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Effects of of 2-hydroxy 4-(methylthio) butanoic acid iso-propyl ester (HMBi) and dl-Met on *in vitro* fermentation characters of high yielding dairy cow diets

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ABSTRACT

An in vitro gas production and degradability study has been conducted to investigate the effects of four treatments including: no supplement (control), 0.065% HMBi/ DM diet (HMBi-1), 0.13% HMBi/ DM diet (HMBi-2) and 0.088% dl-Met/ DM diet (dl-Met) on typical dairy cow diets. Two diets with 17.7% (HCP, high crude protein) and 15.7% (LCP, low crude protein) CP have been formulated by different ingredients for early lactating Holstein dairy cows (DIM 55±7, BW 650 and Milk yield 55±6.4). Resu lts revealed that digestibility of DM were significantly increased by HMBi and dl-Met addition while digestibilities of ADF, NDF and HEMI was decreased for the dl-Met treated diets compared with the diet containing the equivalent amount of Met supplied as HMBi-2. Asymptote gas production (A) has been affected by HBMi and dl-met supplementation (P<0.001) but fractional gas production rate (c) parameter of gas production has not affected by treatments. Regarding fermentation parameters, pH has not been affected by supplements or CP levels. Also, there are linear and quadratic effects of HMBi incremental levels on ammonia-N whereas its concentration decreased with addition of HMBi. It is concluded that supplementation dairy cows' diet with Met sources can alter rumen fermentation and its activity, which is approved by more degradability of DM in both HCP and LCP diets and enhanced cumulative gas production. Low crude protein diets can reduce excess ammonia-N load in rumen and increase N utilization in dairy industry which will modify animals as well as environment friendly farming.

Key words: Dairy cow, degradability, gas production, HMBi, methionine hydroxy analogs, rumen

INTRODUCTION

Metabolizable protein, which is the true protein that digested and absorbed in post-ruminal sites, delivers absorbable amino acids to mammary glands to synthesis of milk and milk protein [1]. There is a growing attention in maximizing efficiency of optimize amino acid delivery to the duodenum to meet the AA requirements of host animal for the highest production levels according its genetic potential. It has been well studied that Methionine (Met) and lysine (Lys) are the first two limiting AA for maximum milk yield and milk protein production [2]. Methionine is very unstable in rumen and when is degraded by rumen microorganisms for carbon and nitrogen, the amount that escapes the rumen might not be adequate for milk production. So, it is one of the most limiting amino acids in dairy rations and playing a considerable role in milk protein synthesis in mammary glands [3, 4]. Appropriate post-ruminal supply of methionine or balancing the metabolizable protein for high levels of methionine, enhanced milk protein synthesis [5]. Therefore, analogues have been produced to decrease degradation, increasing the supply of methionine to the cow and lengthening the supply of methionine to the rumen environment [6]. Remarkable finding to increasing post-ruminal Met supply is to feed rumen protected Met (RPM) [7]. Different technologies have been applied in order to supplying post-ruminal methionine that including physically methods by coating Met with lipid/pH-sensitive polymers or by some layers of ethylcellulose and stearic acid [8]. Studies have shown that these forms of RPM were effective for increasing both milk and component yield [7, 9-11] Other methods to provide post-ruminal Met is to supply derivatives and analogs of Met that resist microbial breakdown [7].

When hydroxylated analogs of Met [2-hydroxy-4-(methylthio)-butanoic acid, HMB] esterifies to various alcohols, this can affect its rumen degradability. The isopropyl ester of HMB (HMBi) was shown to have 40 to 58% bioavailability based on blood kinetics of a pulse ruminal dose [12]. Several studies have demonstrated the benefits of adding different types and forms of Met analogs to the diet to improve milk and milk component production. These analogues are including HMB and HMBi. Overall results of recent studies, suggested that feeding HMBi or Rumen protected methionine (RPM) would give similar improvements in milk production and N utilization. Most of in vitro studies about effects of HBMi on digestibility of nutrients and rumen fermentative characters have been investigated by continuous culture techniques. Investigation effects of HMBi and other methionine analogues on carbohydrate and protein ruminal digestion kinetics can be affected by dynamic characteristic of continuous culture and absence of protozoa. Protozoa have important role in protein and carbohydrate digestion [13, 14]. Objective of this study was to investigation and comparison the effects of two source of Met in forms of HMBi and dl-Met on ruminal nutrient digestibility and fermentation kinetics of two typical diets of high yielding dairy cows including different levels of crude protein and rumen degradable protein.

MATERIALS AND METHODS

The chemical analysis and *in vitro* assays were carried out at the advanced ruminant nutrition lab of dept. of animal sciences at faculty of agriculture, university of Tabriz, Iran.

Diets ingredients: All diets ingredients including forage and concentrate components have been sampled and purchased freshly in appropriate amounts from a local dairy farm and transferred to advanced animal nutrition lab and oven dried immediately for further use.

Chemical analysis: Dry matter (DM, method ID 934.01), ash (method ID 942.05), ether extract (method ID 920.30) and crude protein (CP, method ID 984.13) of each diets ingredients and TMR samples and the residues remaining after ruminal incubation were determined by procedures of [15]. Neutral-detergent fiber (NDF) and acid detergent fiber (ADF) were determined with an ANKOM^{200} (Ankom Technology, USA) Fiber Analyzer using the manufacturer recommended reagents and filter bags (#F57). Analysis of NDF was conducted with a heat stable α-amylase and without sodium sulphite and expressed exclusive of residual ash as ADF.

Diets and samples preparation: Two diets with 17.7% and 15.7% CP have been formulated by different ingredients (Table 1) for early lactating Holstein dairy cows (DIM 55±7, BW 650 and Milk yield 55±6.4). Cows' information (750 head) has been provided by statistics and records unit of a local dairy farm (FKA Co., Isfahan, Iran) for simulate the cows' input data in software. Data from chemical analysis and *in situ* incubation of ingredients (our unpublished data) for each ingredient have been transferred to CPM-Dairy (ver. 3.0.10) software feed library.

In vitro gas production test: Gas production was measured by Fedorak and Hrudey method [16]. A 300 mg portion of oven dried and ground (2 mm) diets weighted into 60 ml fermentation serum bottles with six replications for each treatment. Treatments were the treatment contained no supplement (control), 0.065% HMBi/ DM diet (HMBi-1), 0.13% HMBi/ DM diet (HMBi-2) and 0.088% dl-Met/ DM diet (dl-Met). The amounts of supplementation of HMBi (Meta Smart, Adisseo, France) and dl-Met (Rhodimet, Adisseo, France) were chosen similar to the previous continuous culture studies of Noftsger [17], Fowler [6] assuming the amount of Met supplied by each treatment diet to be 22 g/d at 25 kg/d DMI for dairy cows. A diet comprised of 800 g DM alfalfa hay and 200 g DM commercial concentrate was offered to the animals twice daily at 09.00 a.m and 16.00 p.m in equal sized meals. The animals had access to fresh water and mineral lick ad libitum. Rumen fluid, obtained 2 h after morning feeding from three canulated wethers (38±1.5 Kg), mixed, strained through four layers of cheesecloth and kept at 39^{oC} under a continuous CO_2 stream. Buffered rumen fluid (1:2 v/v) with McDougall's buffer (20 ml) was pipetted into the each pre-warmed (39^{oC}) serum bottles [18] then sealed serum bottles were immediately placed into rotary incubator and were gently shaked during the incubation period and the temperature has been kept at 39^{oC}. Negative controls containing buffered rumen fluid but no substrate, were also included in triplicate for correction of gas produced from small particles present in the ruminal fluid. Cumulative gas production (ml/g DM) was recorded after 2, 4, 6, 8, 12, 16, 24, 36, 48, 72 and 96 h of incubation. Cumulative gas production was fitted iteratively to the exponential model Y =A {1- exp [-c (t-Lag)]}, where y is the cumulative gas production (in ml) at time t, A, the asymptote (ml), c, rate constants (h^{-1}) and L, the lag time (in h) [19].

Digestibility and rumen fermentation: in order to determine the *in vitro* digestibility of the diets components and rumen fermentation (pH and Ammonia-N) parameters, upon end of the incubation period After 94 h of the incubation and for determination of digestibility of dry matter (DMD), NDF (DNDF) and ADF (DADF) six serum bottles have been used. Digestibility of hemicellulose has calculated through differences between NDF and ADF and its content in diets and residua. The bottle content was transferred to 50 ml falcon tube and the pH of the each tube was recorded immediately. The falcon tubes centrifuged at 3000 g for 10 min. A 5 ml aliquot from the supernatant were transferred into eppendorf tubes and stored at -20^{oC} until analyzed for Ammonia-N. Ammonia concentration was phenol-hypochlorite reaction adapted from Broderick and Kang (1980). Then falcon tubes centrifuged twice at similar way and contents washed thoroughly one time by distilled water and another time by saline buffer, to ensure removal any microbial contamination of the contents by rumen microorganisms. After this, falcon tubes containing the incubated diets residua were transfers to oven and the contents have been dried at 60^{oC} for two nights. So, chemical analysis and calculation of *in vitro* digestibility of nutrient components of treated diets have been performed using the mentioned dried residua pellets in falcon tubes.

Statistical analyses: Gas production parameters of *A*, *c*, and *LAG* were estimated using Marquardt option with NLIN procedure of SAS ver. 9.1 [20]. Effects of two levels of CP, methionine levels and theirs interaction with factorial arrangement on gas production parameters, rumen fermentation parameters and nutrients *in vitro* digestibility were subjected to the GLM procedure of SAS software. Significant differences were declared at P <0.05 using Tukey adjust of LS means. Orthogonal polynomial contrast was used to examine their responses (linear and quadratic) to increasing the levels of HMBi.

RESULTS AND DISCUSSION

Ingredients and nutrient composition of two diets are shown in Table 1. Forage to concentrate ratio (F:C) was 60:40 for both diets and reduction in CP has been formulated by replacement of corn grain instead of soybean meal. Replacement of soybean meal by corn grain resulted in reduction in rumen degradable protein (RDP) form 10.5% to 9.5%. Two diets have similar ADF and NDF contents and diets have been formulated as isoenergetic diets.

Table 1. Ingredients and nutrient composition of two diets.

Diet ingredients (% of DM)	High Crude protein diets	Low crude protein diet
Alfalfa	17.89	17.89
Corn Silage	22.11	22.11
Beet Pulp	4.00	4.00
Cottonseed	2.06	2.06
Soybean Meal	8.41	3.28
Soybean Extrude	6.80	6.80
Fish Meal	0.80	0.80
Canola Meal	5.24	5.24
Corn Grain	14.35	19.47
Barley Grain	12.69	12.69
Corn Gluten	2.32	2.32
Salt	0.27	0.27
SodiumBicarbonate	0.72	0.72
CalciumCarbonate	0.76	0.76
CalciumPhosDi	0.23	0.23
MagOx	0.11	0.11
MinVit †	0.24	0.24
Megalac	1.00	1.00
Nutrients (%)		
aNDF	31.38	31.44
ADF	18.75	18.71
Forage NDF	20.81	20.81
NFC ††	40.38	41.98
CP	17.70	15.70
RDP†††	10.5	9.33
RUP	7.20	6.36
NE_L (Mcal/kg)	1.72	1.71

† 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 0.7% Ca and 0.4% P (DM) based on NRC (2001)

††† NFC = 100 – (% NDF – NDIN × 6.25) – % CP – % fat – % ash

††† Estimated by CPM-Dairy software in based of NRC 2001 Models.

Digestibilities of hemicellulose, NDF, ADF and DM were significantly affected by HMBi and dl-Met addition (Table 2). Addition of HMBi or dl-Met significantly has affected DM digestibility of both HCP and LCP diets (P<0.01). Also, there was a significant difference between HCP and LCP diets in DM digestibility (P<0.01). Inclusion of HMBi or dl-Met has significant effects on ADF and NDF digestibility of diets as incremental levels HMBi decreased NDF digestibility in both HCP and LCP diets as well as ADF digestibility. Digestibilities of ADF, NDF and HEMI was decreased for the dl-Met treated diets compared with the diet containing the equivalent amount of Met supplied as HMBi-2. Over all effects of HMBi or dl-Met addition to HCP and LCP diets was tended to decrease the digestibilities of ADF, NDF, DM and HEMI. Also, digestibilities of ADF and NDF were affected quadratically by concentrations of HMBi (Table 2). Fowler et al [6] observed supplementation of methionine and HMBi had no effect on digestibilities of ADF and true OM using continuous culture technique. On that experiment NDF and hemicellulose digestibility were linearly affected supplementation. In contrast, Noftsger [17] used Four dietary treatments consisted of a control, two concentrations of HMB (0.055%, 0.110% of DM) and one concentration of dl-Met (0.097%) and reported that digestibility of ADF showed a quadratic effect to HMB in the diet, being highest at 0 and 0.11% HMB. Gas production technique can apply in order to study the ruminal fermentation kinetics well as methane emission studies [21]. Regarding of increased asymptote gas production due to HMBi supplemnetion as well as increased dry matter digestibility and remembering the decreased ADF and NDF digestibilities, we hypothesized that HMBi can enhance the groups of rumen microorganisms that require non-structural carbohydrate for their growth like amylolytic bacteria. Bach and Stern [22] designed a 2x2 factorial arrangement of 35 and 43% RUP and high or low dietary methionine and observed that high dietary methionine increased apparent NFC digestibility. So, it can concluded that increased dry matter digestibility and decreased ADF and NDF digestibilities might resulted from the requirement of some amylolytic and saccharolytic bacteria for amino acids [23]. We suggest that this sudden decline in ADF, NDF digestibilities might be indicative of a rumen microbial population shift.

Gas production techniques used extensively for monitoring of rumen fermentation kinetics as well as feedstuff energy content and organic matter digestibility [24, 25]. Asymptote gas production (A) has been affected by HBMi and dl-met supplementation (P<0.001) but *c* parameter of gas production has not affected by treatments. Besides this, reduction in CP levels of diets significantly decreased asymptote gas production (A) and increased fractional rate of gas production (c) (P<0.001). The highest A parameters has observed for HCP diets that supplemented by 0.088% HMBi/ DM (HMBi-1) and the lowest one was for LCP diet that supplemented by dl-Met. Lag time of the gas production has not been affected significantly with HMBi, dl-Met and CP levels have not significant effects on Lag time of gas production. Gas production curves of HCP and LCP diets are show in Figure 1.

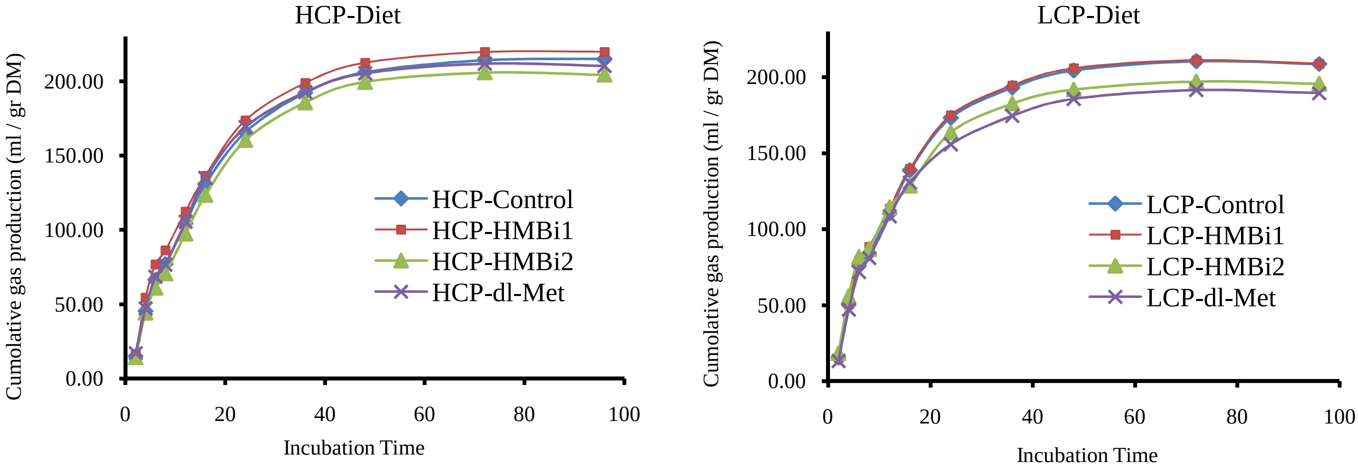

Figure 1. Gas production curves of HCP and LCP diets supplemented affected by treatments

In HCP diet, HMBi-1 and HMBi-2 treatments resulted in the highest and lowest cumulative gas production through all incubation times, respectively. In contrast, dl-Met contained LCP diet has the lowest cumulative gas production in all incubation times. Regarding fermentation parameters, pH has not been affected by treatment or CP levels (P<0.05). Also, there are linear and quadratic effects of HMBi incremental levels on Ammonia-N where as its concentration decreased with addition of HMBi. Significant differences have been observed in Ammonia-N concentration with HMBi-2 and dl-Met supplementation (P<0.05). Unlike HMBi, diets CP content has not affect the Ammonia-N concentration (Table 2). Increased synthesis of AA de novo by rumen microbes with HMBi supplementation may be necessary to counter that HMBi is a less available Met source compared to dl-Met [26].

Table 2. Nutrient digestibility, gas production and fermentation parameters of two diets in batch culture vials including two concentrations of HMBi or dl-Met.

Item	HMBi (%)					Contrasts				*P*-value		
	0	0.065	0.13	dl-met	SEM	L	Q	HMBi2 *vs* dl-met	Control *vs* All	M	CP	M×CP
Nutrient Digestibility												
DM Digestibility (%)												
HCP	44.36	48.83	49.83	50.22	1.12	0.831	0.888	0.007	0.193	0.008	0.006	0.104
LCP	49.55	51.91	50.94	50.04								
NDF Digestibility (%)												
HCP	44.86	40.02	40.02	36.58	0.63	0.09	<0.001	0.839	0.180	<0.001	<0.001	<0.001
LCP	38.18	35.91	42.59	37.18								
ADF Digestibility (%)												
HCP	53.24	47.37	46.47	41.45	1.47	0.265	0.027	0.005	0.022	0.006	<0.001	<0.001
LCP	53.77	54.61	49.80	45.37								
HEM Digestibility (%)												
HCP	51.65	52.61	51.94	43.33	1.18	0.087	0.127	<0.001	0.208	<0.001	<0.001	<0.001
LCP	61.35	49.97	51.75	55.11								
GP parameters												
A (ml)												
HCP	214.64	224.68	212.94	223.09	0.96	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
LCP	217.17	221.69	199.60	187.65								
c (%/h)												
HCP	0.061	0.063	0.060	0.063	0.003	0.989	0.594	0.251	0.510	0.616	<0.001	0.721
LCP	0.068	0.075	0.075	0.075								
Lag (h)												
HCP	0.30	0.25	0.56	0.63	0.16	0.117	0.457	0.201	0.407	0.368	0.916	0.266
LCP	0.51	0.46	0.20	0.61								
Fermentation parameters												
pH												
HCP	5.39	5.38	5.40	5.37	0.02	0.164	0.426	0.164	0.564	0.404	0.323	0.400
LCP	5.36	5.37	5.42	5.38								
Ammonia-N (mg/dl)												
HCP	5.57	5.31	4.91	5.30	0.40	0.016	0.312	0.002	0.031	0.012	0.607	0.850
LCP	5.37	5.25	4.93	5.37								

CONCLUSION

It has concluded that isopropyl ester of HMB can provide more rumen resistant source of Met in comparison with dl-Met. Results of cumulative gas production showed that HMBi can alter rumen fermentation and its activity, which is approved by more degradability of DM in both HCP and LCP diets. The modes of action of the different sources of Met on rumen microbial ecosystem especially on protozoa and fibrolytic populations still require further investigation. Reduction in ruminal ammonia-N concentration can eliminate dairy cows' requirements to more energy for conversion of BUN in liver. Besides this, diets with lower crude protein and supplemented with HMBi can improve nitrogen utilization in dairy farms that decrease N inputs and increase efficiency of N utilization is one of the most crucial goals of dairy industry.

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