

Article

Effect of Source and Level of Dietary Supplementary Copper on In Vitro Rumen Fermentation in Growing Yaks

Xinsheng Zhao ¹, Lizhuang Hao ^{1,*}, Yanfeng Xue ^{1,2,*}, Allan Degen ³ and Shujie Liu ¹

¹ Key Laboratory of Plateau Grazing Animal Nutrition and Feed Science of Qinghai Province, Qinghai Plateau Yak Research Center, Qinghai Academy of Science and Veterinary Medicine of Qinghai University, Xining 810016, China

² Department of Animal Science, College of Animal Science and Technology, Anhui Agricultural University, Hefei 230036, China

³ Desert Animal Adaptations and Husbandry, Wyler Department of Dryland Agriculture, Blaustein Institutes for Desert Research, Ben-Gurion University of the Negev, Beer Sheva 8410500, Israel

* Correspondence: lizhuanghao1122@foxmail.com (L.H.); xueyanfeng1990@163.com (Y.X.)

Abstract: Copper (Cu) is essential for the health of livestock, however, the optimal source and level of dietary Cu for yaks are uncertain. To fill this important gap, we designed an in vitro study to examine the effects of three Cu sources, namely Cu methionine (Met-Cu), Cu chloride (CuCl₂) and tribasic Cu chloride (TBCC), at five levels, namely 5, 10, 15, 20 and 25 mg/kg DM (includes Cu in substrate), on rumen fermentation in yaks. In vitro dry matter degradability (IVDMD) and amylase activity were greater ($p < 0.05$) with added Met-Cu than the other two Cu sources, and ammonia nitrogen (NH₃-N), microbial protein (MCP) and propionate contents were greater with Met-Cu and CuCl₂ than with TBCC. Total gas production and lipase activity were greater with Met-Cu and TBCC than CuCl₂ ($p < 0.05$), which meant that the metabolizable energy yield was greater in the two former Cu sources than the latter, but CH₄ production did not differ ($p = 0.92$) among Cu sources. IVDMD and lipase activity were greatest ($p < 0.05$) at 15 mg Cu/kg DM in the substrate and MCP, isobutyrate, butyrate and isovalerate contents, and amylase and trypsin activities were greatest or second greatest at 10 and 15 mg Cu/kg DM. It was concluded that Met-Cu was the best source of Cu and 10 to 15 mg Cu/kg DM was the optimal level for yaks, at least under in vitro conditions.

Keywords: yak; copper source; copper level; in vitro gas production technique; rumen fermentation



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1. Introduction

The yak (*Poephagus grunniens*), an indigenous herbivore raised at 3000 to 5000 m above sea level across the Asian highlands, is one of the main livestock grazing on the Qinghai-Tibetan Plateau (QTP) [1,2]. At present, there are approximately 17.6 million yaks in the world, of which more than 95% are raised in China. Yaks play a vital role in ecosystem stability, livelihood security, socio-economic development and ethnic cultural traditions [1,2].

Copper (Cu) is essential for the health of the animal, as it is involved in the activity of numerous enzymes, including caeruloplasmin, cytochrome c oxidase, lysyl oxidases, superoxide dismutases and tyrosinase, cofactors and reactive proteins. It is also involved in iron metabolism, the electron transport chain of cellular respiration, connective tissue development, the antioxidant system and pigmentation [3,4]. A study in dairy bulls reported that the addition of 7.68 mg Cu/kg DM increased the activities of ruminal cellulolytic enzymes and protease but did not affect amylase activity [5].

Copper deficiency of grazing yaks on the QTP can be severe and widespread throughout the year. Yaks grazing in the eastern region of the QTP often suffer from secondary Cu deficiency, known colloquially as “swayback disease”, as a result of the high iron content in forage [6]. The prevalence has been estimated at 50 to 60% and mortality can reach 70% [6], making it necessary to provide supplementary Cu to yaks. The nutritional requirement

of Cu for beef cattle, as recommended by NRC, is approximately 10 mg/kg DM [7], but this also depends on the breed of cattle [8,9]. Limited research indicated that the oral administration of Cu sulfate [6,10] and soluble glass Cu bolus [11] can improve the Cu balance of grazing yaks.

Copper supplements used commonly in ruminants can be categorized broadly as inorganic, including Cu sulfate, copper chloride (CuCl_2), Cu acetate, Cu carbonate, Cu citrate, and tribasic copper chloride (TBCC), or organic, including Cu proteinate and amino acid complexes (e.g., copper methionine (Met-Cu)) [3]. Cattle offered either organic or inorganic Cu did not differ in growth rate or Cu status [12,13]. The bioavailability of Cu lysine and Cu sulfate were similar in vitro [14], and the bioavailability of Cu glycinate was greater than that of Cu sulfate in diets with high sulfur and molybdenum contents [15].

Although Cu is essential for the health of animals, the effects of the source and level of Cu on rumen fermentation in yaks are still uncertain. To fill this gap, we fed growing yaks three sources and five levels of Cu and examined rumen fermentation parameters by employing an in vitro gas production system.

2. Materials and Methods

2.1. Animals and Feeding

All animal procedures followed the Guidelines for the Care and Utilization of Laboratory Animals of Qinghai Province (Qinghai Agriculture and Animal Husbandry Bureau, 2002), and were approved by the Committee of Animal use of the Academy of Science and Veterinary Medicine of Qinghai University (QHU20150301).

Three healthy rumen-fistulated Datong steer yaks of similar body condition and approximately 150 kg were housed individually and were used as rumen fluid donors. They were offered 4 kg/d DM of feed (Table 1) that, according to the Beef Cattle Breeding in China (NY/T815-2004) [16] and yak nutrition monograph [17], should provide slightly above energy and nutrient requirements. Yaks were fed 2 kg at 8:00 and 2 kg at 18:00, had free access to fresh water, and were allowed a 15-day adaptation period to the conditions.

Table 1. Composition and nutrient levels of diet/substrate (dry matter basis).

Item ¹	g/100 g DM	Component ²	g/100 g DM
Corn flour	36.00	DM (g/100 g FM)	90.98
Wheat bran	12.00	CP	11.03
Soybean meal	6.60	NDF	31.12
Rapeseed meal	2.13	ADF	17.60
Stone powder	0.90	Ca	0.88
CaHPO_4	0.17	P	0.19
NaCl	0.58	Cu (mg/kg)	4.39
Oat hay	40.00		

¹ CaHPO_4 : calcium hydrogen phosphate, NaCl: sodium chloride. ² DM: dry matter; FM: fresh matter; CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; Ca: calcium; P: phosphorus.

The substrate was prepared at a 60:40 ratio of concentrate to forage. The concentrate and hay were ground, sieved through a 40 mesh (0.45 mm) screen, and then mixed thoroughly to the desired ratio.

2.2. Experimental Design and In Vitro Measurements

A 3×5 two-factor design was used with 3 sources and 5 levels of Cu. The 3 sources were Cu methionine (Met-Cu), Cu chloride (CuCl_2) and tribasic Cu chloride (TBCC) (Xingjia Bioengineering Company, Changsha, China), which were added to the substrate so that the total Cu content would equal 5, 10, 15, 20 or 25 mg/kg.

Before morning feeding, approximately 400 mL of rumen fluid were collected from each yak by stomach tube and vacuum pump (2 XZ-1, Kewei Yongxing Instrument Company, Beijing, China). The sample from each yak was filtered through four layers of gauze

into a pre-warmed 39 °C thermos, mixed evenly and sealed. A buffer solution was prepared according to Menke and Steingass [18], and the rumen fluid and the buffer solution were mixed at a ratio of 1:2 (*v/v*) as a culture solution. The solution was stored in a water bath at 39 °C, with a continuous flow of CO₂.

The *in vitro* gas production system followed Menke and Steingass [18]. Briefly, 200 mg of fermentation substrate, with the added Cu, were placed in a 100 mL glass syringe (DE-89173, Häberle Labortechnik, Lonsee-Ettlenschieß, Germany), 30 mL of culture solution were added and the mixture was shaken gently. The air in the glass syringe was removed, and the syringe was placed in a vibrating incubator at an oscillation speed of 40 r/min at 39 °C for 48 h. There were 3 replicates for each treatment, 3 blanks without substrate for each treatment and 3 hay standards from the University of Hohenheim (Germany) for gas correction for each incubation run, as suggested by Menke and Steingass [18]. At the end of 48 h incubation, the total gas production was recorded and stored in vacuum tubes (Xinle Company, Shijiazhuang, China) to determine gas composition. Then, the syringes were placed in ice-water to terminate fermentation. The pH of the fermentation solution was measured immediately. After filtering (filter 101, Beimu Pulp and Paper Company, Hangzhou, China) the content, the filtrate was preserved by adding 5 mL of 0.2 mol/L HCl and 0.2 mL of 25% (*w/v*) metaphosphoric acid, and stored at −20 °C for measurements of ammonia nitrogen (NH₃-N, 5 mL) and volatile fatty acids (VFAs, 2 mL). The filtrate for the measurement of microbial protein (MCP, 2 mL) was stored at −20 °C, and for digestive enzyme activities (5 mL) at −80 °C. The fermentation residue was collected and dried to a constant weight at 105 °C to measure the *in vitro* dry matter degradability (IVDMD).

2.3. Analytical Procedures and Measurements

The Cu content in the fermented substrate was determined by a flame atomic absorption spectrophotometer (TAS-990 Super, Beijing Puxi Instrument Company, Beijing, China), using an air-acetylene flame, 324.9 nm wavelength, 3 mA lamp current, 0.4 nm slit, 6.0 mm burner height, and 2 000 mL/min acetylene gas flow. According to the method described in GB/T13885-2003 [19], approximately a 5.0 g substrate sample was heated in an electromagnetic oven (K-98-2, MITR Instrument and Equipment Company, Changsha, China) until carbonized. The carbonized sample was combusted completely for 4 h at 550 °C in a muffle furnace (SX-4-10, Taisite Instrument Company, Tianjin, China). The ash was cooled to room temperature, dissolved in 5 mL of 6 mol/L HCl, and diluted to 50 mL with ultra-pure water (20.4 mΩ) in a volumetric flask.

The volume of CH₄ in the total gas produced was determined by gas chromatography (GC-2014, Shimadzu, Kyoto, Japan), using a free fatty acid phase (FFAP) capillary column (30 m × 0.32 mm × 0.5 µm) and flame ionization detection (FID). The column and injector temperatures were 100 °C, and the detector temperature was 110 °C. Nitrogen was used as the carrier gas.

The pH was determined by a pH meter (Hanna HI221, Ann Arbor, MI, USA). The contents of NH₃-N and MCP were measured separately by spectrophotometry (TU-1810, Beijing Puxi Instrument Company, Beijing, China) following Feng and Gao [20], and the instructions of the commercial kit (A045-1-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Detailed operating steps were described in our previous publication [21]. VFAs were determined by gas chromatography (GC-2014, Shimadzu, Kyoto, Japan), using a FFAP capillary column (30 m × 0.32 mm × 0.5 µm) and FID, as described by Cao et al. [22] and Wang [23]. The initial column temperature was 60 °C, followed by an increase of 10 °C/min to 120 °C, which was held for 2 min and further increased by 15 °C/min to 180 °C, which was held for 5 min. The injector and detector temperatures were 250 °C and nitrogen was used as the carrier gas.

The fermentation solution was homogenized in ice-water using a magnetic heating agitator (79-1, Dadi Automation Instrument Company, Jintan, China). The homogenate was centrifuged at 2 500 r/min at 4 °C for 10 min to measure the digestive enzyme activities, except for cellulase, which was centrifuged at 8 500 r/min for 10 min. The digestive enzyme

activities in the supernatant were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China): amylase activity with an amylase kit (C016-1-1), lipase activity with a lipase kit (A054-1-1), trypsin activity with a trypsin kit (A080-2-1), and cellulase activity with a cellulase kit (A138-1-1).

2.4. Statistical Analyses

A two-way ANOVA (SPSS version 19.0, SPSS Inc., Chicago, IL, USA) was used to compare means among Cu sources and among Cu levels. When the source $\times$ interaction was significant, then a one-way ANOVA was used to compare means within a Cu level and among Cu sources. In addition, a one-way ANOVA was used to compare the Cu levels within each Cu source. Where significance existed, Duncan's range test separated the means. Orthogonal polynomial contrasts were used to determine whether the effect of Cu on the variable measured was linear or quadratic with an increase in Cu level. A level of $p < 0.05$ was accepted as significant and values are expressed as means $\pm$ SE.

3. Results

3.1. In Vitro Gas Production, Degradability and Ammonia Nitrogen and Microbial Protein Contents

The interaction between source $\times$ level of Cu did not affect total gas production ($p = 0.67$), IVDMD ($p = 0.61$) or $\text{NH}_3\text{-N}$ ($p = 0.11$), but did affect ($p < 0.01$) CH_4 production and MCP (Table 2). The total gas productions in Met-Cu and TBCC were greater ($p < 0.01$) than in CuCl_2 , but was not affected ($p = 0.49$) by the level of Cu. The CH_4 production decreased linearly ($p < 0.05$) with an increase in CuCl_2 level, but was not affected by the source ($p = 0.92$) or level ($p = 0.11$) of Cu. IVDMD was greater ($p < 0.001$) with Met-Cu than with the other two sources; $\text{NH}_3\text{-N}$ content was greater ($p < 0.001$) with Met-Cu than with TBCC but not greater than with CuCl_2 (Table 2). IVDMD at 15 mg Cu/kg DM was greater ($p < 0.05$) than at the other Cu levels and changed quadratically ($p < 0.05$) with an increase in Cu level. The MCP content with 10 mg Cu/kg DM was greater ($p < 0.05$) than with 5, 20 and 25 mg Cu/kg DM, but not greater than 15 mg Cu/kg DM (Table 2).

Table 2. In vitro total gas and methane (CH_4) productions, dry matter degradability (IVDMD), ammonia nitrogen ($\text{NH}_3\text{-N}$) and microbial protein (MCP) from substrate with added copper (Cu) from different sources at different levels ¹.

Cu Source	Cu (mg/kg DM) ²	Total Gas Production (mL)	CH_4 Production (mL)	IVDMD (%)	$\text{NH}_3\text{-N}$ (mg/dL)	MCP (g/L)
Met-Cu	5 mg/kg	63.5	6.69	44.6 ^c	11.00	2.89 ^b
	10 mg/kg	68.8	5.54	59.8 ^b	13.66 ^A	5.49 ^{Aa}
	15 mg/kg	71.7 ^A	7.88 ^A	74.1 ^a	12.19 ^A	2.60 ^b
	20 mg/kg	64.8	5.97	65.1 ^{Aab}	12.07 ^A	2.31 ^b
	25 mg/kg	61.3	6.92	61.6 ^b	12.84 ^A	2.60 ^b
	Treat	0.689	0.079	0.004	0.383	0.012
p -value ³	L	0.643	0.606	0.008	0.487	0.055
	Q	0.214	0.983	0.002	0.493	0.347
TBCC	5 mg/kg	65.5	6.37 ^b	38.0 ^c	10.55	1.01 ^c
	10 mg/kg	66.5	6.09 ^b	49.6 ^{bc}	9.01 ^B	3.27 ^{Aa}
	15 mg/kg	68.2 ^A	6.43 ^{Bb}	68.0 ^a	8.20 ^B	2.32 ^b
	20 mg/kg	70.0	7.21 ^a	49.2 ^{Bbc}	9.92 ^B	1.25 ^c
	25 mg/kg	67.8	6.23 ^b	56.0 ^{ab}	10.58 ^B	1.13 ^c
	Treat	0.183	0.042	0.026	0.082	0.001
p -value	L	0.063	0.263	0.056	0.628	0.066
	Q	0.212	0.283	0.037	0.013	0.001
CuCl_2	5 mg/kg	61.5	7.49	45.6	11.91	1.16
	10 mg/kg	63.5	7.50	56.2	12.57 ^A	2.31 ^B
	15 mg/kg	60.5 ^B	6.74 ^B	62.2	12.32 ^A	4.33
	20 mg/kg	56.7	4.37	59.5 ^{AB}	12.28 ^A	3.47
	25 mg/kg	58.3	6.32	53.4	12.33 ^A	2.60

Table 2. Cont.

Cu Source	Cu (mg/kg DM) ²	Total Gas Production (mL)	CH ₄ Production (mL)	IVDMD (%)	NH ₃ -N (mg/dL)	MCP (g/L)
<i>p</i> -value	Treat	0.183	0.078	0.097	0.535	0.073
	L	0.056	0.044	0.161	0.533	0.096
	Q	0.839	0.438	0.017	0.326	0.024
Source	Met-Cu	66.0 ^a	6.60	61.0 ^a	12.35 ^a	3.17 ^a
	TBCC	67.6 ^a	6.47	52.2 ^b	9.65 ^b	1.80 ^b
	CuCl ₂	60.1 ^b	6.48	55.4 ^b	12.28 ^a	2.77 ^a
Level	5 mg/kg	63.5	6.85	42.7 ^c	11.16	1.68 ^c
	10 mg/kg	66.3	6.38	55.2 ^b	11.75	3.69 ^a
	15 mg/kg	66.8	7.02	68.1 ^a	10.90	3.08 ^{ab}
	20 mg/kg	63.8	5.85	57.9 ^b	11.42	2.34 ^{bc}
	25 mg/kg	62.5	6.49	57.0 ^b	11.91	2.11 ^c
<i>p</i> -value	L	0.485	0.225	<0.001	0.324	0.661
	Q	0.127	0.724	<0.001	0.414	<0.001
SEM ⁴		0.97	0.17	1.68	0.26	0.21
<i>p</i> -value	Source	0.004	0.916	0.010	< 0.001	0.001
	Level	0.493	0.111	<0.001	0.324	0.001
	S × L ⁵	0.670	0.007	0.609	0.106	0.003

¹ Means with different lowercase letters within sources and among levels for each variable differ significantly from each other ($p < 0.05$). Means with different uppercase letters within levels and among sources for each variable differ significantly from each other ($p < 0.05$). ² Includes added Cu and Cu in substrate. ³ L: Linear effect; Q: Quadratic effect. ⁴ SEM: Standard error of the mean. ⁵ S × L: Source × Level interaction.

3.2. In Vitro pH and Volatile Fatty Acids

The interaction between source × level of Cu did not affect ($p = 0.20$) the A:P ratio, but did affect pH ($p < 0.001$), total VFAs ($p < 0.05$), acetate ($p < 0.05$), propionate ($p < 0.01$), isobutyrate ($p < 0.01$), butyrate ($p < 0.001$), isovalerate ($p < 0.01$) and valerate ($p < 0.01$) contents (Table 3). The isovalerate content with TBCC was greater ($p < 0.001$) than with the other sources; valerate content with CuCl₂ was greater ($p < 0.001$) than with the other two sources and the A:P ratio was greater ($p < 0.05$) with Met-Cu and TBCC than with CuCl₂ (Table 3). The contents of isobutyrate and isovalerate were greatest at 15 mg Cu/kg DM, while the content of butyrate was greater ($p < 0.05$) at 10 and 15 mg Cu/kg DM than at the other levels. Mainly quadratic changes were observed with an increase in Cu level for two sources of Cu. For Met-Cu, pH ($p < 0.05$), propionate ($p < 0.05$), butyrate ($p < 0.01$), valerate ($p < 0.01$), isobutyrate ($p < 0.01$) and isovalerate ($p < 0.01$) changed quadratically; for TBCC, total VFAs ($p < 0.05$), acetate ($p < 0.05$) and butyrate ($p < 0.01$) changed quadratically. In these cases, there was an increase in the variable measured and then a decrease. However, with CuCl₂, concentrations of total VFAs ($p < 0.01$), propionate ($p < 0.01$), butyrate ($p < 0.001$), acetate ($p < 0.05$), isobutyrate ($p < 0.01$) and isovalerate ($p < 0.01$) all decreased linearly with an increase in Cu level (Table 3).

Table 3. In vitro pH, total volatile fatty acids (VFAs) and individual VFAs and acetate:propionate (A:P) ratio from substrate with added copper (Cu) from different sources at different levels ¹.

Cu Source	Cu (mg/kg DM) ²	pH	Total VFA (mmol/L)	Individual VFAs (mmol/L)						A:P Ratio
				Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	
Met-Cu	5 mg/kg	7.23 ^{Aa}	60.9 ^B	36.5 ^B	16.8 ^B	0.29 ^{Bb}	5.98 ^{Bb}	0.76 ^{Cb}	0.60 ^{Bb}	2.18 ^a
	10 mg/kg	6.99 ^{bc}	80.2	44.7	24.6	0.44 ^a	8.50 ^{Ba}	1.13 ^{Ba}	0.94 ^{Ba}	1.82 ^c
	15 mg/kg	6.95 ^c	93.0	55.5	25.8	0.53 ^a	9.96 ^a	1.30 ^a	1.10 ^a	1.92 ^{abc}
	20 mg/kg	7.06 ^{ABbc}	85.9	48.8	25.6	0.48 ^a	8.89 ^a	1.17 ^a	0.97 ^a	1.91 ^{bc}
	25 mg/kg	7.10 ^{Ab}	87.4	52.4 ^A	24.2	0.43 ^a	8.32 ^a	1.05 ^a	0.88 ^{Ba}	2.17 ^{Aab}
<i>p</i> -value ³	Treat	0.005	0.214	0.324	0.073	0.006	0.016	0.010	0.010	0.034
	L	0.180	0.076	0.106	0.043	0.014	0.032	0.039	0.032	0.833
	Q	0.001	0.142	0.293	0.035	0.002	0.005	0.002	0.002	0.004

Table 3. Cont.

Cu Source	Cu (mg/kg DM) ²	pH	Total VFA (mmol/L)	Individual VFAs (mmol/L)						A:P Ratio
				Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	
TBCC	5 mg/kg	7.00 ^B	58.6 ^{Bb}	33.9 ^{Bb}	16.1 ^B	0.36 ^B	6.65 ^{Bbc}	1.03 ^B	0.59 ^B	2.11
	10 mg/kg	7.03	91.3 ^a	54.5 ^a	23.4	0.55	10.26 ^{Aa}	1.55 ^A	0.98 ^B	2.33
	15 mg/kg	6.93	77.2 ^{ab}	42.6 ^{ab}	22.1	0.55	9.59 ^{ab}	1.51	0.92	1.96
	20 mg/kg	6.84 ^B	65.4 ^b	39.2 ^b	17.8	0.36	6.46 ^c	1.03	0.59	2.27
	25 mg/kg	6.72 ^B	68.5 ^b	38.1 ^{Bb}	20.7	0.44	7.22 ^{bc}	1.24	0.73 ^B	1.85 ^B
<i>p</i> -value	Treat	0.167	0.043	0.048	0.169	0.087	0.037	0.092	0.054	0.521
	L	0.023	0.772	0.611	0.604	0.902	0.369	0.829	0.729	0.420
	Q	0.415	0.042	0.050	0.169	0.094	0.035	0.092	0.061	0.479
CuCl ₂	5 mg/kg	6.84 ^{Cc}	96.4 ^{Aa}	51.3 ^A	30.5 ^{Aa}	0.61 ^{Aa}	11.18 ^{Aa}	1.46 ^{Aa}	1.29 ^{Aa}	1.69
	10 mg/kg	7.02 ^b	83.0 ^b	46.0	24.8 ^b	0.48 ^b	9.03 ^{Bb}	1.14 ^{Bb}	1.60 ^{Aa}	1.86
	15 mg/kg	7.05 ^b	84.3 ^b	46.4	26.0 ^b	0.49 ^b	9.17 ^b	1.16 ^b	1.17 ^{ab}	1.79
	20 mg/kg	7.26 ^{Aa}	67.4 ^c	37.9	20.7 ^c	0.34 ^c	6.99 ^c	0.85 ^c	0.71 ^b	1.84
	25 mg/kg	7.10 ^{Ab}	80.0 ^b	44.7 ^{AB}	24.0 ^{bc}	0.43 ^{bc}	8.29 ^{bc}	1.01 ^{bc}	1.54 ^{Aa}	1.85 ^B
<i>p</i> -value	Treat	<0.001	0.005	0.079	0.002	0.004	0.002	0.004	0.027	0.783
	L	<0.001	0.002	0.038	0.001	0.001	<0.001	0.001	0.497	0.384
	Q	0.004	0.040	0.179	0.020	0.051	0.029	0.065	0.141	0.627
Source	Met-Cu	7.07 ^a	81.5	47.6	23.4 ^a	0.43	8.33	1.08 ^b	0.90 ^b	2.00 ^a
	TBCC	6.91 ^b	72.2	41.7	20.0 ^b	0.45	8.04	1.27 ^a	0.76 ^b	2.10 ^a
	CuCl ₂	7.05 ^a	82.2	45.3	25.2 ^a	0.47	8.93	1.12 ^b	1.26 ^a	1.81 ^b
Level	5 mg/kg	7.02	72.0	40.6	21.1	0.42 ^{bc}	7.94 ^b	1.08 ^{bc}	0.83 ^{bc}	2.00
	10 mg/kg	7.01	84.9	48.4	24.3	0.49 ^{ab}	9.27 ^a	1.27 ^{ab}	1.17 ^a	2.00
	15 mg/kg	6.98	84.9	48.1	24.6	0.52 ^a	9.57 ^a	1.32 ^a	1.06 ^{ab}	1.89
	20 mg/kg	7.05	72.9	42.0	21.3	0.39 ^c	7.44 ^b	1.02 ^c	0.76 ^c	2.01
	25 mg/kg	6.98	78.6	45.1	23.0	0.43 ^{bc}	7.94 ^b	1.10 ^{bc}	1.05 ^{ab}	1.96
<i>p</i> -value	L	0.612	0.916	0.776	0.808	0.375	0.165	0.267	0.886	0.788
	Q	0.877	0.089	0.142	0.113	0.303	0.010	0.022	0.263	0.728
SEM ⁴		0.02	2.28	1.41	0.70	0.02	0.27	0.04	0.05	0.04
<i>p</i> -value	Source	<0.001	0.054	0.155	0.001	0.442	0.133	0.029	<0.001	0.012
	Level	0.454	0.071	0.179	0.090	0.006	0.002	0.007	0.001	0.862
	S × L ⁵	<0.001	0.011	0.048	0.003	0.001	<0.001	0.002	0.007	0.197

¹ Means with different lowercases letters within sources and among levels for each variable differ significantly from each other ($p < 0.05$). Means with different uppercase letters within levels and among sources for each variable differ significantly from each other ($p < 0.05$). ² Includes added Cu and Cu in substrate. ³ L: Linear effect; Q: Quadratic effect. ⁴ SEM: Standard error of the mean. ⁵ S × L: Source × Level interaction.

3.3. In Vitro Digestive Enzyme Activities

The interaction between source × level of Cu affected lipase ($p < 0.001$) and trypsin ($p < 0.05$) activities (Table 4). The Cu source affected amylase ($p < 0.001$) and lipase ($p < 0.001$) activities, while the Cu level affected amylase ($p < 0.001$), lipase ($p < 0.001$), trypsin ($p < 0.001$) and cellulase ($p < 0.01$) activities. The amylase activity with Met-Cu > TBCC > CuCl₂ ($p < 0.05$), and the lipase activities with Met-Cu and TBCC were greater ($p < 0.05$) than with CuCl₂ (Table 4). The amylase ($p < 0.001$), lipase ($p < 0.001$), trypsin ($p < 0.001$) and cellulase ($p < 0.05$) activities changed quadratically with an increase in Cu level. The amylase activity at 10 mg Cu/kg DM was greater ($p < 0.05$) than at 5, 20 and 25 mg Cu/kg DM but not at 15 mg Cu/kg DM, while the lipase activity at 15 mg Cu/kg DM was greater ($p < 0.05$) than at the other levels. The trypsin activity at 10 and 15 mg Cu/kg DM was greater ($p < 0.05$) than at the other levels, while the cellulase activity at 5 mg Cu/kg DM was lesser ($p < 0.05$) than at the other levels (Table 4).

Table 4. In vitro digestive enzyme activities from substrate with added copper (Cu) from different sources at different levels ¹.

Cu Source	Cu (mg/kg DM) ²	Amylase (U/mL)	Lipase (U/mL)	Trypsin (U/mL)	Cellulase (U/mL)
Met-Cu	5 mg/kg	1.99 ^{Ac}	0.46 ^{Bc}	46.8 ^b	56.8
	10 mg/kg	2.25 ^{Aa}	0.58 ^{Aa}	88.8 ^{Aa}	70.7
	15 mg/kg	2.20 ^{Ab}	0.52 ^{Ab}	46.8 ^b	78.8
	20 mg/kg	1.99 ^{Ac}	0.43 ^c	37.4 ^b	80.0
	25 mg/kg	1.97 ^{Ac}	0.28 ^{Bd}	32.7 ^b	88.1
	Treat	<0.001	<0.001	0.004	0.112
<i>p</i> -value ³	L	<0.001	<0.001	0.009	0.013
	Q	<0.001	<0.001	0.065	0.525
TBCC	5 mg/kg	1.49 ^{AB}	0.45 ^{Aab}	32.7	55.6 ^b
	10 mg/kg	2.12 ^B	0.41 ^{Bb}	51.4 ^B	74.2 ^a
	15 mg/kg	1.76 ^B	0.52 ^{Aa}	42.1	81.1 ^a
	20 mg/kg	1.67 ^A	0.46 ^{ab}	37.4	84.6 ^a
	25 mg/kg	1.37 ^B	0.41 ^{Ab}	37.4	81.1 ^a
	Treat	0.122	0.039	0.598	0.019
<i>p</i> -value	L	0.271	0.664	0.863	0.004
	Q	0.047	0.046	0.320	0.036
CuCl ₂	5 mg/kg	0.98 ^{Bc}	0.22 ^{Ad}	32.7 ^b	59.1
	10 mg/kg	1.23 ^{Ca}	0.36 ^{Bc}	46.8 ^{Bb}	64.9
	15 mg/kg	1.31 ^{Ca}	0.42 ^{Ba}	70.1 ^a	69.5
	20 mg/kg	1.21 ^{Bab}	0.39 ^{ab}	42.1 ^b	76.5
	25 mg/kg	1.10 ^{Bbc}	0.37 ^{Abc}	37.4 ^b	64.9
	Treat	0.001	<0.001	0.014	0.466
<i>p</i> -value	L	0.136	<0.001	0.818	0.291
	Q	<0.001	<0.001	0.004	0.217
Source	Met-Cu	2.08 ^a	0.45 ^a	50.5	74.9
	TBCC	1.68 ^b	0.45 ^a	40.2	75.3
	CuCl ₂	1.17 ^c	0.35 ^b	45.8	67.0
Level	5 mg/kg	1.49 ^c	0.37 ^c	37.4 ^b	57.2 ^b
	10 mg/kg	1.87 ^a	0.45 ^b	62.3 ^a	69.9 ^a
	15 mg/kg	1.76 ^{ab}	0.49 ^a	53.0 ^a	76.5 ^a
	20 mg/kg	1.62 ^{bc}	0.42 ^b	39.0 ^b	80.3 ^a
	25 mg/kg	1.48 ^c	0.35 ^c	35.9 ^b	78.0 ^a
	Treat	0.200	0.034	0.064	<0.001
<i>p</i> -value	L	0.200	0.034	0.064	<0.001
	Q	<0.001	<0.001	<0.001	0.027
SEM ⁴		0.07	0.01	2.75	2.05
<i>p</i> -value	Source	<0.001	<0.001	0.114	0.092
	Level	<0.001	<0.001	<0.001	0.001
	Source × Level	0.293	<0.001	0.011	0.729

¹ Means with different lowercases letters within sources and among levels for each variable differ significantly from each other ($p < 0.05$). Means with different uppercase letters within levels and among sources for each variable differ significantly from each other ($p < 0.05$). ² Includes added Cu and Cu in substrate. ³ L: Linear effect; Q: Quadratic effect. ⁴ SEM: Standard error of the mean.

4. Discussion

4.1. In Vitro Digestive Enzyme Activities

In the present study, added Met-Cu resulted in the greatest IVDMD and amylase activity among sources, and Met-Cu and CuCl₂ had greater NH₃-N, MCP and propionate contents than TBCC. The high propionate content was consistent with the high amylase activity, indicating that the addition of Met-Cu had a positive effect on rumen fermentation, and the fermentation pattern was directed to a greater propionate production, which can provide high effective energy for ruminants [24]. The high NH₃-N content with the Met-Cu

addition reflected the greater catabolism of protein and non-protein N compounds [25,26]; thus, an adequate supply of substrate and N could explain the increase in MCP synthesis. The total gas productions with Met-Cu and TBCC were greater than with CuCl₂. The in vitro gas production technique is based on the positive correlation between gas production and organic fermentation or metabolizable yield [18]. This would indicate that added Met-Cu and TBCC fermented the substrate to a greater extent and produced a greater yield of metabolizable energy than CuCl₂. These findings were consistent with those of Katulski [27], who reported that the total gas production with TBCC was greater than with CuCl₂ at 24 h. Based on the above results on IVDMD, total gas production and MCP content, Met-Cu proved to be the best supplementary Cu source for yaks.

4.2. Effects of Different Copper Levels on pH and In Vitro Rumen Fermentation

The pH in the rumen was slightly acidic [28]. The solubility of the three Cu sources at pH 6.5 was CuCl₂ > Met-Cu > TBCC due to different chemical bonds [29]. The high solubility of CuCl₂ in the rumen could increase the potential for Cu to interact with molybdenum, sulfur, iron, manganese and zinc [3,28]. The ruminal pH in the present study was within the optimal range of 6.2–7.2 [30], and the Cu level did not affect ruminal pH and total VFA content. This result was consistent with the in vitro findings of Katulski [27], who reported that supplementing the diets of Jersey steers with 10 to 70 mg Cu/kg DM had no effect on the ruminal pH and total VFA content. Similarly, in vivo results in Angus steers reported that the addition of 10 or 20 mg Cu/kg DM had no effect on the ruminal pH and VFA molar proportions [31]. However, in an in vitro study in lactating Holsteins, ruminal pH decreased with the supplementation of 60 mg Cu/kg DM when compared with 0, 20, 80 or 100 mg Cu/kg DM [32]. Differences between donor animals, donor animal diets, culture substrates and methodology could be the reason for these differences. Ruminal NH₃-N is primarily a consequence of the microbial degradation of protein and non-protein N compounds, and is used for MCP synthesis [25,26]. High or low concentrations of NH₃-N are not conducive to rumen fermentation [33]. In the current study, ruminal NH₃-N content fell within the optimal range of 6.3–27.4 mg/dL [34].

The gases released during rumen fermentation are primarily CO₂, CH₄ and hydrogen produced by carbohydrate fermentation [35,36]. The Cu level had no effect on total gas and CH₄ productions, which was consistent with Katulski [27], who reported no difference in total gas production at 24 h with 10, 20, 30 and 40 mg Cu/kg DM added to the diet. However, the total gas production decreased with a Cu level above 40 mg/kg DM. The unaltered CH₄ production in the present study was consistent with the unaltered contents of acetate and propionate, as the productions of acetate and propionate are accompanied by an increase and decrease, respectively, of hydrogen formation, which is the main energy source for the growth of methanogens, and is ultimately converted into CH₄ [26,37]. Shang et al. [5] observed that the addition of 7.68 mg Cu/kg DM increased the populations of methanogens and protozoa in the rumen of dairy bulls; however, other studies reported that high levels of Cu can decrease ruminal CH₄ production [32] or decrease the trend of enteric CH₄ production [38]. Kholif et al. [39] reported that supplementary 9 mg Cu/kg DM in the diet of Boer goats decreased CH₄ production by approximately 5%. The equivocal results among studies could be due to different populations of microbiota or the composition of the fermentation substrate [26] as well as different Cu sources and levels.

Rumen fermentation variables reflect the fermentation conditions and environmental changes in the rumen [40]. The greater IVDMD at 15 mg Cu/kg DM was consistent with the greater butyrate, isobutyrate and isovalerate contents, and the positive effects on ruminal digestive enzyme activities. Similarly, total tract DM digestibility, as well as the activities of cellulolytic enzymes and protease, increased with the addition of 7.68 mg Cu/kg DM as Cu sulphate to a diet containing 7.5 mg Cu/kg DM in dairy bulls [5]. This indicates that 15 mg Cu/kg DM (dietary and supplementary Cu) had a positive effect on rumen fermentation, as was also observed in the present study.

Differences in the VFA profile are most probably related to differences in digestive enzyme activities caused by different levels of Cu additives. In the present study, the Cu source affected the pattern of the profile. With the supplementary CuCl₂, the concentrations of total VFAs, propionate, acetate, butyrate, isobutyrate and isovalerate all decreased linearly with an increase in Cu level. This meant that CuCl₂ had a detrimental effect on the production of the VFAs. However, with Met-Cu and TBCC, these VFAs changed either quadratically or did not change with an increase in Cu level. There was an initial increase in the concentration of VFAs to 10 or 15 mg Cu/kg DM and then a decrease, which demonstrated a positive effect on the Cu sources, at least initially. Ruminal acetate is produced mainly by fiber degradation, whereas propionate and butyrate are produced mainly by starch degradation [26,41]. The unaltered acetate content was linked to cellulase activity. Cellulase was lowest at 5 mg Cu/kg DM, as was acetate content, albeit the low acetate content was not significant. The content of butyrate was greatest at 10 and 15 mg Cu/kg DM, which corresponded to the high amylase activity at these Cu levels. Moreover, the unaltered A:P ratio indicated that the addition of 5 to 25 mg Cu/kg DM did not alter the rumen fermentation pattern. Ruminal isobutyrate and isovalerate are mainly the degradation products of four branched-chain amino acids and are important growth factors for some rumen bacteria [42,43]. The contents of isobutyrate and isovalerate were greatest at 10 and 15 mg Cu/kg DM, and the NH₃-N content was unaltered. The results were consistent with the increase in trypsin activity, and the unaltered NH₃-N content could be attributed to the increase in MCP synthesis.

5. Conclusions

The source and level of Cu affected *in vitro* rumen fermentation in growing yaks. Substrate with Met-Cu had the greatest IVDVD, and high gas production and propionate content, which indicated high metabolizable energy yield and fermentation directed towards propionate production. Met-Cu proved to be the best supplementary Cu source, and 10 to 15 mg Cu/kg DM, which included Cu in the substrate, was the optimal level for growing yaks.

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