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Evaluation of dietary supplementation of B vitamins and HMBi on fermentation kinetics, ruminal or post-ruminal diet digestibility using modified *in vitro* techniques

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ABSTRACT

The effects of dietary supplementation of HMBi, alone or in combination with B vitamins were investigated on fermentation characteristics and *in vitro* ruminal and post-ruminal digestion using three modified techniques. A total-mixed ration was formulated using the assumptive animal information by CPM-Dairy software and used as a substrate. Eight dietary treatments arranged in a 2×2×2 factorial design with factors of HMBi (0 and 0.13% DM), folic acid (0 and 0.012% DM), and vitamin B_{12} (0 and 0.0021% DM). In the first experiment, fermentation characteristics and parameters were evaluated for dietary supplements' effects using *in vitro* gas measuring technique. In the second experiment, an *in vitro* modified batch culture technique was performed to study on *in vitro* digestion and fermentation end products. Finally, ruminal and post-ruminal digestibility of crude protein was investigated by a modified three-step technique. In comparison with control, supplementation of HMBi declined cumulative gas production ($P < 0.001$) and fermentation parameters ($P < 0.01$) all over the incubation times. Interaction effects between B_9 and B_{12} were identified ($P \leq 0.01$) that were associated with the decreased cumulative gas production ($P \leq 0.01$), A fraction ($P = 0.01$) and C fraction ($P = 0.02$). Digestion of CP declined at first incubation time by alone supplementation of HMBi, folic acid and B_{12} but it was improved after that. Supplementation of folic acid alone or in combination with HMBi increased ($P \leq 0.05$) NDF digestion for 4 and 24 h of incubation. Ammonia-N was decreased significantly with supplementation compared to intact diet. Total VFA was also enhanced (13 to 46%) by dietary supplementation of B_{12} compared to unsupplemented diet. Highest VFA was associated with HMBi from 24 h and thereafter. Addition of HMBi individually or with B_9 and B_{12} increased post-ruminal CP while B vitamins had not the same effect of supplementing individually. Results suggest that supplementation of diet with HMBi and B vitamins, modify rumen fermentation by decreasing gas production, enhancing production of VFA and increasing utilization of ammonia-N in the rumen.

Key words: folic acid, vitamin B_{12}, HMBi, post-ruminal digestion, *in vitro*

Introduction

It has been well documented that methionine (Met), under some conditions, is the first limiting amino acid for milk and milk protein production for lactating dairy cows (Lee et al., 2015). In order to allow its direct absorption from the small intestine, various strategies have been developed to protect methionine from the extensive metabolism via the rumen microorganisms (Brask et al., 2013). MetaSmart (MS), the isopropyl ester of the hydroxy analogue of methionine (2-hydroxy-4-methylthio-butanoic isopropyl ester; HMBi) has been proposed as a novel methionine supplement for ruminant animals (Breves et al., 2010). About 50% of HMBi are degraded to 2-hydroxy-4-(methylthio) butyric acid (HMB) by microorganisms (Noftsger et al., 2005) in the rumen, therefore, HMBi may affect rumen fermentation via HMB. The exact mode of action of HMBi in the rumen is not clear, Breves et al. (2010) suggested that the isopropyl ester confers a steric hindrance of some bacterial esterase enzymes that are not conferred by other organic esters.

The B vitamins have been well identified as being important over transition period due to critical roles for energy balance (Duplessis et al., 2012), liver health (Pinotti et al., 2002), immune function (Evans et al., 2006) and reproduction (Girard & Matte, 2006). The 5-methyl-tetrahydrofolic acid, the major circulating folate type, has been identified as a cofactor in methionine synthesis (Girard & Matte, 2006). Ruminal bacteria of the ruminants have the

ability to synthesize B vitamins using folic acid (B_9) and B_{12} vitamin (NRC 2001) for their use and use of the host organism. Apparent ruminal synthesis of folates in dairy cows ranges from 13 to 21 mg/d (Schwab et al., 2006). Using the rumen simulation technique, Bouillier-Oudot et al. (1988), reported that the addition of folic acid to mixed bacterial cultures resulted in increased digestibility of straw. Moreover, increased milk and milk component yields have been reported by dietary supplementation of B_9 in dairy cows (Graulet et al., 2007). However, Slyter & Weaver (1977) showed that *Ruminococcus flavefaciens* has folate requirements so that cellulose digestion was increased by supplementary folic acid (Hall, et al. 1955) and vitamin B_{12} (Scott & Dehority, 1965) in mixed cultures. In a study conducted by Wejdemar (1996), the addition of folic acid to clarified rumen fluid promoted growth of *Butyrivibrio fibrisolvens* and its utilization of ammonia-N. These results question the adequacy of the production of B-vitamins by ruminal microorganisms for ruminants with high production levels. Although folic acid and vitamin B_{12} are essential cofactors in the metabolism of certain bacteria (Hungate, 1966), there are no data detailing the effects of supplementing diets of ruminants with B vitamins on ruminal fermentation products, as is proposed in this study. The objective of the present study was whether dietary supplementation of HMBi; alone or in combination with B vitamins, at levels used in previous studies with dairy cows, would affect ruminal fermentation and *in vitro* nutrient digestibility and also subsequent intestinal digestion.

Materials and Methods

Diet preparation and treatments

A total mixed ration (TMR) was formulated by the CPM-Dairy (version 3.0.8.01) software for lactating dairy cows (680 kg body weight) with 70 days in milk and 45 kg milk yield (Table 1). Several samples of prepared TMR were mixed and dried for 48 h at 60°C, milled through a 2 mm sieve, and used as a substrate in the *in vitro* incubations. Eight dietary treatments were arranged in a 2×2×2 factorial design with factors of methionine analogue HMBi (MS, MetaSmart, Adisseo Inc., Antony, France) at two levels of 0 and 0.13% DM (30.42 g/d), folic acid (a powder with 95% B_9; Microvit® B_9 Promix Folic Acid Adisseo, Antony - France) at two levels of 0 and 0.012% DM (2.6 g folic acid/d), and vitamin B_{12} (a powder with 1% cyanocobalamine; Microvit ™ B_{12} Promix 10 000; Adisseo, Antony Cedex-France) as cyanocobalamine (SynCbl) at 0 and 0.0021% DM (0.5 g SynCbl/d). The amounts of supplementation of HMBi and B vitamins were chosen similar to the previous studies of Noftsger et al. (2005) and Graulet et al. (2007), respectively.

In vitro gas production

In the first experiment, the effects of supplements of B vitamins and rumen-protected analogue of methionine on fermentation characteristics were evaluated by 8 treatments using *in vitro* gas measuring technique according to Parnian et al. (2014). Prepared TMR was weighed (300 mg) into 50 ml serum vials based on 8 treatments in 6 replicates. The stock solutions were prepared with distilled water for each of supplements to meet suggested concentrations of supplements in 1 ml of each mixed-solution (by magnetic stirrer) pipetting into vials according to treatments' arrangement. Six extra vials were adjusted as blank vials and loaded only by equal volumes of water instead of supplements. Rumen contents were obtained from 3 adult dairy cows immediately after slaughter (Tabriz industrial

Table 1. *Ingredient and chemical composition of the diet.*

Dietary ingredients [% of diet DM]	
Corn silage	21.35
Alfalfa hay	18.79
Corn grain	11.70
Barley grain	14.09
Soybean Meal	9.82
Heat-treated full fat soybean meal	5.12
Beet pulp	5.34
Fish meal	2.56
Cotton seed	2.56
Cottonseed Meal	2.56
Fat powder	0.43
Corn gluten	2.14
Calcium carbonate	0.81
Sodium bicarbonate	0.76
Mineral†	0.62
Vitamin†	0.62
Salt	0.43
Dicalcium phosphate	0.17
Magnesium oxide	0.11
Chemical composition [% of diet DM]	
Crude protein	17.64
Rumen degradable protein	59.27
Rumen undegradable protein	40.73
Neutral-detergent fiber	31.98
Forage neutral-detergent fiber	20.95
Acid-detergent fiber	19.09
None fiber carbohydrate††	37.59
Ether extract	6.07
NE_L††† [Mcal/kg]	1.73
Ash	9.40

†Provided [kg of DM]: 44 mg of Mn, 58 mg of Zn, 14 mg of Cu, 0.85 mg of I, 0.38 mg of Co, 0.3 mg of Se, 23 mg of Fe, 6,500 IU of vitamin A, 2,000 IU of vitamin D, and 18 IU of vitamin E. Diets contains 0.98% Ca and 0.49% P (DM) based on CPM-Dairy software (version 3.0.8.01)
††NFC=100 – (% NDF–NDIN × 6.25) – %CP – %fat – % ash
†††Estimated by CPM-Dairy software in based of NRC 2001 Models.

slaughterhouse, Iran), and placed in a pre-heated thermos container at 39°C. In the laboratory, the ruminal digesta was mixed, strained through 4 layers of linen-cloth, and maintained at 39°C under O_2-free CO_2. All vials were incubated with 20 ml of rumen liquor and prewarmed McDougall (1948) buffer solution (1:2), capped with rubber and sealed immediately after loading; and were affixed to a rotary shaker platform (Jal Teb instruments, Iran) at 39°C. Gas production of 54 vials was measured after 2, 4, 6, 8, 12, 16, 24, 36, 48, 72 and 96 h of incubation using a water displacement apparatus. Gas production kinetics parameters were estimated using a DUD method with the NLIN procedure of SAS (2003) according to the non-linear exponential model:

$$GP = A\left(1 - e^{(-C(T-Lag))}\right)$$

where *GP* (ml) is a cumulative gas production in incubation time *T* (h), *A* is the maximum gas production (ml) after the asymptote is reached, *C* (/h) is the fractional fermentation rate, and *Lag* is Lag time.

In vitro batch culture digestibility

In order to investigate effects of HMBi and B vitamins on digestibility for DM, CP and NDF, an *in vitro* technique with some modifications was performed similarly to *in vitro* gas measuring technique in the second experiment. Prepared TMR was weighed (300 mg) into 2.5×3 nylon bags (45µm pore size), heat sealed and placed into eighty 50 ml serum vials (8 treatments in 10 replicates) for six times of incubation (4, 8, 12, 24, 36 and 48 h). Serum vials were loaded with prepared stocks of supplements and buffered inoculum based on treatment arrangement, as described above, and then were affixed to a rotary shaker platform at 39°C according to *in vitro* gas production technique. Produced gas was emptied during incubation via needles that installed on the rubber caps of vials. At the end of each incubation time, related vials removed and the bags in the vials were pulled out, rinsed and washed with phosphate buffer (pH 7.4) to ensure removal of any microbial contamination by rumen microorganisms. The phosphate buffer was prepared according to (Parnian-khajehdizaj, et al. 2014). One 16 ml aliquot of vials' rumen fluid was pipetted into a tube containing 4 ml of 25% metaphosphoric acid and another 4 ml rumen fluid was also pipetted into a tube containing 1 ml of 25% metaphosphoric acid, caped and inverted and frozen (-20°C) for subsequent analysis of total volatile fatty acids (VFA) and ammonia-N, respectively. The washed bags were dried at 60°C for 48 h and then weighed.

The residues were analysed for CP and NDF and *in vitro* batch culture digestibility (IVBCD) of CP and NDF were calculated as:

$$IVBCD = \frac{\{[(grams\ of\ TMR)\times(\%of\ nutrient\ in\ TMR)] - [(grams\ of\ residue)\times(\%of\ nutrient\ in\ residue)]\}}{[(grams\ of\ TMR)\times(\%of\ nutrient\ in\ TMR)]}$$

Ruminal and post-ruminal digestibility

An *in* vitro three-step technique was performed with some modification to investigate ruminal and post-ruminal digestibility of dietary crude protein (Gargallo et al., 2006). Approximately 5 g of prepared TMR in 10 replications per treatment weighed into 5×10 cm heat sealed nylon bags (45µm pore size) and placed in eight digestion vessels. The required amounts of each supplement for 50 g of the substrate were pipetted from owning stocks into every vessel according to the treatment levels. Then, 1.6 l buffer solution and 400 ml of the rumen inoculum were added to each vessel, and the vessels were flushed with CO_2 and incubated in a rotary shaker at 39°C for 12 h. Upon ruminal removal, the bags were squeezed and rinsed for 5 min 3 times in an automatic washing machine (or until the runoff is clear) and stored at 18°C. Frozen bags were thawed at room temperature and the content was dried at 60°C for 48 h to determine DM digestibility. The remained digestion residues were adjusted to chemical analysis of N content and the rest was pooled. To evaluate *in vitro* post-ruminal digestibility, 15 nylon bags (5×5 bag size and 45µm pore size) were filled with 625 mg of pooled 12 h incubation-exposed residue according to the treatment arrangement and then heat-sealed. Two litters of a prewarmed 0.1 *N* HCl solution (pH 1.9) containing 1 g/l of pepsin (P-7000, Sigma, St. Louis, MO) was added in vessels (containing 15 bags per vessel) and incubated with constant rotation at 39°C for 1 h rotary shaker. After incubation, all liquid was drained, and the bags were rinsed with tap water until the runoff was clear. To determine abomasal digestibility 5 of 15 bags per vessel were removed and the rest was incubated with 2 l of a prewarmed pancreatin solution (0.5 *M* KH_2PO_4 buffer, pH 7.75, containing 50 ppm of thymol and 3 g/l of pancreatin; Sigma P-7545) for 24 h with constant rotation at 39°C to determine intestinal digestion. After incubation, all liquid drained, and the bags were rinsed again with tap water until the runoff was clear. The bags were drained and dried in an oven at 60°C for 48 h and dry weight was recorded. The residue in all bags was analysed for N content. The amount of CP digestion in each step was calculated as the amount of the sample N minus the N remains after incubation divided by the amount of sample N before incubation.

Laboratory analysis

Analysis of the residue in all bags for N content was done by the Kjeldahl method (AOAC 2005). Ash content was determined following combustion in a furnace at 550°C for 5 h and ether extract of diet ingredients was measured using diethyl ether according to (AOAC 2005). An ANKOM^{200} fiber analyser with a neutral detergent solution as described previously by Van Soest, et al. (1991), was used to determine the remaining fiber in each bag. Total VFA in batch culture

fluids was determined by (Markham 1942). Ammonia-N was measured by phenol-hypochlorite assay using spectrophotometer at 630 nm according to Broderick & Kang (1980).

Mathematical and statistical analysis

The effects of HMBi, folic acid and vitamin B_{12} on *in vitro* gas production and digestibility were analysed using the general linear procedure in PROC GLM of SAS (2003) according to the model:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \delta_k + (\alpha\beta)_{ij} + (\alpha\delta)_{ik} + (\beta\delta)_{jk} + (\alpha\beta\delta)_{ijk} + \varepsilon_{ijkl}$$

where Y_{ijkl} = observation, μ = least squares mean, α_i = main effect of HMBi (i = 1–2), β_j = main effect of folic acid (j = 1–2), δ_k = main effect of vitamin B_{12} (k = 1–2), $(\alpha\beta)_{ij}$ = interaction effect of HMBi and folic acid, $(\alpha\delta)_{ik}$ = interaction effect of HMBi and vitamin B_{12}, $(\beta\delta)_{jk}$ = interaction effect of folic acid and B_{12}, $(\alpha\beta\delta)_{ijk}$ = interaction effect of HMBi, folic acid and vitamin B_{12}; and ε_{ijkl} = residual error.

The probability of difference (PDIFF) option of the least squares means statement in the GLM procedure was applied to compare the treatments means. Differences with $P < 0.05$ were accepted among means to represent as statistically significant differences.

Results and discussion

In vitro gas production and batch culture digestibility

Cumulative gas production of eight individual treatments for 4, 8, 12, 24, 36 and 48 h incubation times, fermentation kinetics parameters and also gas production curves are shown in Table 2 and Figure 1. In comparison with control, supplementation of HMBi declined cumulative gas production ($P < 0.001$) and fermentation parameters ($P<0.01$) all over the incubation times. Although no significant difference was observed among supplemented treatments through all incubation times in cumulative gas production, *A* and also *C* fractions when the dietary supplements of B_9 and B_{12} were individually included in TMR, interaction effects between B_9 and B_{12} were significant ($P \leq 0.01$) and were associated with the decreased cumulative gas production ($P \leq 0.01$), *A* fraction ($P = 0.01$) and *C* fraction ($P = 0.02$). Analysis of the fitted data revealed that the values of lag time ranged from 0.60 to1.21 h, as the highest lag time was associated with decreased gas production which highlights the interaction effects of supplements. *In vitro* batch culture digestibility kinetics of DM, CP and NDF of the diet containing different levels of supplements are shown in Table 3. Approximately 50% of the total DM disappearance occurred after 12 h of incubation in all the investigated treatments (Table 3). *In vitro* digestibility of DM was tended ($P=0.07$) to decrease at the early incubation time by dietary supplementation of B_9 while it was declined significantly by an interaction effect of supplements between HMBi and B_{12} or B_9. Despite the obvious affection of supplementary HMBi and B_9 on *in vitro* digestion of CP, digestion of NDF was not affected by HMBi supplementation at early incubation times while it was improved 7 to 15% by folic acid for 4, 24 and 48 h of incubation compared to unsupplemented treatment. This improvement was also obvious regarding the compound effect of HMBi with folic acid for 4 h and 24 h of incubation time related to NDF digestion. Decreased cumulative gas production ($P < 0.001$) and *A* fraction ($P < 0.01$) by HMBi compared with control was one of the main effects observed in this experiment. These results are in agreement with previous studies *in vitro* batch culture (Baghbanzadeh-Nobari et al., 2017), continuous culture (Noftsger et al., 2003); and also Alstrup et al. (2015) who has shown a tendency ($P=0.07$) for supplementation of HMBi to decrease production of CH_4/kg DMI in dairy cows. However, other studies revealed that HMBi increases the total concentration of VFA and acetate: propionate ratio in the rumen of goats (Brask et al., 2013; Feng et al., 2013) and ewes (Baghbanzadeh-Nobari et al., 2017) giving an indication of more methane gas production. In a research conducted by (Lee et al., 2015), dietary supplementation of HMBi linearly increased the genus composition of *Fecalibacterium* and quadratically decreased that of *Eubacterium* in ruminal contents of dairy cows. Moreover, the increase in the abundance of cellulolytic bacterial representatives in the rumen has been reported previously by Martin et al. (2013), who used methionine analogues HMBi and also HMB in cattle, but observed no direct effects on fiber degradation. While Baghbanzadeh-Nobari et al. (2017) found a significant positive effect on fiber degradation by HMBi in the rumen of ewes. Moreover, the positive effects of HMBi on fiber digestibility in the rumen have also been reported by several *in vitro* and *in vivo* studies (Noftsger et al., 2005; Feng et al., 2013).

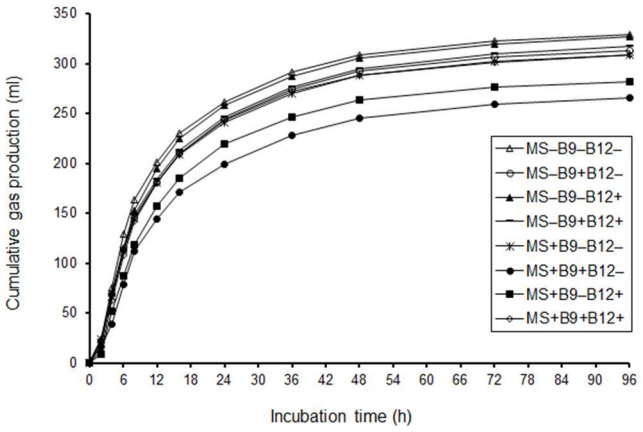

Figure 1. *Effects of dietary supplements of folic acid (B_9), vitamin B_{12} and HMBi (MS) on cumulative gas production curves.*

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Table 2. *Effects of dietary supplementing different levels of HMBi (MS) and B vitamins on cumulative gas production and fermentation kinetic parameters of in vitro gas production.*

	Treatments[1]									P-value						
	MS–				MS+											
Item	B_9–B_{12}–	B_9+B_{12}–	B_9–B_{12}+	B_9+B_{12}+	B_9–B_{12}–	B_9+B_{12}–	B_9–B_{12}+	B_9+B_{12}+	SEM	Met	B_9	B_{12}	MS×B_9	MS×B_{12}	B_9×B_{12}	MS×B_9×B_{12}
Cumulative gas production																
4h	75.4^{d}	70.2^{d}	68.4^{d}	68.0^{d}	66.9^{d}	52.3^{e}	38.6^{e}	62.2^{d}	5.15	<.001	0.81	0.06	0.32	0.52	<.01	0.02
8h	163.5^{d}	152.8^{d}	145.1^{d}	149.1^{d}	145.3^{d}	118.7^{e}	111.9^{e}	141.9^{d}	8.36	<.001	0.88	0.17	0.67	0.61	<.01	0.08
12h	201.6^{d}	194.7^{d}	180.5^{d}	183.7^{d}	181.2^{d}	156.7^{e}	144.3^{e}	181.2^{d}	8.74	<.001	0.72	0.8	0.51	0.43	<.01	0.04
24h	261.4^{d}	258.8^{d}	243.9^{d}	246.8^{d}	240.9^{d}	219.1^{e}	198.9^{e}	243.4^{d}	9.77	<.001	0.41	0.09	0.42	0.67	0.01	0.03
36h	291.6^{d}	287.4^{d}	274.4^{d}	276.1^{d}	270.0^{d}	246.1^{e}	228.1^{e}	271.9^{d}	9.78	<.001	0.53	0.11	0.42	0.65	0.01	0.03
48h	309.1^{d}	306.0^{d}	292.4^{d}	294.7^{d}	288.4^{d}	263.6^{e}	245.7^{e}	288.6^{d}	9.76	<.001	0.53	0.10	0.49	0.70	0.01	0.03
Fermentation parameters																
A[ml]	317.0^{d}	315.3^{d}	303.5^{d}	306.3^{d}	298.3^{d}	273.9^{e}	258.2^{e}	297.7^{d}	9.15	<.01	0.53	0.14	0.58	0.81	0.01	0.02
C[/h]	0.088^{d}	0.084^{de}	0.078^{ef}	0.080^{def}	0.080^{def}	0.076^{ef}	0.072^{f}	0.084^{de}	0.0035	0.08	0.52	0.14	0.29	0.13	0.02	0.24
Lag[h]	0.72^{ef}	0.94^{def}	0.60^{f}	0.63^{f}	0.68^{f}	1.21^{d}	1.07^{de}	1.15^{d}	0.132	<.01	0.02	0.78	0.34	0.04	0.09	0.50

[1] MS–B_9–B_{12}– = no supplement; MS–B_9+B_{12}– = 2.6 g of folic acid/d; MS–B_9–B_{12}+ = 0.5 g of vitamin B_{12} (as SynCbl)/d;
MS–B_9+B_{12}+ = 2.6 g of folic acid/d + 0.5 g of vitamin B_{12} (as SynCbl)/d;
MS+B_9–B_{12}– = 30.42 g of HMBi/d; MS+B_9+B_{12}– = 30.42 g of HMBi/d + 2.6 g of folic acid/d;
MS+B_9–B_{12}+ = 30.42 g of HMBi/d + 0.5 g of vitamin B_{12} (as SynCbl)/d;
MS+B_9+B_{12}+ = 30.42 g of HMBi/d + 2.6 g of folic acid/d 0.5 g of vitamin B_{12} (as SynCbl)/d.
[def] Values within a row with different superscripts differ significantly at P≤0.05.

Table 3. *Effects of dietary supplementing different levels of HMBi (MS) and B vitamins on digestibility of dry matter, crude protein and neutral-detergent fiber during in vitro batch culture incubation.*

	Treatments[1]									P-value						
	MS −				MS +											
Item	B_9–B_{12}–	B_9+B_{12}–	B_9–B_{12}+	B_9+B_{12}+	B_9–B_{12}–	B_9+B_{12}–	B_9–B_{12}+	B_9+B_{12}+	SEM	MS	B_9	B_{12}	MS×B_9	MS×B_{12}	B_9×B_{12}	MS×B_9×B_{12}
Digestibility of dry matter [% of DM]																
4h	42.1^{d}	36.6^{ef}	38.9^{de}	33.7^{f}	36.4^{ef}	38.0^{e}	37.0^{ef}	39.4^{de}	1.27	0.91	0.07	0.27	<.01	0.03	0.76	0.88
8h	44.1	43.3	46.1	42.5	42.9	46.3	43.6	45.8	1.33	0.51	0.27	0.08	0.23	0.25	0.30	0.66
12h	48.2^{e}	51.7^{de}	52.0^{de}	53.0^{d}	50.9^{de}	47.9^{e}	53.5^{d}	50.2^{de}	1.44	0.53	0.67	0.02	0.01	0.94	0.51	0.58
24h	66.6	64.3	65.0	67.2	65.6	64.9	67.6	64.5	1.65	0.91	0.40	0.53	0.44	0.97	0.67	0.14
36 h	70.9^{f}	75.6^{de}	71.8^{ef}	73.5^{def}	74.3^{de}	74.0^{def}	72.9^{def}	76.3^{d}	1.46	0.17	0.02	0.93	0.41	0.62	0.88	0.11
48 h	77.3^{ef}	77.2^{ef}	77.1^{ef}	79.1^{de}	74.9^{f}	77.5^{def}	81.4^{d}	79.4^{de}	1.39	0.52	0.52	0.01	0.73	0.10	0.54	0.09
Digestibility of crude protein [% of DM]																
4h	46.4^{d}	38.4^{f}	47.4^{d}	38.8^{ef}	49.4^{d}	41.3^{ef}	48.1^{d}	42.0^{e}	1.2	<.01	<.001	0.79	0.45	0.53	0.64	0.45
8h	47.2^{efg}	44.2^{gh}	46.4^{fg}	42.6^{h}	50.0^{e}	49.0^{ef}	54.0^{d}	49.7^{ef}	1.27	<.001	<.001	0.52	0.68	0.05	0.27	0.49
12h	48.4^{h}	57.9^{de}	54.8^{ef}	55.0^{ef}	52.5^{fg}	55.1^{ef}	58.5^{d}	50.6^{gh}	1.23	0.90	0.22	0.15	<.001	0.56	<.001	0.74
24h	64.3^{ef}	64.3^{e}	66.1^{def}	66.4^{def}	64.9^{def}	62.0^{e}	69.0^{d}	62.3^{f}	1.68	0.54	0.06	0.09	0.04	0.89	0.46	0.39
36 h	70.3^{g}	77.5^{de}	71.8^{fg}	75.0^{def}	78.8^{d}	73.7^{efg}	75.7^{def}	77.4^{de}	1.41	<.01	0.08	0.90	<.01	0.72	0.48	0.01
48 h	74.3^{gh}	76.4^{fg}	88.8^{d}	71.8^{h}	74.6^{gh}	77.0^{efg}	80.9^{e}	78.9^{ef}	1.45	0.96	<.001	<.001	<.001	0.67	<.001	<.001
Digestibility of neutral-detergent fiber [% of DM]																
4h	35.5^{de}	32.6^{de}	35.4^{de}	29.7^{e}	34.8^{de}	38.0^{d}	37.5^{d}	38.4^{d}	2.13	0.01	0.44	0.98	0.04	0.32	0.40	0.92
8h	36.1^{de}	35.7^{de}	37.0^{de}	34.5^{e}	35.2^{de}	35.5^{de}	36.9^{de}	38.2^{d}	1.33	0.51	0.71	0.29	0.23	0.21	0.77	0.41
12h	40.3^{d}	37.0^{de}	37.4^{de}	33.6^{e}	36.0^{e}	39.2^{d}	37.5^{de}	41.1^{d}	1.54	0.22	0.93	0.52	<.01	0.03	0.98	0.82
24h	41.4	45.5	47.2	37.1	37.2	47.5	38.1	46.5	4.15	0.87	0.29	0.81	0.04	0.83	0.18	0.31
36 h	49.6	46.8	48.4	42.4	44.3	48.0	43.7	45.0	3.32	0.51	0.69	0.33	0.15	0.82	0.56	0.93
48 h	51.0^{def}	46.3^{ef}	49.7^{def}	45.9^{f}	56.2^{d}	56.4^{d}	53.6^{de}	45.3^{f}	2.7	0.02	0.04	0.51	0.97	0.13	0.32	0.23

[1] MS–B_9–B_{12}– = no supplement; MS–B_9+B_{12}– = 2.6 g of folic acid/d; MS–B_9–B_{12}+ = 0.5 g of vitamin B_{12} (as SynCbl)/d;
MS–B_9+B_{12}+ = 2.6 g of folic acid/d + 0.5 g of vitamin B_{12} (as SynCbl)/d; MS+B_9–B_{12}– = 30.42 g of HMBi/d;
MS+B_9+B_{12}– = 30.42 g of HMBi/d + 2.6 g of folic acid/d;
MS+B_9–B_{12}+ = 30.42 g of HMBi/d + 0.5 g of vitamin B_{12} (as SynCbl)/d;
MS+B_9+B_{12}+ = 30.42 g of HMBi/d + 2.6 g of folic acid/d 0.5 g of vitamin B_{12} (as SynCbl)/d.
[def] Values within a row with different superscripts differ significantly at P≤0.05.

Volatile fatty acids are important end-products of fermentation, which are produced in the rumen by microorganisms; and dietary composition, rumen ecosystem and microbial population are factors that influence VFA content and composition in the rumen. In the present study, the total amount of VFA (Table 4) numerically increased 15 to 29% and 10 to 34% by HMBi and folic acid, respectively, when both of them were individually supplemented in diet while decreased or unaffected cumulative gas production and fermentation parameters by these supplements were not in agreement of reported properties of them by literature (Feng et al., 2013). Noftsger et al. (2003) reported that HMB supplementation did not influence the production of total VFA, but resulted in high concentrations of butyrate and low concentration of propionate. However, no consistent modifications in rumen volatile fatty acid profiles were occurred *in vitro* (Vazquez-Anon et al., 2001; Noftsger et al., 2003) and *in vivo* (Noftsger et al., 2005) after supplementation of diets with HMB and HMBi analogues of Met.

Wang et al. (2016) deduced that the quantity of several bacteria, such as *Butyrivibrio fibrisolvens*, *Ruminococcus albus*, *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* and also the activity of several enzymes such as xylanase, pectinase, α-amylase and cellobiase increased quadratically with increasing rumen-protected folic acid supplementation. Moreover, these authors reported 42% increased overall fiber degradability and improved *in vitro* and *in situ* ruminal acid detergent fiber degradability when rations were included with folic acid. The inconsistent results on the fermentation response to HMBi or folic acid may be associated with the dietary composition, the mode of administration of folic acid and methionine analogue (unprotected *vs.* protected), a supplemental dose of supplements and also the ruminal synthesis of folic acid. Girard et al. (1994) revealed that the ruminal synthesis of folic acid was influenced by the concentrate to forage ratio. High-concentrate diets can promote the synthesis of B_9 (Hayes et al., 1966; Girard et al., 1994) and cause that supplementary B_9 has a reduced impact on ruminal fermentation. Ragaller et al. (2010) revealed that folic acid supplementation of diets high in concentrate did not also affect the ruminal fermentation, but increased the ruminal digestibility of acid detergent fiber in diets with a low concentrate proportion. Total VFA was also enhanced (13 to 46%) by dietary supplementation of B_{12} compared to unsupplemented diet, which highlights the vitamin B_{12} requirements of some rumen bacteria. Several *Bacteroides* species of rumen bacteria required vitamin B_{12} to produce propionate (Chen & Wolin 1981). Many propionate-producing bacteria form propionate via a randomizing sequence of reactions that includes conversion of succinyl-CoA to methylmalonyl-CoA (Gottschalk, 1986); this carbon transfer rearrangement is catalyzed by a vitamin B_{12}-dependent methylmalonyl mutase (Strobel, 1992). When *P. ruminicola* was grown by Strobel (1992) in continuous culture in the absence of vitamin B_{12}, acetate and succinate were the major fermentation products and virtually no

Table 4. *Effects of dietary supplementing different levels of HMBi (MS) and B vitamins on total volatile fatty acid production and ammonia concentration during in vitro batch culture incubation.*

	Treatments[1]									P-value						
	MS −				MS +											
Item	B_9–B_{12}–	B_9+B_{12}–	B_9–B_{12}+	B_9+B_{12}+	B_9–B_{12}–	B_9+B_{12}–	B_9–B_{12}+	B_9+B_{12}+	SEM	MS	B_9	B_{12}	MS×B_9	MS×B_{12}	B_9×B_{12}	MS×B_9×B_{12}
Ammonia-N [mg/dl]																
4h	5.2^{d}	4.3^{e}	4.1^{e}	3.2^{g}	3.7^{f}	3.0^{g}	3.0^{g}	3.0^{g}	0.14	<.001	<.001	<.001	0.01	<.001	0.12	0.07
8h	5.9^{d}	5.4^{de}	5.0^{de}	3.5^{g}	3.8^{fg}	4.8^{ef}	3.3^{g}	3.6^{g}	0.36	<.001	0.40	<.001	<.01	0.32	0.11	0.79
12h	7.6^{d}	5.5^{def}	5.5^{ef}	5.1^{f}	4.9^{f}	6.6^{def}	4.8^{f}	6.2^{def}	0.85	0.53	0.92	0.17	0.02	0.30	0.48	0.35
24h	18.8^{d}	16.8^{de}	18.9^{d}	15.6^{e}	17.9^{de}	18.2^{de}	16.5^{de}	17.8^{de}	0.96	0.90	0.19	0.29	0.02	0.81	0.97	0.42
36 h	21.4^{d}	20.1^{e}	19.8^{e}	20.1^{e}	19.9^{e}	20.0^{e}	20.0^{e}	20.0^{e}	0.32	0.15	0.36	0.09	0.29	0.08	0.10	0.08
48 h	21.9^{d}	18.8^{e}	20.0^{e}	19.9^{e}	20.1^{e}	20.0^{e}	20.1^{e}	20.0^{e}	0.35	0.76	0.01	0.47	0.03	0.52	0.02	0.03
Total VFA [mmol/dl]																
4h	3.6^{f}	4.3^{e}	4.5^{de}	4.5^{de}	4.5^{de}	4.9^{d}	4.6^{de}	4.6^{de}	0.18	<.01	0.04	0.09	0.94	0.02	0.04	0.56
8h	4.1^{f}	5.5^{de}	6.0^{d}	5.6^{de}	5.3^{de}	5.7^{de}	5.6^{de}	5.2^{e}	0.27	0.39	0.27	0.02	0.21	0.01	<.001	0.21
12h	6.0^{g}	7.0^{f}	7.4^{de}	6.9^{f}	7.2^{def}	7.1^{ef}	7.1^{ef}	7.5^{d}	0.12	<.001	0.02	<.001	0.37	<.001	<.001	<.001
24h	7.1e	8.7^{d}	8.8^{d}	8.6^{d}	9.1^{d}	8.6^{d}	8.8^{d}	8.6^{d}	0.21	<.01	0.18	0.03	<.01	<.01	0.01	<.01
36 h	8.2^{f}	9.8^{de}	9.5^{de}	10.1^{de}	10.4^{d}	9.9^{de}	9.1^{ef}	9.6^{de}	0.42	0.26	0.07	0.91	0.10	0.01	0.91	0.10
48 h	9.6^{e}	10.6^{de}	10.8^{d}	11.0^{d}	11.6^{d}	10.8^{d}	11.4^{d}	11.0^{d}	0.38	0.02	0.98	0.14	0.04	0.14	0.62	0.34

[1] MS–B_9–B_{12}– = no supplement; MS–B_9+B_{12}– = 2.6 g of folic acid/d; MS–B_9–B_{12}+ = 0.5 g of vitamin B_{12} (as SynCbl)/d; MS–B_9+B_{12}+ = 2.6 g of folic acid/d + 0.5 g of vitamin B_{12} (as SynCbl)/d; MS+B_9–B_{12}– = 30.42 g of HMBi/d; MS+B_9+B_{12}– = 30.42 g of HMBi/d + 2.6 g of folic acid/d; MS+B_9–B_{12}+ = 30.42 g of HMBi/d + 0.5 g of vitamin B_{12} (as SynCbl)/d; MS+B_9+B_{12}+ = 30.42 g of HMBi/d + 2.6 g of folic acid/d 0.5 g of vitamin B_{12} (as SynCbl)/d.

[def] Values within a row with different superscripts differ significantly at P≤0.05.

propionate was detected. Large amounts of propionate and very little succinate were produced when the medium was supplemented with vitamin B_{12}. The influence of B_9 on rumen fermentation has been reported once using eight steers at two different F: C ratios (70:30 and 30:70) by Chiquette et al. (1993) who found no effect of B_9 supplementation on ruminal acetate, propionate and butyrate concentrations up to 23 h after feeding in both diets. Furthermore, the ratio of acetate to propionate was also not affected. While in another study, Hayes et al. (1966) also found that folic acid improved ruminal fermentation, increased microbial activity and reduced ruminal pH in steers. Ruminal ammonia-N content (Table 4) was significantly ($P \leq 0.05$) reduced with supplementing HMBi and B vitamins and was the highest in control and the lowest in unsupplemented treatments. As Santoso et al. (2007) deduced that the balance between the degradation of dietary protein and the uptake of ammonium nitrogen by microorganisms, that use it for microbial protein synthesis, reflects the concentration of ammonia-N in the rumen. The stimulatory effects of HMBi on microbial protein synthesis in the rumen has been well established (Vazquez-Anon et al., 2001; Brask et al., 2013); hence, the lower ammonia-N concentration in our study may strengthen the probability of improved microbial protein synthesis by HMBi, albeit it was not determined. Fowler et al. (2015) suggested that the effects of HMBi on rumen microflora shift and growth probably are due to the modulation of gene expression in influencing micro-organisms and amination HMBi to methionine through the rumen bacteria and incorporated into bacterial protein. Utilization of ammonia-N was also improved by folic acid in rumen liquor (Wejdemar 1996). However, Ragaller et al. (2010) reported that folic acid supplementation reduced the ruminal fermentation rate of organic matter in dairy cows receiving a diet with a high concentrate proportion of 66% and caused a decreased microbial protein synthesis. These results showed that the dietary composition influenced the effects of folic acid on ruminal nutrient degradation and ruminal ammonia-N utilization. In the present study, it was shown that HMBi as an analogue of methionine may have the potential to modify *in vitro* ruminal fermentation with removing further ammonia-N from the rumen environment before 12 h of incubation via its spatial carbon structure.

Ruminal and post-ruminal digestibility

The effects of dietary supplementing different levels of HMBi and B vitamins on *in vitro* three-step digestibility of dry matter and crude protein were reported in Table 5. Results of *in vitro* ruminal DM digestibility in this technique were approximately close to *in vitro* batch culture digestibility of DM but with about 2-3% overestimation, while this comparison between both techniques for CP digestibility was not numerically the same. Supplementation of diet with B_9 had lowest *in vitro* digestibility of DM and CP. Digestibility DM and CP of HMBi-supplemented diet

Table 5. *Effects of dietary supplementing different levels of HMBi (MS) and B vitamins on in vitro ruminal, post-ruminal and total tract digestibility of dry matter and crude protein during in vitro three-step incubation.*

	Treatments[1]									P-value						
	MS −				MS +											
Item	B_9–B_{12}–	B_9+B_{12}–	B_9–B_{12}+	B_9+B_{12}+	B_9–B_{12}–	B_9+B_{12}–	B_9–B_{12}+	B_9+B_{12}+	SEM	MS	B_9	B_{12}	MS×B_9	MS×B_{12}	B_9×B_{12}	MS×B_9×B_{12}
Dry matter																
RID [% of DM]	55.4^{de}	54.4^{e}	57.8^{d}	57.7^{d}	57.9^{d}	55.2^{de}	56.4^{de}	56.6^{de}	1.09	0.82	0.24	0.07	0.60	0.22	0.06	0.52
AID [% of DM]	23.4^{efg}	24.2^{e}	23.7^{ef}	23.0^{fg}	25.6^{d}	23.0^{fg}	22.6^{g}	23.0^{fg}	0.37	0.85	0.03	<.001	0.02	0.04	0.16	<.001
IID [% of DM]	4.3^{de}	6.7^{def}	6.9^{de}	6.9^{de}	5.8^{ef}	8.0^{de}	8.5^{d}	8.2^{d}	0.89	0.03	0.08	0.02	0.88	0.98	0.06	0.90
PID [% of DM]	27.7^{e}	30.8^{d}	30.6^{d}	29.9^{d}	31.4^{d}	31.0^{d}	31.1^{d}	31.2^{d}	0.72	<.01	0.29	0.36	0.18	0.31	0.10	0.04
TID [% of DM]	67.8^{f}	68.5^{de}	70.7^{d}	70.4^{de}	71.2^{d}	69.1^{def}	70.0^{de}	70.1^{de}	0.79	0.19	0.47	0.03	0.28	0.03	0.60	0.14
Crude protein																
RID [% of CP]	58.4^{ef}	56.0^{f}	59.7^{d}	61.0^{de}	60.5^{d}	57.2^{ef}	58.9^{de}	58.8^{de}	1.02	0.88	0.12	0.03	0.44	0.03	0.02	0.83
AID [% of RUP]	39.4^{i}	41.3^{g}	41.0^{g}	42.2^{e}	41.7^{f}	39.2^{i}	42.8^{d}	40.4^{h}	0.11	0.63	<.01	<.001	<.001	0.17	0.11	0.01
IID [% of RUP]	35.9^{f}	33.8^{gh}	37.4^{e}	33.2^{hi}	33.1^{i}	38.3^{d}	34.3^{g}	35.7^{f}	0.31	0.24	0.67	0.48	<.001	0.01	<.001	0.06
PID [% of RUP]	75.3^{g}	75.1^{g}	78.4^{d}	75.4^{fg}	74.8^{g}	77.5^{e}	77.0^{e}	76.1^{f}	0.25	0.11	0.05	<.001	<.001	<.001	<.001	0.24
TID[2] [% of CP]	89.7^{fg}	89.0^{g}	91.3^{d}	90.4^{ef}	90.1^{ef}	90.4^{ef}	90.6^{e}	90.1^{ef}	0.25	0.35	0.02	<.001	0.04	<.001	0.18	0.45

[1] MS–B_9–B_{12}– = no supplement; MS–B_9+B_{12}– = 2.6 g of folic acid/d; MS–B_9–B_{12}+ = 0.5 g of vitamin B_{12} (as SynCbl)/d; MS B_9+B_{12}+ = 2.6 g of folic acid/d + 0.5 g of vitamin B_{12} (as SynCbl)/d; MS+B_9–B_{12}– = 30.42 g of HMBi/d; MS+B_9+B_{12}– = 30.42 of HMBi/d + 2.6 g of folic acid/d; MS+B_9–B_{12}+ = 30.42 g of HMBi/d + 0.5 g of vitamin B_{12} (as SynCbl)/d; MS+B_9+B_{12}+ 30.42 g of HMBi/d + 2.6 g of folic acid/d 0.5 g of vitamin B_{12} (as SynCbl)/d.
RID = ruminal *in vitro* digestibility for 12 h; AID = abomasal *in vitro* digestibility; IID = intestinal *in vitro* digestibility; PID post-ruminal *in vitro* digestibility; TID = total tract *in vitro* digestibility; TDP = total tract digestible dietary protein; IADP intestinally absorbable dietary protein; IDP = intestinal dietary protein digestibility estimated by Gargallo et al. (2006).
[2] TID = (TDP/CP) ×100; TDP = RDP + IADP; IADP = RUP × IDP.
[def] Values within a row with different superscripts differ significantly at P≤0.05.

was more improved in the abomasum than ileum while it could not be seen in post-ruminal digestibilities. As amino acids are absorbed mainly in the small intestine; anyway, when post-ruminal digestion was divided into abomasal and intestinal sections, it revealed this fact that crude protein was extensively degraded in abomasum rather than the intestine so that the values for abomasum are greater than intestine (Table 5). Post-ruminal digestibility of DM was affected by all supplements in comparison with unsupplemented diet but there was no significant difference among supplemented treatments. Addition of HMBi alone or in combination with B_9 and B_{12} increased post-ruminal CP while B vitamins had not the same effect of supplementing alone. However, we used no intestinal fluid to simulate *in vivo* digestion of non-fiber carbohydrate for post-ruminal digestion, where the main post-ruminal-investigation focus of this study was on crude protein digestibility. Despite the substantial increase in the total tract *in vitro* digestibility in response to dietary supplementation of HMBi or B vitamins, this improvement was not large enough to enhance markedly to break down the substrate in the rumen. However, the concentration of supplemental folic acid at current study was approximately two times greater than the one given to the growing steers in the study of Chiquette et al. (1993) who had not observed any effect of folic acid supplementation on digestibility of DM, CP and NDF in both high-forage and high concentrate diets. It was concluded that the effects of addition of HMBi in combination with folic acid on the degradation of NDF were more pronounced than with vitamin B_{12} as confirmed by *in vitro* batch culture technique. Despite the mitigation of *in vitro* cumulative gas production by supplements, the total VFA concentration and ammonia-N uptake by rumen microbes were improved. Future research, including *in vivo* studies, in order to understand the factors that contribute to antimicrobial activity and selection of the optimal dose, is required. The current results spotlight the lack of knowledge about to what extent of B vitamins can suffer the degradation by rumen microbes making the influence on fermentation.

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