press, 2004.
[18] FENG Zongci, GAO Min. Improvement of method for determining ammonia nitrogen content in rumen fluid by colorimetry[J]. Animal Husbandry and Feed Science, 2010, 31(6/7): 37.
[19] CAO Qingyun, ZHOU Wuyi, ZHU Guizhao, et al. Study on determination of volatile fatty acids in sheep rumen fluid by gas chromatography[J]. China Feed, 2006(24): 26-28.
[20] WANG Jiaqi. Research Methods in Ruminant Nutrition[M]. Beijing: Modern Education Press, 2011: 139-141.
[21] MENKE K H, STEINGASS H. Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid[J]. Animal Research and Development, 1988, 28: 7-55.
[22] HA Sihua. Role of *in vitro* fermentation gas production technique in evaluation of feed nutritional value[J]. Contemporary Animal Husbandry, 2013(1): 15-19.
[23] GAO Tianshuang, SUN Haixia, XIE Xiaolai, et al. Effects of replacing corn with sorghum in diets on rumen fermentation in sheep[J]. Heilongjiang Animal Science and Veterinary Medicine, 2014(9): 7-10.
[24] ZHANG Haitao, WANG Jiaqi, BU Dengpan, et al. Effects of *Bacillus subtilis* natto on rumen fermentation and enzyme activity in calves before and after weaning[J]. China Animal Husbandry and Veterinary Medicine, 2009, 36(12): 5-11.
[25] LIU Caijuan, SUN Manji, SUN Jinyan, et al. Effects of dietary compound probiotics on rumen fermentation and cellulase activity in dairy cows[J]. Chinese Journal of Animal Nutrition, 2011, 23(5): 821-827.
[26] MOHARRERY A, BRASK M, WEISBJERG M R. The effect of different physical forms of rapeseed as a fat supplement on the activity of some enzymes in the duodenal chyme of dairy cows[J]. Journal of Animal and Feed Sciences, 2014, 23(4): 293-298.
[27] WANG Jie. Comparison of growth performance and main digestive physiological parameters between Small-tailed Han sheep and Tan sheep[D]. Master' s Thesis. Yangling: Northwest A&F University, 2008.
[28] GOODRICH R D, TILLMAN A D. Effects of sulfur and nitrogen sources and copper levels on the metabolism of certain minerals by sheep[J]. Journal of Animal Science, 1966, 25(2): 484-491.

[29] SAXENA K K, RANJHAN S K. A note on the effect of cobalt and copper supplementation on *in vivo* cellulose digestion by nylon-bag technique in Hariana calves[J]. Indian Journal of Animal Science, 1978, 48: 833-835.
[30] LENG R A, NOLAN J Y. Nitrogen metabolism in the rumen[J]. Journal of Dairy Science, 1984, 67(5): 1072-1089.
[31] SLYTER L L. Monensin and dichloroacetamide influences on methane and volatile fatty acid production by rumen bacteria *in vitro*[J]. Applied and Environmental Microbiology, 1979, 37(2): 283-288.
[32] COLEMAN G S, SANDFORD D C. The uptake and utilization of bacteria, amino acids and nucleic components by rumen ciliate *Eudiplodinium maggii*[J]. Journal of Applied Bacteriology, 1979, 47(3): 409-419.
[33] GRAY G M. Starch digestion and absorption in nonruminants[J]. The Journal of Nutrition, 1992, 122(1): 172-177.
[34] ZHAO Guoqi, JIA Yahong, CHEN Xiaolian, et al. Study on effects of diets with different NDF/NFE ratios on rumen fermentation parameters in goats[J]. Chinese Journal of Animal Science, 2006, 42(13): 29-33.

Note: Figure translations are in progress. See original paper for figures.