Effects of Manganese Content in Fermentation Substrate on In Vitro Rumen Fermentation in Yaks (Postprint)

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Abstract

To determine the manganese requirement as a trace element in yaks, this experiment utilized manganese glycinate as the supplementation form to investigate the effects of varying manganese concentrations in fermentation substrates on in vitro rumen fermentation of yaks. Five manganese concentrations in the fermentation substrate were established at 35.00, 40.00, 50.00, 60.00, and 70.00 mg/kg, and a 48-h fermentation was performed using in vitro rumen fermentation technology. Following fermentation, gas production, rumen fermentation characteristics, and digestive enzyme activities were measured. The results demonstrated: 1) At a manganese concentration of 40.00 mg/kg, the concentrations of ammonia nitrogen, microbial protein, acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, and total volatile fatty acids in the fermentation fluid all reached maximum values of 10.60 mg/dL, 3.90 g/L, 47.12 mmol/L, 19.45 mmol/L, 0.35 mmol/L, 5.41 mmol/L, 0.96 mmol/L, 0.50 mmol/L, and 72.24 mmol/L, respectively; 2) At a manganese concentration of 50.00 mg/kg, lipase activity in the fermentation fluid reached its maximum value of 0.50 U/mL, while the acetate/propionate ratio was lowest at 2.05. In summary, for growing yaks, if manganese glycinate is used as the manganese supplementation form, a dietary manganese concentration of 40.00–50.00 mg/kg is recommended to promote rumen fermentation and forage degradation.

Full Text

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Abstract

To determine the manganese requirement of yaks, this study investigated the effects of different manganese contents in fermentation substrate on in vitro rumen fermentation of yaks using manganese bisglycinate as the additive form. Five manganese levels were designed in the fermentation substrate: 35.00, 40.00, 50.00, 60.00, and 70.00 mg/kg. In vitro rumen fermentation was conducted for 48 hours, after which gas production, rumen fermentation characteristics, and digestive enzyme activities were measured. The results showed: (1) When manganese content was 40.00 mg/kg, the contents of ammonia nitrogen, microbial protein, acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, and total volatile fatty acids in the fermentation fluid all reached their maximum values at 10.60 mg/dL, 3.90 g/L, 47.12 mmol/L, 19.45 mmol/L, 0.35 mmol/L, 5.41 mmol/L, 0.96 mmol/L, 0.50 mmol/L, and 72.24 mmol/L, respectively. (2) When manganese content was 50.00 mg/kg, lipase activity in the fermentation fluid reached its maximum value of 0.50 U/mL, while the acetic acid/propionic acid ratio reached its minimum value of 2.05. In conclusion, for growing yaks, when using manganese bisglycinate as the manganese source, a dietary manganese content of 40.00–50.00 mg/kg is recommended to promote rumen fermentation and forage degradation.

Keywords: yak; manganese bisglycinate; in vitro rumen fermentation; digestive enzyme activity

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The yak is a unique species on the plateau, characterized by high immunity, stress resistance, and rapid adaptability to harsh natural environments [1]. Its wool, milk, and meat provide essential material resources for herders. Traditionally, yaks have relied primarily on natural grasslands, but low calving rates, slow growth rates, and severe weight loss during cold seasons have seriously constrained the development of the yak industry. Consequently, cold-season supplementation has attracted increasing attention from researchers and herders, making scientific diet formulation particularly important. While considerable research has been conducted on energy and protein nutrition in yaks [2-3], studies on trace element manganese remain largely absent. As an essential trace element, manganese plays crucial roles in animal life activities, promoting bone development [4-5], participating in hematopoiesis [6], enhancing cellular immune function [7-8], and serving as a component of many enzymes [9]. Xi Lilan [10] reported that cattle require 46.40–48.40 mg/kg manganese in their diet. Zhuang

Huaifei et al. [11] studied the effects of different dietary manganese and copper levels on antioxidant indices in Holstein bulls, finding that the appropriate dietary manganese level was 50.00 mg/kg. Ji Shoukun et al. [12] demonstrated that the maintenance requirements for manganese were 0.29 and 0.22 g/d for male and female lambs, respectively, equivalent to approximately 116 and 88 mg/kg DM in the diet. This study employed in vitro rumen fermentation technology to investigate the effects of manganese bisglycinate at 35–70 mg/kg on rumen fermentation in growing yaks, aiming to determine the appropriate dietary manganese level for growing yaks, improve yak feeding standards, and provide a theoretical basis for scientifically formulated supplementation diets to promote the development of the yak industry.

1 Materials and Methods

1.1 Experimental Animals and Management

Three healthy, similar-bodied, permanently rumen-fistulated Datong castrated yaks were selected as experimental animals. The experimental diet consisted of concentrate and roughage (oat hay) at a 60:40 ratio, fed individually twice daily (08:00 and 18:00) with free access to water. Rumen fluid was collected in the morning after a 15-day adaptation period.

1.2 Fermentation Substrate

Based on China' s "Feeding Standard of Beef Cattle" (NY/T 815-2004) and literature [13], a basal diet for yaks weighing 150 kg with a daily gain of 500 g was formulated using oat hay as roughage at a 60:40 concentrate-to-roughage ratio. This diet served as the fermentation substrate, with its composition and nutrient levels shown in Table 1 .

1.3 Experimental Design

A single-factor experimental design was adopted with five treatments and three replicates per treatment. Manganese bisglycinate (provided by Changsha Xingjia Bioengineering Co., Ltd., purity 21%, product number 2015071810) was added to the fermentation substrate to achieve manganese contents of 35.00, 40.00, 50.00, 60.00, and 70.00 mg/kg (DM basis). Other trace elements (iron, zinc, selenium, copper, and iodine) were maintained at consistent levels of 5.26, 6.27, 0.04, 4.40, and 0.00 mg/kg (DM basis), respectively.

1.4 In Vitro Rumen Fermentation

Artificial rumen fluid was prepared according to Menke et al. [14]. Individual solutions were prepared following the formulations in Table 2 : micro-element solution (Solution A), buffer solution (Solution B), macro-element solution (Solution C), indicator, and reducing solution. The artificial rumen fluid was prepared by sequentially adding 667 mL ultrapure water, 0.17 mL Solution A, 333

mL Solution B, 333 mL Solution C, 1.70 mL indicator, and 67 mL reducing solution to a 2 L glass jar. Thirty milliliters of artificial rumen fluid and 200 mg of fermentation substrate were placed in each fermentation tube and incubated in an artificial rumen incubator at (39±0.5) °C [15-16]. Gas production readings were recorded at 2, 4, 6, 8, 12, 14, 16, 24, 30, 36, and 48 hours.

1.5 Analytical Methods

1.5.1 Manganese Content Determination Manganese content was determined according to GB/T 13885-2003 [17] using a TAS-990 atomic absorption spectrophotometer. The standard curve for manganese content was:

Abs = 0.18290Conc. + 0.0031400 (r = 0.9997, n = 4)

where Conc. is manganese content (g/mL) and Abs is absorbance value.

1.5.2 Gas Production, pH, Dry Matter Disappearance (DMD), Microbial Protein (MCP), and Ammonia Nitrogen (NH -N) Gas production and DMD were calculated using the following formulas:

Gas production (mL) = Gas volume in culture tube at a given time point (mL) − Gas volume in blank tube at the corresponding time point (mL)

DMD (%) = [(Sample DM weight − Residue DM weight + Blank tube DM weight) / Sample DM weight] × 100

Fermentation fluid pH was measured using a HANNA-HI221 high-precision pH meter. MCP content was determined using kits from Nanjing Jiancheng Bioengineering Institute. NH -N content was measured using the improved colorimetric method of Feng Zongci et al. [18].

1.5.3 Methane Production and Volatile Fatty Acid (VFA) Content Methane production was determined according to literature [19-20] using a gas chromatograph (GC-2014, Shimadzu, Japan) to measure methane content in gas produced during in vitro rumen fermentation. Conditions: flame ionization detector (FID), capillary column (FFAP, 30.00 m × 0.32 mm, 0.50 m); column temperature 100 °C (isothermal); injector temperature 100 °C; FID temperature 110 °C; injection volume 100 L; carrier gas high-purity nitrogen (99.99%) at 0.7 MPa; hydrogen pressure 0.4 MPa; air pressure 0.4 MPa; capillary column pressure 65 kPa; split ratio 40:1. Methane standard gas was produced by Lanzhou Huaté Chemical Supply Station, with composition shown in Table 3 .

The methane standard curve was: Y = 249833X + 78225.5 (r = 0.999) [Y is peak area, X is methane content (mmol/L)].

VFA content was determined according to literature [21-22]. Sample pretreatment: fermentation fluid was filtered through four layers of gauze, 5 mL was placed in a clean centrifuge tube and centrifuged at 3000 r/min for 10 min. Two milliliters of supernatant was transferred to a centrifuge tube, 0.2 mL of

25% metaphosphoric acid was added, mixed well, reacted for 10 min, then centrifuged at 12000 r/min at 4 °C for 10 min. The supernatant was transferred to a new tube and stored at -80 °C.

VFA content was measured by gas chromatography. Conditions: FID, capillary column (FFAP, 30.00 m × 0.32 mm, 0.50 m); temperature program: initial 60 °C, increased to 120 °C at 10 °C/min (held 2 min), then to 180 °C at 15 °C/min (held 5 min); injector temperature 250 °C; FID temperature 250 °C; injection volume 1 L; carrier gas high-purity nitrogen (99.99%) at 0.7 MPa; hydrogen pressure 0.4 MPa; air pressure 0.4 MPa; capillary column pressure 0.6–0.8 MPa; split ratio 40:1. Standard curves for VFA contents are shown in Table 4 .

1.5.4 Amylase (AMS), Lipase (LPS), Trypsin (TYS), and Cellulase (CLS) Activities Activities of AMS, LPS, TYS, and CLS were determined using kits from Nanjing Jiancheng Bioengineering Institute. Unit definitions: One AMS unit is defined as the amount of enzyme that hydrolyzes 10 mg starch in 30 min at 37 °C per mL of enzyme solution. One LPS unit is defined as the consumption of 1 mol substrate per minute per mL of enzyme solution at 37 °C. One TYS unit is defined as the change in absorbance of 0.003 per minute per mL of enzyme solution at pH 8.0 and 37 °C.

1.6 Statistical Analysis

Data were initially processed using Excel 2007. One-way ANOVA was performed using the ANOVA procedure in SAS 9.1.3 software, with Duncan' s multiple comparison test. All data are expressed as mean ± standard deviation.

2 Results

2.1 Gas Production, Methane Production, DMD, and Fermentation Fluid pH

As shown in Table 5 , gas production tended to decrease while fermentation fluid pH tended to increase with increasing manganese content. DMD showed an initial increase followed by a decrease. Gas production reached its highest value of 73.80 mL at 35.00 mg/kg manganese, with the second-highest value of 72.80 mL at 40.00 mg/kg. No significant differences were observed among 35.00, 40.00, 50.00, and 60.00 mg/kg treatments ($P > 0.05$), but these four treatments were all significantly higher than the 70.00 mg/kg treatment ($P < 0.05$). Methane production peaked at 8.01 mL at 60.00 mg/kg manganese, significantly higher than at 35.00, 40.00, and 50.00 mg/kg ($P < 0.05$), but not significantly different from 70.00 mg/kg ($P > 0.05$). DMD reached its maximum of 68.73% at 60.00 mg/kg, significantly higher than at 35.00 and 70.00 mg/kg ($P < 0.05$), but not significantly different from 40.00 and 50.00 mg/kg ($P > 0.05$). Fermentation fluid pH reached its maximum of 7.49 at 70.00 mg/kg ($P < 0.05$), significantly higher than at 35.00 and 40.00 mg/kg ($P < 0.05$), but not significantly different from 50.00 and 60.00 mg/kg ($P > 0.05$).

2.2 NH -N and MCP Contents in Fermentation Fluid

As shown in Table 6 , NH -N and MCP contents in fermentation fluid showed an initial increase followed by a decrease with increasing manganese content. NH -N content peaked at 10.60 mg/dL at 40.00 mg/kg manganese, significantly higher than at 50.00, 60.00, and 70.00 mg/kg ($P < 0.05$), but not significantly different from 35.00 mg/kg ($P > 0.05$). MCP content reached its maximum of 3.90 g/L at 40.00 mg/kg, significantly higher than at 35.00, 60.00, and 70.00 mg/kg ($P < 0.05$), but not significantly different from 50.00 mg/kg ($P > 0.05$).

2.3 Digestive Enzyme Activities in Fermentation Fluid

As shown in Table 7 , AMS, LPS, and TYS activities in fermentation fluid showed an initial increase followed by a decrease with increasing manganese content, while CLS activity showed an increasing trend. AMS activity peaked at 0.51 U/mL at 60.00 mg/kg manganese, significantly higher than at 35.00, 40.00, and 50.00 mg/kg ($P < 0.05$), but not significantly different from 70.00 mg/kg ($P > 0.05$). LPS activity reached its maximum of 0.50 U/mL at 50.00 mg/kg, significantly higher than all other treatments ($P < 0.05$); no significant differences were observed among 40.00, 60.00, and 70.00 mg/kg ($P > 0.05$), but these were all significantly higher than 35.00 mg/kg ($P < 0.05$). TYS activity peaked at 65.46 U/mL at 60.00 mg/kg, significantly higher than at 35.00 and 70.00 mg/kg ($P < 0.05$), but not significantly different from 40.00 and 50.00 mg/kg ($P > 0.05$). No significant differences in CLS activity were observed among treatments ($P > 0.05$), with the maximum value of 81.12 U/mL at 70.00 mg/kg.

2.4 VFA Contents in Fermentation Fluid

As shown in Table 8 , contents of acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, and total volatile fatty acids all showed an initial increase followed by a decrease with increasing manganese content. Acetic acid content peaked at 47.12 mmol/L at 40.00 mg/kg manganese, significantly higher than at 50.00, 60.00, and 70.00 mg/kg ($P < 0.05$), but not significantly different from 35.00 mg/kg ($P > 0.05$). No significant differences in propionic acid content were observed among treatments ($P > 0.05$), with the maximum of 19.45 mmol/L at 40.00 mg/kg. No significant differences in isobutyric acid content were observed among treatments ($P > 0.05$), with the maximum of 0.35 mmol/L at 40.00 mg/kg. No significant differences in butyric acid content were observed among treatments ($P > 0.05$), with the maximum of 5.41 mmol/L at 40.00 mg/kg. No significant differences in isovaleric acid content were observed among treatments ($P > 0.05$), with the maximum of 0.96 mmol/L at 40.00 mg/kg. No significant differences in valeric acid content were observed among treatments ($P > 0.05$), with the maximum of 0.50 mmol/L at 40.00 mg/kg. Total volatile fatty acid content peaked at 72.24 mmol/L at 40.00 mg/kg, significantly higher than at 60.00 and 70.00 mg/kg ($P < 0.05$), but not significantly different from 35.00 and 50.00 mg/kg ($P > 0.05$). The

acetic acid/propionic acid ratio reached its minimum of 2.05 at 50.00 mg/kg, significantly lower than at 35.00, 40.00, and 60.00 mg/kg ($P < 0.05$), but not significantly different from 70.00 mg/kg ($P > 0.05$).

3 Discussion

Gas production from rumen fermentation reflects the degradation degree of dietary nutrients [23]; higher gas production indicates more complete fermentation, more energy provided to the animal, and better growth performance. In this study, gas production was highest at 35.00 mg/kg manganese and gradually decreased with increasing manganese content, suggesting that higher manganese levels were detrimental to rumen fermentation. Methane production peaked at 60.00 mg/kg manganese. The decrease in gas production coupled with increased methane production indicates greater energy loss. At 60.00 mg/kg manganese, methane production reached its maximum of 8.01 mL, while propionic acid content (an efficient acid) reached its minimum of 12.71 mmol/L and the acetic acid/propionic acid ratio reached its maximum of 2.87, indicating that 60.00 mg/kg manganese was unfavorable for animal growth. The MCP content results support this conclusion, as MCP content was relatively low at 60.00 mg/kg, indicating low rumen microbial activity.

pH is a comprehensive indicator of rumen fermentation, influenced by substrate type and organic acid precipitation [24]. Normal rumen fermentation and feed degradation can only proceed when pH is within the normal range. In this study, fermentation fluid pH ranged from 7.03 to 7.49 across different manganese levels, which is within the normal range (5.60–7.50).

DMD directly reflects the degradation degree of dietary nutrients in the rumen. In this study, DMD showed an initial increase followed by a decrease with increasing manganese content, peaking at 68.73% at 60.00 mg/kg manganese. This indicates that 60.00 mg/kg manganese was most beneficial for feed degradation. Manganese can significantly promote cellulose degradation by rumen microorganisms. Martinez et al. [25] reported that manganese concentrations of 5.00–30.00 mg/L in substrate could promote cellulose degradation by rumen microorganisms, with 15.00 mg/L being optimal. Huang Jinglong [26] found that apparent digestibility was significantly higher in the 40.00 mg/kg manganese group than in the 120.00 and 160.00 mg/kg groups. The DMD results were generally consistent with digestive enzyme activity measurements, as AMS, LPS, and TYS activities showed an initial increase followed by a decrease with increasing manganese content, while CLS activity showed a continuous increase. All enzyme activities were at relatively high levels at manganese concentrations of 40.00–60.00 mg/kg. Therefore, from the perspective of rumen digestive enzyme activities and DMD, manganese concentrations of 40.00–60.00 mg/kg were most conducive to dietary degradation.

NH -N originates from protein degradation and is primarily used for microbial MCP synthesis [27], maintaining a dynamic balance in the rumen. In this

study, NH -N content ranged from 6.42 to 10.60 mg/dL, which is within the normal range (0.35–29.00 mg/dL [28-29]). NH -N content showed an initial increase followed by a decrease with increasing manganese content, peaking at 40.00 mg/kg. MCP provides 40%–60% of protein requirements for ruminants. MCP content also showed an initial increase followed by a decrease, reaching its maximum at 40.00 mg/kg manganese. These results indicate that 40.00 mg/kg manganese was most favorable for NH -N and MCP formation.

VFAs are important energy substances for ruminants, providing 60%–80% of digestible energy [30-31]. Contents of acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, and total volatile fatty acids all showed an initial increase followed by a decrease, reaching maximum values at 40.00 mg/kg manganese and maintaining relatively high levels at 35.00–50.00 mg/kg. For ruminants, glucose produced through hepatic gluconeogenesis is the main energy source, and propionic acid is an important precursor for gluconeogenesis. A lower acetic acid/propionic acid ratio indicates a higher proportion of propionic acid, which is more favorable for ruminant growth. In this study, the acetic acid/propionic acid ratio reached its minimum of 2.05 at 50.00 mg/kg manganese, which was most conducive to propionic acid production. Therefore, from the perspective of volatile fatty acids, manganese concentrations of 35.00–50.00 mg/kg were beneficial for energy production during rumen fermentation.

4 Conclusion

1. For growing yaks, when using manganese bisglycinate as the manganese source, a dietary manganese content of 40.00–50.00 mg/kg is recommended to promote rumen fermentation and forage degradation.
2. According to existing data, the manganese content in yak diets is only 33.82 mg/kg, which is far below the required 40.00–50.00 mg/kg and indicates severe deficiency. Additional manganese supplementation is necessary to improve rumen fermentation and growth performance in yaks.

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