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Research article

Effect of combination of vitamin-mineral supplementation and concentrate-to-roughage ratio diets on ruminal fermentation, gas kinetics and microbial biomass production using *in vitro* gas production technique

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Abstract

A vitamin-mineral combination consisting of betaine, biotin and chromium picolinate (BBC) was developed and the effects of BBC supplementation were investigated using an *in vitro* gas production technique. The effects were examined on the gas kinetics, microbial biomass production (MBP) and rumen fermentation. The experimental design was a 3×4 factorial arrangement in a completely randomized design. Factor A was the concentrate (C)-to-roughage (R) ratio of diets with three levels (50C:50R, 60C:40R, 70C:30R) and factor B was the amount of BBC supplementation with four levels (0 g/kg dry matter (DM); 3 g/kg DM; 6 g/kg DM; 9 g/kg DM). The gas production was recorded at 1 hr, 2 hr, 4 hr, 6 hr, 8 hr, 12 hr, 16 hr, 24 hr and 48 hr of incubation. The gas kinetics, truly digestible organic matter (TDOM), MBP and efficiency of MBP were measured. Ammonia nitrogen (NH_3-N) and total volatile fatty acids (TVFA) were analyzed in fluid. The results revealed that the gas kinetics (*a* and *b*) did not differ among treatments, but the gas production rate constants (c) increased when the C:R ratio in diets increased ($p < 0.05$). TDOM, MBP and EMBP differed ($p < 0.05$) among treatments, and the C:R ratios influenced ($p < 0.05$) the extent of the difference, but the amount of BBC supplementation did not. The NH_3-N concentration was greater ($p < 0.05$) in diets supplemented with BBC than in a diet without BBC supplementation. Increasing the C:R ratio in the diet resulted in lower acetic acid and higher propionic acid proportions ($p < 0.01$). In conclusion, increasing the C:R ratio from 50C:50R to 60C:40R or 70C:30R resulted in enhanced TDOM, MBP and TVFA. BBC inclusion had no positive effect.

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Introduction

To date, no single feed additive or management practice has been able to enhance animal growth cost-effectively. Alternative feed additives such as dietary acidifiers, plant extracts, essential oils, probiotics and prebiotics have been introduced as potential replacements for antibiotics (Mohammed et al., 2013). However, the limitations of such feed additives in effectively increasing the production efficiency have resulted in little or limited application (Benchaar et al., 2016). Commercial feed additives have been developed to enhance the performance of ruminants, such as betaine, biotin, chromium picolinate and other substances to maximize the effectiveness of each substance combination.

There are a number of feed additives that are applied in ruminant feed. A mixture of feed additives involving metabolism in rumen microbes and animals is being considered. Betaine, biotin and chromium have been shown to improve nutrient utilization and animal performance. For example, betaine is one feed additive that is favored for use in heat-stressed animals where it has been shown to decrease the susceptibility of microbial populations to stress (Lai and Lai, 2011) and has been demonstrated to increase milk production (Wang et al., 2010). Osmoregulation by betaine is also seen in microbial populations and has been shown to promote favorable bacterial growth under osmotic stress conditions (Diamant et al., 2001; Wdowiak-Wrobel et al., 2013) including fluctuations in pH (Laloknam et al., 2006). Biotin is an important nutrient for both rumen bacteria and animals as this vitamin is an essential co-enzyme for the activation of a range of carboxylase enzymes (Hausmann et al., 2018) that catalyze key reactions in gluconeogenesis, amino acid catabolism and fatty acid synthesis (Baur and Baumgartner, 2019). In addition, biotin is important for cellulolytic rumen bacterial growth (Baldwin and Allison, 1983). Recent study indicated that there are specific requirements for vitamins such as biotin, vitamin B12, folic acid and riboflavin for major cellulolytic organisms in the rumen, including bacteria such as species of *Ruminococcus* and *Bacteroides*, and anaerobic fungi such as *Neocallimastix* (Ashwin and Srinivas, 2019). An *in vitro* study reported that biotin synthesis by microbes was reduced by 50% as the concentration in diets increased from 20% to 50% (Gomez et al., 1998). Similarly, Abel et al. (2001) demonstrated that the synthesis of ruminal biotin declined by half when the proportions of barley to hay increased from equal to 83% and 17%, respectively, most likely due to an imbalance among microbial species influenced by the reduced ruminal pH. Supplemental biotin may be required depending on dietary factors that affect biotin synthesis and destruction (Peterson et al., 2004). Furthermore, chromium is involved in various important metabolic functions in animals and plays a role in increasing glucose tolerance and improving animal performance. For example, chromium supplementation has been shown to increase animal production response (Keshri et al., 2017). Deka (2013) and Keshri et al. (2017) reported that there was no adverse effect on rumen fermentation by supplementing with an inorganic chromium source at up to 2.5–3 mg/kg. Besong et al. (2001) found that chromium could alter volatile fatty acid (VFA) production and was not toxic to rumen microorganisms at concentration up to 25.6 mg/kg.

There have been a few studies on the combination of betaine, biotin, and chromium picolinate for ruminants. The role of many enhancers is to provide synergistic effects to boost ruminant productivity and efficiency. A combination of betaine, biotin and chromium (BBC) has been established in high-production ruminants that use high concentrate ratios to increase rumen fermentation, feed efficiency and animal performance. To achieve these goals, the current study used an *in vitro* gas production technique to explore the impact of BBC supplied at various ratios of concentrate to roughage in diets on efficient microbial biomass synthesis and ruminal fermentation.

Materials and Methods

Ethical approval

The experimental procedure was approved by the Animal Ethics Committee of Khon Kaen University (Record no. ACUC-KKU-83/2562) based on the Ethics of Animal Experimentation of the National Research Council of Thailand.

Treatments and experimental design

The experiment was carried out with a 3×4 factorial arrangement in a completely randomized design. Factor A was three ratios of concentrate to roughage (C:R) in the diet: 50:50, 60:40 and 70:30. Factor B was different levels of BBC supplementation (0 g/kg dry matter, DM, 3 g/kg DM, 6 g/kg DM and 9 g/kg DM). The feeds were dried in an oven at 60°C for 48 hr and ground to pass through a screen with a mesh size of 1 mm for use in the *in vitro* gas production technique. Each air-dried sample (200 mg) was incubated in a 50 mL serum bottle containing 20 mL of buffer mineral solution and

10 mL of rumen fluid for 96 hr at 39°C. The quantity of gas production was measured in 48 bottles (4 bottles/treatment × 12 treatments). Degradability was analyzed with another set of 72 bottles for 24 hr and 48 hr of incubation (3 bottles/treatment × 12 treatments × 2 sampling times).

The BBC product was obtained from Animal Supplement & Pharmaceutical Co., Ltd., Thailand. BBC consisted of 100 g, 0.04 g and 0.04 g of betaine, biotin, and chromium picolinate, respectively, in 1 kg.

Chemical analyses of dry matter (method 967.03), ash (method 942.05), crude protein (CP; method 984.13), and ether extract (EE; method 920.39) were done according to the Association of Official Analytical Chemists (2005). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed according to the method of Van Soest et al. (1991).

Animals and preparation of rumen fluid inoculum

The rumen fluid was collected from two crossbred beef bulls (380 ± 40.2 kg body weight (BW)). The animals were adapted to a 1% concentrate diet (16% CP and 2.4 Mcal ME/kg DM) with rice straw as roughage, which was fed ad libitum. The animals were individually penned with fresh water and mineral blocks available ad libitum. The animals were fed for 20 d before collecting the rumen fluid using a stomach tube technique. The rumen fluids from the two animals were mixed with artificial saliva solution according to the method of Menke and Steingass (1988). Briefly, 0.2 g of air-dried sample was incubated in a 50 mL serum bottle containing 20 mL of buffer mineral solution and 10 mL of rumen fluid for 96 hr at 39°C.

Measurement of in vitro gas kinetics

During the incubation, the gas production was recorded at 1 hr, 2 hr, 4 hr, 6 hr, 8 hr, 10 hr, 12 hr, 18 hr, 24 hr and 48 hr. The model of Ørskov and McDonald (1979) was applied to estimate the cumulative gas production using Equation 1:

$$GP = a + b(1 - e^{(-ct)}) \quad (1)$$

where, a is the gas production from the immediately soluble fraction (measured in milliliters), b is the gas production from the insoluble fraction (in milliliters), c is the gas production rate constant for the insoluble fraction (b) (as percentage per hour), t is the incubation time (in hours), ($a + b$) is the potential extent of gas production (in milliliters), GP is the gas produced at time t (in milliliters).

The effective gas production potential (EGP) was calculated using Equation 2:

$$EGP = \frac{a+bc}{c+k} \quad (2)$$

where, k is the rate of particulate outflow from the rumen (0.05/hr) according to the equation of Ørskov and McDonald (1979) and other parameters are the same as in Equation (1).

Measurement of in vitro apparent, true substrate degradation

Three replications each using 200 mg dry weight of feed samples were weighed into 50 mL calibrated serum bottles and incubated with 30 mL of mixed rumen inoculum at 39°C with parallel incubation of blanks, as described by Blümmel et al. (1997b). The incubation was terminated at the chosen time. After 24 hr of incubation, the gas volume was recorded and the whole contents of the serum bottle were transferred into pre-weighed ANKOM bags. The bags were rinsed three times with solution and then dried at 105°C for 3 hr in a forced-drying oven. The bags were weighed to determine the apparent undigested residue and corrected with the mass of the blank incubations. Next, undigested residues continued to be extracted in 100 mL of neutral detergent solution (NDS) by boiling for 1 hr, followed by filtration on pre-weighed Gooch crucibles and washing in hot distilled water and acetone to recover the true undigested residue (Van Soest et al., 1991). Crucibles with undigested residue were dried at 100°C overnight and weighed to determine the true undigested residue. The true dry matter degradability (TDDM) was calculated as the weight of substrate incubated minus the weight of the residue after NDS treatment (Blümmel et al., 1997b). Crucibles with undigested residue were ashed at 500°C for 3 hr to determine the true undigested OM, which was corrected for the appropriate blanks. The true degradability of organic matter (TDOM) was calculated as the difference between OM incubated and the undigested OM recovered in the residue of NDS extraction.

The microbial biomass production (MBP) and efficiency of microbial biomass production (EMBP) were calculated as described by Blümmel et al. (1997a). The ratio of TDOM and gas production (Gv) at 24 hr of incubation (Gv_{24hr}) was used to identify the partitioning factor (PF) according to Blümmel et al. (1994) using MBP (in milligrams) = TDOM – ($Gv_{24\ hr}$×2.2); EMBP (in milligrams per milligram) = MBP/TDOM and PF (in milligrams per milliliter) = TDOM/$Gv_{24\ hr}$, where TDOM is in milligrams and $Gv_{24\ hr}$ is in milliliters per 0.2 g DM.

The metabolizable energy (ME, measured in megajoules per kilogram DM) was estimated from 200 mg of dry sample after 24 hr of incubation using the equation for concentrate feeds (Menke et al., 1988) of ME = (0.157Gv + 0.0084CP + 0.022EE - 0.0081Ash + 1.06), where Gv is in milliliters and CP, EE and ash are calculated as percentages.

After 24 hr and 48 hr, the supernatant was removed and stopped using 2 mL of sulfuric acid (H_2SO_4) added to 18 mL of incubation medium, which was centrifuged at 16,000×*g* for 15 min. The supernatant was stored at -20°C before ammonia-nitrogen (NH_3-N) and volatile fatty acid (VFA) analysis. NH_3-N was measured according to the Kjeldahl method (Association of Official Analytical Chemists, 2005) and VFA was analyzed using high performance liquid chromatography (model RF-10AXmugiL; Shimadzu; Japan), according to Mathew et al. (1995).

Ruminal methane (CH_4) production using VFA proportions was estimated according to Moss et al. (2000) as: CH_4 production (micro moles) = 0.45 (acetate) - 0.275 (propionate) + 0.4 (butyrate).

Statistical analysis

Data on *in vitro* gas production, nutrient degradability and rumen fermentation parameters were analyzed using a factorial experiment and the PROC GLM option of Statistical Analysis System (2002) as: $Yijk = \mu + Ri + Aj + (R \times A)ij + Eijk$, where Yijk is the observation of the i^{th} C:R ratio (*Ri*) with the j^{th} BBC level (*Aj*), μ is the general mean, $(R \times A)ij$ is the interaction between the C:R ratio and BBC levels, and *Eijk* is the experimental error. Linear and quadratic polynomial contrasts were used to examine responses of different C:R ratios to increasing addition levels of BBC. The least square means were presented and significance was determined at $p < 0.05$.

Results

Chemical composition of diets

Table 1 shows the ingredients in the concentrate and the chemical compositions of the concentrate, rice straw and BBC mixtures. The chemical compositions of diets containing different C:R ratios are presented in Table 2. The concentrate contained 18.30% CP and 12.84 MJ ME/kg DM, while the rice straw contained 3.33% CP and 5.76 MJ ME/kg DM. BBC contained 7.53% CP and 43.55% ash. When the concentrate and roughage were mixed at a certain ratio, the CP and EE contents increased but the NDF and ADF contents decreased as the C:R ratio increased. The chemical composition of diets was unaffected by the level of BBC inclusion.

Table 1 Ingredients and chemical composition of concentrate, rice straw and betaine-biotin-chromium (BBC)

Item	Concentrate	Rice straw	BBC
Ingredient, % dry matter basis			
Cassava chip	57.00	-	
Rice bran	8.50	-	
Soybean meal	15.00	-	
Palm kernel meal	14.00	-	
Salt	0.40	-	
Dicalcium phosphate	1.50	-	
Premixed	0.50	-	
Sulfur	0.30	-	
Molasses	1.50	-	
Urea	1.30	-	
Total	100.00		
Chemical composition			
Dry matter (DM), %	94.30	91.20	97.98
		---------- % Dry matter ----------	
Organic matter	93.40	88.20	56.45
Crude protein	18.30	3.33	7.53
Ether extract	5.70	0.62	1.45
Neutral detergent fiber	40.10	81.10	-
Acid detergent fiber	14.50	57.70	-
Ash	6.57	11.80	43.55
Metabolizable energy (MJ/kg DM)	12.84	5.76	-

Table 2 Chemical composition of different treatment ratios of concentrate (C) to roughage (R)

Diet C:R ratio	Chemical composition						ME (MJ/kg DM)
	DM	OM	CP	EE	NDF	ADF	
	%	% DM					
50C:50R	92.77	90.65	10.80	3.15	60.33	35.94	8.71
60C:40R	93.08	91.16	12.29	3.66	56.25	31.64	9.30
70C:30R	93.39	92.68	13.78	4.16	52.17	27.34	9.89

DM = dry matter; OM = organic matter; CP = crude protein; EE = ether extract; NDF = neutral detergent fiber; ADF = acid detergent fiber; ME = metabolizable energy

Effect on gas kinetics and gas production

Table 3 shows the gas kinetics, total gas production at 24 hr of incubation and effective gas production potential (EGP) of each treatment combination. The gas kinetics results showed that the intercept value (*a*) of gas production from the insoluble fraction (*b*) and rate constants for the insoluble fraction (*c*) were not different ($p > 0.05$) among treatment combinations. There was no interaction effect between the C:R ratio and BBC level ($p > 0.05$) on gas kinetics. BBC inclusion did not have any effects on gas kinetics, except for the *a* value that was lowest in the diet with the inclusion of BBC at 6 g/kg. Increases in the C:R ratio from the 50C:50R ratio to 60C:40R and 70C:30R resulted in progressively higher rates of degradability ($p < 0.05$). The total gas production at 24 hr was significantly ($p < 0.05$) different among treatment combinations. The BBC level had no effect on this parameter, but the C:R ratio linearly influenced ($p < 0.05$) the total gas as the C:R ratio increased from 50C:50R to 70C:30R, with the highest value occurring at 70C:30R. Similarly, the best EGP was from the diet comprising 70C:30R ($p < 0.01$).

Table 3 Effect (mean ± SD) of concentrate to roughage (C:R) ratio and betaine-biotin-chromium (BBC) supplementation on gas kinetics, cumulative gas production and effective gas production

C:R ratio	BBC (g/kg)	Gas kinetics				Cumulative gas (mL/200 mg DM)	EGP (mL/200 mg DM)
		a	b	c	d		
Treatment combination							
50C:50R	0	-2.67±0.59	54.27±0.50	0.042±0.00	51.61±0.09	36.62±0.45^{a}	22.08±0.42^{a}
50C:50R	3	-1.77±1.02	54.70±6.58	0.040±0.00	52.94±5.55	36.94±2.42^{a}	22.62±1.75^{a}
50C:50R	6	-1.10±0.35	51.45±4.18	0.043±0.00	50.36±3.83	35.69±0.49^{a}	22.53±0.34^{a}
50C:50R	9	-1.32±0.39	58.72±1.57	0.034±0.00	57.41±1.19	35.45±2.72^{a}	22.37±2.06^{a}
60C:40R	0	-2.78±0.04	53.52±0.89	0.055±0.00	50.74±0.93	41.11±1.21^{b}	25.14±0.52^{ab}
60C:40R	3	-2.51±0.13	60.84±1.44	0.046±0.00	58.33±1.57	42.79±1.32^{bc}	26.70±1.35^{b}
60C:40R	6	-1.12±0.33	55.48±4.68	0.046±0.00	54.36±5.01	39.45±0.53^{a}	25.52±1.27^{ab}
60C:40R	9	-2.10±0.09	51.06±8.35	0.051±0.01	48.96±8.26	37.58±3.02^{a}	23.62±2.60^{a}
70C:30R	0	-1.82±0.70	50.19±1.94	0.058±0.00	48.38±1.24	40.31±2.04^{b}	25.07±1.14^{ab}
70C:30R	3	-1.16±0.23	59.60±1.66	0.048±0.00	58.45±1.89	44.95±0.60^{c}	28.09±0.22^{bc}
70C:30R	6	-1.25±0.96	54.58±5.49	0.050±0.00	53.33±4.53	42.50±0.45^{bc}	26.09±2.24^{ab}
70C:30R	9	-2.34±0.48	61.37±2.80	0.053±0.01	59.03±3.28	45.80±0.60^{c}	29.06±0.56^{c}
SEM		0.38	2.90	0.004	2.74	1.14	1.02
p-values		0.20	0.18	0.17	0.16	<0.05	0.21
C:R ratio effect							
50C:50R		-1.71±0.81	54.79±4.09	0.040±0.00^{a}	53.08±3.85	36.18±1.55^{a}	22.40±1.07^{a}
60C:40R		-2.13±0.69	55.22±5.32	0.051±0.01^{b}	53.10±5.35	40.23±2.46^{a}	25.25±1.70^{b}
70C:30R		-1.64±0.71	56.44±5.32	0.054±0.01^{b}	54.79±5.15	43.39±2.46^{a}	27.07±1.95^{c}
SEM		0.19	1.45	0.002	1.37	0.57	0.51
p-values		0.19	0.71	< 0.01	0.61	< 0.01	< 0.01
L		0.80	0.44	< 0.01	0.39	< 0.01	< 0.01
Q		0.08	0.83	0.07	0.63	0.53	0.43

Table 3 Continued

C:R ratio	BBC (g/kg)	Gas kinetics				Cumulative gas (mL/200 mg DM)	EGP (mL/200 mg DM)
		a	b	c	d		
BBC effect							
0		-2.42±0.63[a]	52.66±2.18	0.052±0.01	50.24±1.65	39.34±2.40	24.10±1.67
3		-1.81±0.77[ab]	58.38±4.25	0.047±0.00	56.57±3.91	41.56±3.91	25.80±2.73
6		-1.15±0.49[b]	53.84±4.18	0.047±0.00	52.68±3.94	39.21±3.07	24.71±2.06
9		-1.92±0.55[a]	57.05±6.24	0.048±0.01	55.13±6.29	39.61±5.23	25.02±3.52
SEM		0.22	1.68	0.002	1.58	0.66	0.59
p-values		< 0.05	0.10	0.31	0.07	0.09	0.27
L		0.05	0.27	0.29	0.15	0.61	0.54
Q		< 0.01	0.47	0.13	0.24	0.19	0.26
C		0.16	< 0.05	0.72	< 0.05	0.03	0.14

C:R ratio = concentrate-to-roughage ratio; SEM = standard error of mean; L = linear effect; Q = quadratic effect; C = cubic effect

Effective gas production potential (EGP) = $a+bc / (k+c)$, where $k = 0.05$/hr, a = gas production from immediately soluble fraction; b = gas production from insoluble fraction; c, gas production rate constant for insoluble fraction (b)

Means in the same column superscripted with different lowercase letters are significant ($p < 0.05$) different whereas ones without superscription denote non-significant ($p > 0.05$) difference.

Effect on substrate degradability, microbial biomass production and efficiency

Table 4 shows the apparent dry matter degradability (ADDM), TDDM and TDOM of diets containing different C:R ratios and levels of BBC addition. There were no interaction effects between the C:R ratio and BBC supplementation on these parameters ($p > 0.05$). The BBC supplementation did not influence the parameters ($p > 0.05$). Increasing the C:R ratio from 50C:50R to 70C:30R in diets resulted in increased ADDM, TDDM, TDOM and percentage degradability of TDDM and TDOM ($p < 0.01$).

Table 5 shows the values for MBP, EMBP, PF and ME of the treatment combinations. There was no interaction effect between the C:R ratio and BBC addition on MBP and EMBP. BBC supplementation had no effect ($p > 0.05$) on either MBP or EMBP. Furthermore, MBP and EMBP linearly increased ($p < 0.01$) as the C:R ratio in the diet increased.

Partitioning factors were significantly ($p < 0.01$) different among treatment combinations. Increasing the C:R ratio in diets resulted in reduced PF ($p < 0.01$).

Effect on ruminal NH_3-N and volatile fatty acid production

The NH_3-N, TVFA and individual VFA profiles are provided in Table 6. There was an interaction effect between the C:R ratio and BBC inclusion on NH_3-N, TVFA and butyric acid ($p < 0.05$), whereas acetic acid, propionic acid and the A:P ratio did not show any interaction effect ($p > 0.05$). The NH_3-N concentration was significantly ($p < 0.01$) different among treatment combinations and was in the range 25.95–28.87 mg/dL. TVFA was influenced by the C:R ratio and was higher in the treatments with a higher ratio (70C:30R) ($p < 0.05$). Although BBC inclusion had no influence on VFA profiles, the C:R ratio did. When the C:R ratio increased from 50C:50R to 70C:30R, the proportion of acetate acid (C2) reduced ($p < 0.05$), but propionic acid (C3) and butyric acid (C4) increased ($p < 0.05$). However, the BBC supplementation had no effect. The A:P ratio was lower ($p < 0.05$) in the diet containing 70C:30R.

CH_4 production was also influenced by the C:R ratio. Increasing the concentrate level decreased ($p < 0.01$) methane production, with the lowest value for the 70C:30R ratio (Table 5). BBC addition had a quadratic effect ($p < 0.05$) on CH_4 production; with the inclusion of 9 g/kg, DM, the production decreased compared to BBC at other levels.

Discussion

Effect on gas kinetics and gas production

Gas production and its kinetics, which reflect the apparent substrate degradability (Pashaei et al., 2010), were not different among treatments. However, total gas production increased linearly in proportion to the concentrate (70%) in the diet. Similarly, Anantasook and Wanapat (2012), Polyorach et al. (2014) and Kholif et al. (2017) indicated that the gas production and fermentation rates increased as the proportion of concentrate increased in diets with various roughage sources. In the current study, degradability of insoluble fractions in diets was not different among treatment combinations, but the rate of

fermentation was high at 70C:30R and 60C:40R which differed from 50C:50R. Unfortunately, a response in fermentation was not observed with BBC inclusion. Despite the fact that biotin is a specific, necessary vitamin for major cellulolytic organisms (Ashwin and Srinivas, 2019) and most cellulolytic rumen bacteria require biotin for growth (Baldwin and Allison, 1983), no evidence of gas production was found. This was probably due to the narrow range of C:R ratios in the current study. However, Peterson et al. (2004) found that diets with concentrate levels of 52–77% had the greatest synthesis of biotin in the total tract of sheep. Cruywagen and Bunge (2004) found that adding biotin at 20 mg/kg in roughage substrate resulted in increased gas production. Nakai et al. (2013) reported that betaine may be degraded in the rumen and another report suggested that a large proportion of the betaine may escape during the microbial degradation and pass through the rumen without being degraded (Monteiro et al., 2016). Thus, betaine had no significant role in rumen degradation. Deka (2013) reported that the supplementation of an inorganic chromium source at up to 3.0 mg/kg had no effect on rumen fermentation. Similarly, Keshri et al. (2017) found that chromium at 2.5 mg/kg had no effect on total gas production.

Table 4 Effect (mean ± SD) of concentrate to roughage (C:R) ratio and betaine-biotin-chromium (BBC) supplementation on apparent dry matter (DM) degradability (ADDM), truly DM degradability (TDDM), truly OM degradability (TDOM)

C:R ratio	BBC g/kg	ADDM (mg)	TDDM (mg)	TDOM (mg)	TDDM (%)	TDOM (%)
Treatment combination						
50C:50R	0	89.41±0.84	131.65±1.64	121.36±2.26	65.82±0.81	66.83±1.24
50C:50R	3	88.34±1.97	130.16±0.25	119.51±0.22	65.08±0.13	65.81±0.12
50C:50R	6	87.64±0.42	131.54±0.61	120.87±0.17	65.77±0.30	66.56±0.10
50C:50R	9	88.52±2.39	131.43±0.88	120.73±0.39	65.71±0.44	66.48±0.21
60C:40R	0	95.89±7.62	135.71±5.35	126.51±4.87	67.85±2.68	69.27±2.67
60C:40R	3	105.25±1.81	144.51±3.30	134.33±2.71	72.25±1.65	73.55±1.48
60C:40R	6	99.98±0.36	137.32±1.71	128.49±2.27	68.66±0.85	70.35±1.24
60C:40R	9	100.94±3.86	138.90±4.63	129.32±3.32	69.45±2.31	70.80±1.82
70C:30R	0	116.79±3.89	150.49±1.07	142.40±0.81	75.25±0.53	77.52±0.44
70C:30R	3	122.79±5.18	152.15±2.93	141.36±3.31	76.08±1.47	76.96±1.80
70C:30R	6	114.55±3.30	145.89±6.06	140.19±0.73	72.94±3.03	76.32±0.40
70C:30R	9	117.11±5.91	148.08±3.32	138.78±4.38	74.04±1.66	75.56±2.38
SEM		2.70	2.28	1.87	1.14	1.02
p-values		0.55	0.32	0.22	0.32	0.22
C:R ratio effect						
50C:50R		88.48±1.40[a]	131.19±0.99[a]	120.62±1.14[a]	65.60±0.49[a]	66.42±0.63[a]
60C:40R		100.51±4.85[b]	139.11±4.66[b]	129.66±4.03[b]	69.56±2.33[b]	70.99±2.21[b]
70C:30R		117.81±4.80[c]	149.15±3.83[c]	140.68±2.56[c]	74.58±1.91[c]	76.59±1.39[c]
SEM		1.35	1.14	0.93	0.57	0.51
p-values		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
L		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Q		0.14	0.46	0.40	0.46	0.43
BBC effect						
0		100.70±13.37	139.28±9.23	130.09±10.10	69.64±4.62	71.21±5.19
3		105.39±15.63	141.21±10.18	130.67±10.16	70.61±5.09	71.53±5.22
6		100.13±12.14	136.99±7.05	128.17±8.77	68.49±3.53	70.16±4.44
9		101.31±13.25	140.26±7.89	130.32±8.44	70.13±3.95	71.33±4.28
SEM		1.56	1.32	1.08	0.66	0.59
p-values		0.16	0.22	0.52	0.22	0.52
L		0.97	0.57	0.50	0.57	0.51
Q		0.31	0.51	0.40	0.51	0.40
C		0.04	0.06	0.31	0.06	0.31

C:R ratio = concentrate-to-roughage ratio; BBC = betaine-biotin-chromium; SEM = standard error of mean; L = linear effect; Q = quadratic effect; C = cubic effect

Means in the same column superscripted with different lowercase letters are significant ($p < 0.05$) different whereas ones without superscription denote non-significant ($p > 0.05$) difference.

Table 5 Effect (mean ± SD) of concentrate to roughage (C:R) ratio and betaine-biotin-chromium (BBC) supplementation on microbial biomass production (MBP), efficacy of MBP (EMBP), partitioning factors (PF), metabolizable energy (ME) and methane (CH_4)

C:R ratio	BBC (g/kg)	MBP (mg)	EMBP (mg/mg)	PF (mg/mL)	ME (MJ/kg DM)	CH_4 (μmol)
Treatment combination						
50C:50R	0	40.11±0.80^{a}	0.305±0.00^{a}	3.29±0.04^{a}	7.717±0.00	31.45±0.57
50C:50R	3	38.31±1.72^{a}	0.294±0.01^{a}	3.24±0.01^{a}	7.705±0.00	32.18±0.37
50C:50R	6	39.73±1.03^{a}	0.302±0.01^{a}	3.28±0.01^{a}	7.693±0.01	31.81±0.23
50C:50R	9	42.99±1.52^{a}	0.327±0.01^{a}	3.42±0.03^{b}	7.443±0.00	31.51±0.06
60C:40R	0	35.67±2.27^{a}	0.262±0.03^{b}	3.06±0.13^{c}	8.680±0.01	31.45±0.45
60C:40R	3	40.50±1.49^{a}	0.280±0.00^{b}	3.15±0.08^{a}	8.887±0.01	31.78±0.60
60C:40R	6	41.80±2.07^{a}	0.304±0.01^{a}	3.26±0.04^{a}	8.369±0.00	31.61±0.30
60C:40R	9	47.03±0.76^{b}	0.338±0.00^{a}	3.48±0.13^{c}	8.047±0.00	31.28±0.09
70C:30R	0	50.37±2.83^{b}	0.335±0.02^{a}	3.40±0.03^{c}	9.045±0.01	31.06±0.35
70C:30R	3	42.72±2.24^{a}	0.281±0.02^{b}	3.15±0.07^{a}	9.509±0.01	31.07±0.57
70C:30R	6	48.35±2.76^{b}	0.332±0.01^{a}	3.36±0.14^{ab}	9.016±0.00	31.34±1.07
70C:30R	9	38.77±2.60^{a}	0.262±0.02^{b}	3.05±0.07^{c}	9.592±0.00	29.65±0.20
SEM		1.90	0.01	0.05	0.004	0.34
p-values		< 0.01	< 0.01	< 0.01	< 0.01	0.35
C:R ratio effect						
50C:50R		40.28±1.30^{a}	0.31±0.01	3.30±0.08	7.64±0.12^{a}	31.73±0.41^{a}
60C:40R		41.25±1.69^{a}	0.30±0.02	3.24±0.19	8.50±0.34^{b}	31.53±0.36^{a}
70C:30R		45.05±2.58^{b}	0.29±0.02	3.23±0.15	9.29±0.28^{c}	30.78±0.86^{b}
SEM		0.95	0.01	0.02	0.002	0.17
p-values		< 0.01	0.32	0.11	< 0.01	< 0.01
L		< 0.01	0.50	0.09	< 0.01	< 0.01
Q		0.24	0.18	0.19	< 0.01	0.22
BBC effect						
0		42.05±4.27	0.30±0.05^{ab}	3.25±0.17^{ab}	8.48±0.61^{a}	31.31±0.41^{ab}
3		40.51±6.05	0.29±0.06^{a}	3.18±0.09^{a}	8.70±0.82^{b}	31.67±0.65^{b}
6		43.30±5.84	0.31±0.05^{b}	3.30±0.08^{b}	8.36±0.59^{c}	31.58±0.55^{b}
9		42.93±5.54	0.31±0.05^{b}	3.31±0.25^{b}	8.36±0.99^{c}	30.81±0.91^{a}
SEM		1.09	0.01	0.03	0.002	0.20
p-values		0.32	< 0.05	< 0.05	< 0.01	< 0.05
L		0.29	< 0.05	< 0.05	< 0.01	0.09
Q		0.60	0.34	0.16	< 0.01	< 0.05
C		0.15	< 0.05	< 0.05	< 0.01	0.79

C:R ratio = concentrate-to-roughage ratio; SEM = standard error of mean; L = linear effect; Q = quadratic effect; C = cubic effect

Means in the same column superscripted with different lowercase letters are significant ($p < 0.05$) different whereas ones without superscription denote non-significant ($p > 0.05$) difference.

Table 6 Effect (mean ± SD) of concentrate to roughage (C:R) ratio and betaine-biotin-chromium (BBC) supplementation on ammonia nitrogen (NH_3-N) and volatile fatty acids (VFA) incubation at 24 hr

C:R ratio	BBC (g/kg)	NH_3-N (mg/dL)	TVFA (mM)	Acetic acid (%)	Propionic acid (%)	Butyric acid (%)	A:P ratio
Treatment combination							
50C:50R	0	28.23±0.11^{ac}	96.11±7.57^{a}	70.42±1.10^{a}	17.88±0.77	11.69±0.33^{ab}	3.94±0.23
50C:50R	3	27.49±0.01^{a}	97.57±4.75^{a}	72.13±0.82^{a}	16.93±0.49	10.93±0.33^{a}	4.26±0.17
50C:50R	6	28.23±0.28^{ac}	87.40±5.24^{a}	71.41±0.26^{a}	17.42±0.32	11.17±0.06^{ab}	4.10±0.09
50C:50R	9	27.88±0.32^{ac}	88.22±4.71^{a}	70.48±0.17^{a}	17.79±0.07	11.73±0.10^{b}	3.96±0.03
60C:40R	0	28.13±0.55^{ac}	99.85±6.49^{ab}	70.40±0.67^{a}	17.88±0.62	11.72±0.05^{b}	3.94±0.17
60C:40R	3	28.45±0.38^{ac}	117.80±5.67^{cd}	71.15±0.87^{a}	17.46±0.82	11.40±0.05^{b}	4.08±0.24
60C:40R	6	28.37±1.07^{ac}	123.18±2.09^{d}	70.73±0.57^{a}	17.67±0.40	11.60±0.16^{b}	4.00±0.12
60C:40R	9	28.47±0.29^{ac}	110.85±8.86^{bc}	70.47±0.77^{a}	18.14±0.08	11.39±0.70^{b}	3.89±0.06
70C:30R	0	25.95±0.28^{b}	109.44±7.91^{b}	69.40±0.55^{b}	18.38±0.48	12.21±0.06^{b}	3.78±0.13
70C:30R	3	28.43±0.10^{ac}	124.82±5.45^{d}	69.48±0.90^{b}	18.38±0.78	12.15±0.13^{b}	3.79±0.21
70C:30R	6	28.74±0.29^{c}	125.32±0.48^{d}	69.83±1.48^{b}	18.01±1.47	12.16±0.01^{b}	3.90±0.40
70C:30R	9	28.87±0.75^{c}	102.73±.56^{ab}	66.30±0.56^{c}	20.24±0.25	13.46±0.31^{c}	3.28±0.07
SEM		0.33	4.04	0.57	0.47	0.19	0.13
p-values		< 0.01	< 0.05	0.10	0.39	0.01	0.42
C:R ratio effect							
50C:50R		27.96±0.37	92.32±6.50^{a}	71.11±0.92^{a}	17.51±0.54^{a}	11.38±0.41^{a}	4.07±0.18^{a}
60C:40R		28.35±0.51	112.92±10.46^{b}	70.69±0.63^{a}	17.79±0.50^{a}	11.53±0.31^{a}	3.98±0.15^{a}
70C:30R		28.00±1.32	115.57±11.16^{b}	68.75±1.69^{b}	18.75±1.15^{b}	12.50±0.61^{b}	3.68±0.31^{b}
SEM		0.17	2.02	0.28	0.233	0.10	0.07
p-values		0.22	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
L		0.86	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Q		0.09	< 0.01	0.06	0.25	< 0.01	0.23
BBC effect							
0		27.43±1.19^{a}	101.80±8.38^{a}	70.08±0.81^{ab}	18.05±0.55^{ab}	11.88±0.30^{ab}	3.89±0.17^{ab}
3		28.13±0.52^{b}	113.39±13.31^{b}	70.92±1.38^{a}	17.59±0.86^{a}	11.49±0.57^{c}	4.04±0.27^{a}
6		28.45±0.56^{b}	111.96±19.22^{b}	70.66±1.01^{a}	17.70±0.74^{a}	11.65±0.45^{ac}	4.00±0.21^{a}
9		28.41±0.59^{b}	100.60±11.31^{a}	69.08±2.20^{b}	18.73±1.19^{b}	12.19±1.05^{b}	3.71±0.34^{b}
SEM		0.19	2.33	0.33	0.27	0.11	0.08
p-values		< 0.01	< 0.01	< 0.01	0.05	< 0.01	0.04
L		< 0.01	0.64	0.05	1.00	< 0.05	0.11
Q		0.08	< 0.01	< 0.01	0.02	< 0.01	< 0.05
C		1.00	0.77	0.89	0.78	0.78	0.87

A:P ratio = acetic acid-to-propionic acid ratio; C:R ratio = concentrate-to-roughage ratio; SEM = standard error of the mean; L = linear effect; Q = quadratic effect; C = cubic effect

Means in the same column superscripted with different lowercase letters are significant ($p < 0.05$) different whereas ones without superscription denote non-significant ($p > 0.05$) difference.

Effect on substrate degradability, microbial biomass production and efficiency

There was a close relationship between gas production and apparent DM degradability (correlation coefficient (r) = 0.86, $p < 0.01$), true DM degradability ($r = 0.87$, $p < 0.01$) and true OM degradability ($r = 0.85$, $p < 0.01$), which was similar to the report of Blümmel et al. (1997b). There was no interaction effect between the C:R ratio and BBC addition on degradability *in vitro*. These parameters linearly increased as the C:R ratio increased. Similarly, Polyorach et al. (2014) observed an increase in IVOMD in complete rations when increasing the concentrate proportion from 20% to 80%. With an increasing concentrate proportion, TDMD and TDOM increased ($p < 0.05$). These findings agreed with those of Anantasook and Wanapat (2012) and Phesatcha et al. (2020), regarding improved *in vitro* digestibility when increasing the proportion of concentrates in the diet. This could be attributed to an increase in microbial growth due to the increased C:R ratio, which enhanced digestibility. In the current study, MBP was highest for 70C:30R and lowest for 50C:50R. This could have been due to the ability of rumen bacteria to synchronize ammonia and fermentable carbohydrates, which could be assisted by BBC inclusion. However, Reddy et al. (2016) found that diets containing C:R ratios of 50C:50R to 70C:30R had similar MBP values. In contrast, Zicarelli et al. (2008) in a trial involving six diets with a forage-to-concentrate ratio (C/R) in the range 0–50:50 found that the organic matter digestibility rose as the percentage of concentrate increased up to the 30:70 diet while the diets with 40:60 and 50:50 C/R did not follow this trend. Keshri et al. (2017) showed that the supplementation of different chromium sources did not affect adversely *in vitro* rumen fermentation parameters.

The highest EMBP was observed for 50C:50R followed by 60C:40R and 30C:70R. As observed by Kumari et al. (2012) and Reddy et al. (2015), EMBP was favorably linked with the PF of the diets. Generally, the PF is an indicator of a higher proportion of OM degradability toward microbial biomass production than gas production (Blümmel et al. 1997b). Therefore, the PF provides meaningful information for the prediction of MBP and voluntary intake in ruminants (Blümmel et al., 1997b). Thirumalesh and Krishnamoorthy (2009) noticed a positive correlation between microbial biomass flow to the duodenum and the PF of the total mixed ration. In the current study, the highest PF occurred for 50C:50R, followed by 60C:40R and 30C:70R.

BBC addition had no favorable effect on MBP, EMBP and the PF. There is a paucity of literature on the supplementing of a mixture of BBC on rumen fermentation *in vitro*. According to Li et al. (2021), at a biotin concentration of 1.0 mg/L, the total nitrogen removal rate increased, showing that biotin addition can effectively stimulate anammox bacterial activity, resulting in biomass reaching maximum levels. There is no published mention of this issue regarding the inclusion of chromium and betaine.

The ME of diets increased as the C:R ratio increased, but not with BBC inclusion. The ME in diets that contained 70C:30R was higher than for the other ratios. This agreed with Reddy at al. (2016) and Kholif et al. (2017). The C:R ratio was the most important factor regarding the differences in ME between treatment combinations, while BBC supplementation had only a minor effect.

Effect on ruminal NH_3-N and volatile fatty acid production

The rumen NH_3-N concentrations were different ($p < 0.01$) among treatments. However, the C:R ratio had no effect on NH_3-N. Nagadi (2019) also found no significant difference in diets that contained concentrate at 0–100%. Other studies reported that NH_3-N concentrations increased in proportion to the concentrate in a complete diet, which might have been due to the active degradation of protein (Anantasook and Wanapat, 2012; Polyorach et al., 2014). The mean range of the NH_3-N concentration was suitable for rumen bacteria synthesis and led to supporting nutrient digestion (Hummel et al., 2006). During incubation, ammonia is incorporated into microbial protein synthesis (Blümmel et al., 1997b), which resulted in higher MBP in high C:R diets in the current study. When BBC was included, the concentration of NH_3-N was higher than when BBC was not included. As a result, one or more elements in the BBC combinations may aid in the breakdown of protein in the diet. Keshri et al. (2017) reported that NH_3-N increased upon supplementation with Cr at 2.5 mg/kg, which supported the current results. However, Rikhari et al. (2010) found that the supplementation of chromium picolinate at 0.5 mg/kg and 1.0 mg/kg did not have any significant effect on NH_3-N.

Similarly, TVFA production linearly increased as the level of concentrate increased in the diet, whereas BBC addition had a quadratic effect. There was a relationship between gas production and TVFA ($r = 0.58$, $p < 0.05$) that was consistent with the findings of Getachew et al. (2004), who concluded that TVFA production was positively correlated with *in vitro* gas production. Reddy et al. (2016) found that total VFA

production increased as the level of concentrate increased from 50C:50R to 100C:0R. A high level of concentrate in another study also appeared to increase the propionate concentration and decrease the A:P ratio (Ding et al., 2014). Similar results have been reported using buffalo rumen fluid (Zicarelli et al., 2011). However, Pangsai (2009) reported that supplementation of biotin had no effect on VFA. More recently, Hausmann et al. (2017) reported that supplementation of biotin at 20 mg/d increased propionate and reduced the acetate proportion compared to the results with no biotin supplementation (control), which coincided with a lower A:P ratio. Nakai et al. (2013) reported that there were no effects of betaine supplementation, incubation time or an interaction between betaine supplementation and the incubation time on VFA, propionic acid and butyric acid.

CH_4 production was reduced when the C:R ratio increased. This result might have been due to an increased proportion of concentrate in the diet, which changed the ruminal concentrations of VFAs in such a way that less acetic acid and more propionic acid were formed. Hence, the supply of hydrogen for methanogenesis would be limited. A positive response to high levels of concentrate of methane reduction has been reported by Polyorach et al. (2014) and Phesatcha et al. (2020). However, no published literature to date shows any depression in CH_4 production by supplementation with betaine, biotin and chromium. In the current study, BBC supplementation at low and medium doses (3 g/kg DM and 6 g/kg DM, respectively) appeared to increase CH_4 production, but it had no effect at high levels (9 g/kg DM) which was the similar to the control.

Based on the findings in the current study, it can be concluded that the ratio of C:R at 70:30 and the BBC level at 6 g/kg DM resulted in better rumen characteristics in terms of MBP, EMBP and TVFA production. However, further in vivo experimentation is warranted to determine whether BBC supplementation improves nutrient utilization in ruminants.

Conflict of Interests

The authors declare that there are no conflicts of interests.

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