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Methionine precursor effects on lactation performance of dairy cows fed raw or heated soybeans

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ABSTRACT

Two experiments were conducted to evaluate the effect of isopropyl ester of 2-hydroxy-4-(methylthio) butanoic acid (HMBi) on lactation performance of dairy cows. Experiment 1 evaluated the effect of HMBi in diets with 15.3% crude protein (CP) and with different proportions of rumen-degradable and undegradable protein. Variation in rumen-degradable and undegradable protein was achieved by replacing raw with heated soybeans. Experiment 2 was an on-farm trial to evaluate HMBi with a large number of observations and using a farm-formulated diet (17.2% CP). In experiment 1, 20 Holsteins at 100 ± 41 d in milk were allocated to 5 replicated 4 × 4 Latin squares with 21-d periods. Treatments were formed by a 2 × 2 factorial arrangement of raw or heated soybeans with or without HMBi. Paper capsules with HMBi were orally administered twice daily to each cow. Dosage of HMBi was 7.6 g of digestible Met/cow per day. There was no interaction between soybean type and HMBi. Heat-treated soybeans increased the yields of milk, protein, fat, and lactose, and reduced urea N in milk and plasma (PUN) compared with raw soybeans. Rumen microbial yield, dry matter intake (DMI), and the total-tract apparent digestibility of nutrients did not differ between soybean types. There was no evidence for HMBi-driven effects on DMI, milk and components yield, or diet digestibility. Urinary purine derivative excretion and PUN concentration were reduced in HMBi-fed cows compared with cows fed diets without HMBi. In experiment 2, 294 Holstein cows were blocked by parity and milk yield, and randomly assigned to HMBi (8.9 g of digestible Met/cow per day) or control. The final data set had 234 cows (215 ± 105 days in milk; 96 primiparous and 138 multiparous; 114 on control and 120 on HMBi) housed in 4 freestall groups (1 group/treatment per parity). The freestall group was the experimental unit for DMI, diet and orts composition, and feed availability. The HMBi supplement was top dressed for 28 d on the first daily meal of each cow, immediately after feed delivery of the same batch of feed to all 4 freestall groups (3 times per day). Sample collection and feed analysis occurred during the last 5 d. Spot urine samples and blood samples from each cow were obtained for analysis of the urinary allantoin to creatinine ratio and PUN. Feed availability, the contents of CP and neutral detergent fiber in diets and orts, and DMI did not differ. Cows fed with HMBi had greater milk protein yield and concentration compared with control and had no change in milk fat and lactose. Rumen microbial yield was greater and PUN was lower in HMBi-fed cows compared with control. In experiment 1, HMBi decreased rumen microbial yield and did not affect lactation performance, but it increased ruminal microbial yield and the secretion of milk protein in experiment 2. These results suggest that lactation response to HMBi may be partially mediated by ruminal events. Heated soybeans increased the efficiency of N utilization and the yields of milk, protein, fat, and lactose, but did not interact with HMBi supplementation.

Key words: isopropyl ester of 2-hydroxy 4-(methylthio)-butanoic acid, milk protein, plasma urea nitrogen, rumen microbial synthesis

INTRODUCTION

The consideration of AA absorption and requirements for fine tuning protein supply in lactating cow diets may improve cow performance, efficiency of dietary N utilization, and the finances of dairy farming. Several nutritional models used in practice estimate protein digestion and AA absorption from the intestinal lumen (NRC, 2001; Fox et al., 2004). Although mathematics in such models are known to not truly describe the complex biology of ruminants (Arriola Apelo et al., 2014), they have sufficient accuracy to be used under

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field conditions (Pacheco et al., 2012). Model predictions suggest that Met is the first limiting AA for milk protein synthesis in corn-based diets formulated with soybeans as the main RUP source, justifying, under this widespread dietary scenario, the supplementation of dairy cows with rumen-protected Met (**RPMet**). Supplementing diets with RPMet may increase milk protein yield and content, although responses often lack consistency (Patton, 2010; Robinson, 2010). Among other factors, the magnitude and direction of response depends on the N profile of the basal diet (Phipps et al., 2008), stage of lactation (Schwab et al., 1992), type of product (Patton, 2010), and the synthetic capacity of the mammary gland (Burgos et al., 2010). The meta-analysis of Zanton et al. (2014) suggests that the supplementation of RPMet results in increased yield of both milk protein and fat. The nutrigenomic role of AA in the regulation of milk protein, fat, and lactose synthesis has been described in the literature (Osorio et al., 2016).

The Met hydroxyl analog supplement, 2-hydroxy-4-(methylthio)-butanoic acid (**HMTBa**), is a Met analog in which the α-amino group is substituted with a hydroxyl group. The possibility of HMTBa being a source of Met to rumen microbes and to tissues, including the mammary gland, is well documented (Belasco, 1980; Lapierre et al., 2011). The isopropyl ester of HMTBa (**HMBi**) was developed in an attempt to increase the rumen protection of HMTBa (Graulet et al., 2005; Noftsger et al., 2005). After ingestion, HMBi is hydrolyzed to HMTBa and isopropanol during and before absorption by the rumen wall (McCollum et al., 2000; Breves et al., 2010). Little HMTBa or HMBi is found in omasal digesta (Noftsger et al., 2005), and only HMTBa is found in the blood following HMBi infusion into the rumen (Graulet et al., 2005). A fraction of the rumen-released HMTBa is oxidized to 2-keto-4-(methylthio) butanoic acid and further transaminated into L-Met (Dibner and Knight, 1984) for incorporation into microbial protein (Belasco, 1980).

The heat treatment of protein sources may improve protein quality for ruminants because of reduction in the rate of protein hydrolysis in the rumen, which may favor more rumen escape of digestible RUP (Van Soest, 1994). Heat-treated whole soybeans can maximize the amount of digestible AA passing to the small intestine of dairy cows in comparison to raw soybeans (Faldet and Satter, 1991). However, ruminal protein degradability and postruminal digestibility can be affected by temperature and steeping time during heat processing, and the protein dispersibility index (**PDI**) can been used to monitor heat processing practices (Hsu and Satter, 1995). The capacity of heat-treated soybeans to improve lactation performance relative to raw soybeans is a product-dependent variable. A commercial brand of heat-treated whole soybeans (Alfa Nutrisoja, Cooperativa Agroindustrial Alfa, Chapecó, Brazil) elicited positive responses in milk (+1.1 kg/d) and lactose (+60 g/d) yields of dairy cows when it replaced raw soybeans at 3.7% and at 10.7% of diet DM, without affecting DMI (Dias Júnior et al., 2017).

In this study, 2 experiments were conducted to evaluate HMBi supplementation of soybean-based diets. Experiment 1 evaluated the response to the Met precursor when added to low protein diets that differed in the proportion of RDP and RUP from raw or heated soybeans; both diets were predicted to provide adequate RDP, and thus yield similar amounts of ruminally synthesized microbial protein. We hypothesized that the variation in dietary RDP concentration would alter the effect of HMBi on indirect markers of rumen function, and the variation in dietary RUP would be used to evaluate the response to HMBi under increased flow of RUP from soybeans. Experiment 2 was a controlled on-farm experiment to evaluate supplementation of HMBi. Our overall objective of the 2 experiments was to evaluate the effects of HMBi supplementation on lactation performance of dairy cows.

MATERIALS AND METHODS

Experimental procedures were approved by the University of Lavras Bioethic Committee in Utilization of Animals (Protocol 040/2010).

Experiment 1

Twenty Holstein cows (100 ± 41 DIM, 8 primiparous) were assigned to 5 groups based on parity and milk yield. Within a group, cows were randomly assigned to a sequence of 4 treatments in concurrently run 4 × 4 Latin Squares with 21-d periods (first 14 d for adaptation), and balanced for carry-over effects. Treatments were formed by a 2 × 2 factorial arrangement of coarsely ground raw or heated whole soybeans (Alfa Nutrisoja) with or without HMBi (30 g/d of MetaSmart; Adisseo Inc., Antony, France; assumed as: 51% CP equivalent, 100% Met in CP equivalent, 50% bioavailability; Graulet et al., 2005; Whitehouse et al., 2019). Heated and raw soybeans originated from the same batch of seeds. The PDI of the heated soybeans was 10.5% of the CP (Hsu and Satter, 1995). The dry HMBi was orally given in paper capsules to each cow twice daily at a predicted CP equivalent dosage of 7.7 g/d of digestible Met. Controls received the paper capsule only. The daily dosage per cow was based on NRC (2001) prediction to achieve a Lys:Met ratio in MP of 3:1 (Whitehouse et al., 2013).

Table 1. Composition of the offered TMR and the consumed diet (% of DM) in experiment 1

Item	Treatment[1]			
	R	RHB	HT	HHB
Offered TMR				
Corn silage	38.5	38.5	38.4	38.4
Tifton hay	7.8	7.8	7.8	7.8
Raw soybeans	12.9	12.9		
Heated soybeans			13.1	13.1
Soybean meal	5.0	5.0	4.9	4.9
Finely ground mature corn	24.2	24.2	24.1	24.1
Citrus pulp	8.3	8.3	8.2	8.2
Urea	0.4	0.4	0.4	0.4
Premix[2]	2.9	2.9	2.9	2.9
CP	15.3	15.3	15.3	15.3
NDF	34.7	34.7	35.7	35.7
Ether extract	5.8	5.8	5.8	5.8
Ash	7.9	7.9	7.9	7.9
NFC	36.4	36.4	35.3	35.3
Consumed diet				
CP	15.8	15.8	15.8	15.8
NDF	34.1	34.1	35.2	35.1
Ether extract	6.0	6.0	6.0	6.0
Ash	7.8	7.8	7.8	7.8
NFC	36.3	36.3	35.2	35.3

[1]Treatments: R = raw soybeans; RHB = raw soybeans + HMBi; HT = heated soybeans; HHB = heated soybeans + HMBi. HMBi = 2-hydroxy-4-(methylthio) butanoic acid.

[2]% of diet DM: 0.4 MgO, 1.0 sodium bicarbonate, 0.3 salt, 0.8 limestone, 0.4 minerals and vitamins (per kg): 200 g of Ca; 156 g of P; 35 g of S; 30 g of Mg; 150 mg of Co; 2,000 mg of Cu; 200 mg of I; 82 mg of Se; 5,000 mg of Mn; 11,900 mg of Zn; 1,000 kIU of vitamin A; 220 kIU of vitamin D; 6.2 kIU of vitamin E.

Diets were formulated with the NRC (2001) dairy model for a cow producing 34.0 kg/d of milk with 3.50% fat and 3.10% CP, 620 kg of BW, and 23.2 kg/d of DMI. Urea was added to all diets to ensure adequate RDP (i.e., 0 RDP balance in the rumen) for the heated soybean diets and a positive RDP balance for the raw soybean diets. By substituting raw soybeans for heated soybeans, the goal was to increase the flow to the intestine of RUP from soybeans, thereby creating a situation where HMBi supplementation could be evaluated in diets differing in supplies of MP or RDP and provide similar supplies of MP from rumen bacteria.

Cows were individually fed in sand-bedded tiestalls. The TMR were mixed in a stationary mixer and offered twice daily at approximately 0600 h and 1400 h. Refusals from each cow and feed ingredients were sampled daily, and composite samples were formed and analyzed by period. Corn silage and refusals were dried in a forced air oven at 55°C for 72 h and ground through a 1-mm mesh screen. The DM content was determined by drying at 100°C for 24 h, and CP was determined by micro-Kjeldahl analysis (method 990.03 of AOAC International, 2012). Ether extract (**EE**) was analyzed after hydrolysis with hydrochloric acid (method 920.39 of AOAC International, 2012). Ash was analyzed by incineration at 550°C for 8 h. The NDF was analyzed using a TE–149 fiber analyzer (TECNAL Equipamentos para Laboratórios, Piracicaba, Brazil) including amylase and sodium sulfide. The nutrient composition of the offered TMR (Table 1) was calculated from the composition of the individual feeds (Table 2) multiplied by the amount of consumed feed DM. The nutrient composition of the consumed diet considered the nutrient composition of the refusal from each cow and was calculated for each treatment by the ratio of total nutrient intake to total DMI.

Cows were milked 3 times per day, starting at 0430, 1230, and 2000 h. Milk sampling occurred from d 15 to 17 of each period. Components and MUN concentration of 9 consecutive milk samples were measured by midinfrared analysis (Bentley Instruments Inc., Chaska, MN) at the Laboratory of the Paraná State Holstein Breeders Association (APCBRH, Curitiba, Brazil). Milk energy secretion (**MES**, Mcal/d) was calculated as: [(0.0929 × % fat) + (0.0547 × % protein) + (0.0395 × % lactose)] × kilogram of milk (NRC, 2001). After the morning milking, BW was determined on d 19 and 20, and BCS (1–5; Wildman et al., 1982) was evaluated by 3 independent appraisers.

Total-tract apparent digestibility of DM, OM, NDF, and non-NDF OM was determined on d 18 to 20 by collection of feces in buckets by 1 person for every 2 cows concurrent to defecation during 3 continuous 8-h

Table 2. Composition of feed ingredients in experiment 1 (mean ± SD, n = 4)

Item	% of as-fed DM	Nutrient, % of DM			
		CP	NDF	Ether extract	Ash
Corn silage	34.6 ± 1.3	7.8 ± 0.2	51.2 ± 1.2	4.6 ± 0.2	5.9 ± 0.3
Tifton hay	90.1 ± 1.1	13.5 ± 1.1	74.5 ± 0.9	3.0 ± 0.5	7.8 ± 0.1
Raw soybeans	88.3 ± 1.1	40.3 ± 0.3	26.1 ± 1.3	17.7 ± 0.5	5.6 ± 0.3
Heated soybeans	89.7 ± 1.2	39.8 ± 0.6	33.8 ± 1.2	18.1 ± 0.7	5.8 ± 0.3
Soybean meal	88.8 ± 0.7	48.5 ± 0.3	16.6 ± 1.6	3.6 ± 0.6	7.1 ± 0.5
Finely ground mature corn	86.6 ± 1.0	7.6 ± 0.3	12.3 ± 1.4	3.9 ± 0.1	1.4 ± 0.1
Citrus pulp	87.4 ± 1.2	7.0 ± 0.2	24.1 ± 1.6	4.3 ± 0.2	7.0 ± 0.1

sampling periods and weighed. The second and third sampling periods were each delayed by 8 h to avoid a major disturbance to the animals and still represent a 24-h collection period. Fecal aliquots (equal fresh-weight basis) were immediately frozen during the collection period and a composite sample was formed. The concentrations of DM, NDF, and ash in feces were determined as previously described for feed analysis. The urinary output was also collected in buckets, simultaneously to fecal sampling, to estimate rumen microbial synthesis based on purine derivative excretion. A 10% sulfuric acid solution was immediately added to the urine samples (1:9) before refrigeration at 4°C. Composite urine samples were diluted 1:3 with distilled water and frozen at −20°C. Allantoin (**Alla**) was analyzed as in Young and Conway (1942), and creatinine (**Crea**) and uric acid (**UA**) were analyzed using laboratory kits (Doles Reagentes e Equipamentos para Laboratórios Ltda, Goiânia, Brazil). The relative rumen microbial yield was estimated by the excretion (mmol/d) of purine derivatives in urine and by the purine derivative to Crea ratio (Chizzotti et al., 2008).

Blood samples from the coccygeal vessels were obtained on d 21 to determine plasma urea N (**PUN**). Samples were obtained immediately before the first daily feeding and 1, 2, 3, 6, 9, 12, 15, 18, and 21 h after feeding. The blood, collected with EDTA, was immediately refrigerated, centrifuged at 1,000 × *g* for 15 min at room temperature, and then the plasma was frozen at −20°C. The PUN concentration was analyzed with a laboratory kit (Labtest Diagnóstica SA, Lagoa Santa, Brazil). Plasma glucose concentration at 12 h postfeeding was analyzed with a laboratory kit (Doles Reagentes e Equipamentos para Laboratórios Ltda).

Experiment 2

In a controlled farm experiment, 294 cows were paired and blocked based on parity, milk yield, and DIM. Within parity, cows in each block were split between 2 freestall groups located in the same building. All 4 groups were fed the same batch of TMR 3 times daily. One group of primiparous and one of multiparous cows were supplemented with HMBi (35 g/d of MetaSmart), and the other 2 groups acted as controls. A final data set of 234 Holstein cows (96 primiparous and 138 multiparous) was generated based on the cow's presence during the entire experimental period and on the availability of data for all measured variables. Milk yield during 3 consecutive days before the start of the experiment was used for blocking and as a covariate in the statistical model. The supplied amount of HMBi (8.9 g/d of digestible Met) was top dressed to each cow once per day on the first daily meal immediately after feed delivery. Treatments were offered for 28 d and the response was evaluated on d 24 to 28.

The composition of the diet was defined by the farm manager. The formulated diet contained (% of DM) the following: 38.8% corn silage, 5% green chop Tifton (*Cynodon* spp.), 10.4% soybean meal, 8.6% heated soybeans (Alfa Nutrisoja), 16.9% mature ground corn, 8.6% citrus pulp, 4.7% whole cottonseed, 3.6% corn gluten feed, and 3.5% minerals, vitamins, and additives. To ascertain that diet composition and availability was not a factor in the response to HMBi, samples from each batch of TMR (3 times per day) were obtained at 5 locations of the feed bunk of both groups on d 24 to 28. A composite group sample was formed each day and frozen until analysis of DM, CP, and NDF, as previously described. On d 25 to 29, feed refusals were weighed and sampled for analysis. The DMI of the group was calculated, as well as the quantity of feed refusal as a proportion of the offered TMR.

All cows were evaluated for BCS and girth perimeter (**GP**) by 4 independent appraisers. Cows were milked 3 times per day. Milk yield of individual cows was measured on d 24 to 28, and the concentrations of solids and MUN were analyzed on 3 consecutive milk samples obtained on d 26. A spot urine sample from each cow was randomly obtained within d 24 to 28 for analysis of Alla and Crea. The relative rumen microbial yield was estimated with the Alla:Crea ratio and GP or GP raised to the 0.75 power ($GP^{0.75}$) as measures of body size, an adaptation of the methodology of Chen et al. (1995). Simultaneously with urine sampling, a blood sample was obtained from the coccygeal vessels for the analysis of PUN. Sample processing and laboratory methods were the same as previously described for experiment 1.

Statistical Analysis

All data were analyzed using PROC MIXED of SAS (version 9.4, SAS Institute Inc., Cary, NC). A $P \leq 0.05$ was interpreted as statistically significant and a $0.05 < P \leq 0.10$ as a trend. Analyses of studentized residuals were performed to check that model assumptions were reasonably met.

Experiment 1. The model for variables with 1 value per cow on each experimental period had the random effect of cow (1–20) and the fixed effects of period (1–4), soybean type (raw vs. heated), HMBi (effect of HMBi, HB, vs. control), and the interaction between soybean type and HMBi. For the variable obtained over time (PUN), the effects of sampling time (1–10) and its 2- and 3-term interactions with soybean and HMBi were added to the model. The mean square for the interaction of cow, period, soybean, and HMBi was the error

term to evaluate the effects of soybean and HMBi and for the interaction. The covariance structures evaluated were first order autoregressive, unstructured, and compound symmetry. The best covariance structure was defined by the Schwarz's Bayesian Criterion.

Experiment 2. The response in milk yield used a model containing the continuous covariate effect (milk yield before treatment allocation), the random effect of block, and the fixed effect of HMBi. For the other variables measured only once during the experiment, a similar model was used, but without the covariate term. Each freestall group received a treatment for variables measured per cow. Data on DMI and diet and orts availability and composition used the freestall group as the experimental unit (n = 2/treatment) and were analyzed as repeated measures over time to evaluate possible undesirable interactions between treatment and the daily feeding routine of the farm. The model contained the fixed effects of HMBi, sampling day (1–5), and the interaction of HMBi and sampling day. The mean square for the effect of freestall group nested within HMBi was defined as the error term to evaluate the effect of treatment. Covariance structures evaluated were the same as in experiment 1.

RESULTS

Experiment 1

Heated and raw soybeans had similar concentrations of CP and EE, whereas NDF concentrations were 7.7 percentage units of DM higher for heated soybeans (Table 2). Model simulation of the diets (Table 3) used the intake of analyzed ingredients (Table 2) in the offered TMR (Table 1) and the observed cow data (DMI, production data, and BW) for each treatment (Table 4). The NRC (2001) model estimated that the replacement of raw soybeans with heated soybeans increased diet RUP from 4.9 to 5.4% of DM (Table 3). Rumen RDP balance of the heated soybean diets was slightly negative; however, predicted bacterial MP flow was essentially equal for raw and heated soybeans (1,293 vs. 1,292 g/d, respectively). Because of an increased flow of predicted RUP, the predicted flow of MP increased 122 g/d with heated soybeans (mean value for the 2 diets with each soybean source).

Basal diets appeared to be deficient in Met relative to Lys. Supplementing the basal diets with HMBi resulted in a predicted Lys:Met ratio in MP of 3.10:1 for raw soybeans and 3.13:1 for heated soybeans (Table

Table 3. Predicted protein and AA supplies (NRC, 2001) based on actual intake, cow performance and size, and feed composition in experiment 1

	Treatment[2]			
Item[1]	R	RHB	HT	HHB
MP allowable milk, kg/d	38.0	37.8	40.2	41.9
NE_L allowable milk, kg/d	45.9	45.3	45.5	47.7
RUP, % of DM	4.9	4.9	5.4	5.4
RDP, % of DM	10.4	10.4	9.9	9.9
RDP balance, g/d	62	62	−44	−47
MP required, g/d	2,206	2,227	2,307	2,373
MP supplied, g/d	2,348	2,355	2,413	2,534
MP balance, g/d	141	121	106	155
MP–Bacterial, g/d	1,294	1,293	1,267	1,317
MP–RUP, g/d	943	944	1,035	1,095
MP–Endogenous, g/d	110	110	110	116
Lys, % of MP	6.70	6.69	6.58	6.55
Met,[2] % of MP	1.88	2.16	1.84	2.08
Lys/Met	3.57	3.10	3.58	3.13
d Arg, g/d	115	115	116	121
d His, g/d	53	53	54	56
d Ile, g/d	116	116	117	122
d Leu, g/d	205	205	209	218
d Lys, g/d	157	157	159	166
d Met,[3] g/d	44	51	44	53
d Phe, g/d	117	117	118	124
d Thr, g/d	116	116	117	122
d Val, g/d	128	128	130	136
Total d essential AA, g/d	1,301	1,301	1,314	1,372

[1]Designation d before amino acid indicates "digestible."

[2]Treatments: R = raw soybeans; RHB = raw soybeans + HMBi; HT = heated soybeans; HHB = heated soybeans + HMBi. HMBi = 2-hydroxy-4-(methylthio) butanoic acid.

[3]Assume that 30 g of HMB_i would provide 7.6 g of digestible Met.

Table 4. Feed intake, lactation performance, and feed efficiency in experiment 1

Item	Treatment[1]				SEM	*P*-value[2]		
	R	RHB	HT	HHB		SB	HB	SB × HB
DMI, kg/d	23.4	23.4	23.4	24.5	0.39	0.16	0.13	0.17
Milk, kg/d	34.5	34.8	37.5	38.0	0.50	<0.01	0.46	0.91
Fat, kg/d	1.039	1.080	1.163	1.162	0.0256	<0.01	0.45	0.43
Fat, %	3.02	3.10	3.11	3.06	0.052	0.64	0.71	0.24
CP, kg/d	0.994	1.012	1.070	1.087	0.0160	<0.01	0.29	0.98
CP, %	2.89	2.91	2.85	2.87	0.025	0.13	0.57	0.96
Lactose, kg/d	1.552	1.558	1.714	1.727	0.0279	<0.01	0.74	0.91
Lactose, %	4.51	4.48	4.58	4.55	0.037	0.05	0.47	0.98
Solids, kg/d	3.878	3.938	4.278	4.304	0.0697	<0.01	0.54	0.81
Solids, %	11.26	11.32	11.42	11.35	0.097	0.33	0.95	0.55
MUN, mg/dL	13.3	12.9	12.5	12.5	0.28	0.04	0.43	0.48
PUN, mg/dL	15.6	15.3	14.5	14.0	0.21	<0.01	0.05	0.74
MES,[3] Mcal/d	21.2	21.7	23.4	23.5	0.39	<0.01	0.46	0.66
Milk/DMI	1.48	1.50	1.61	1.56	0.026	<0.01	0.51	0.19
MES/DMI, Mcal/kg	0.91	0.94	1.01	0.97	0.179	<0.01	0.71	0.10
MES/DOMI,[4] Mcal/kg	1.42	1.46	1.60	1.52	0.041	<0.01	0.60	0.17
N in milk[5]/N intake	0.26	0.27	0.28	0.28	0.005	0.01	0.86	0.28
BW, kg	616	614	614	617	2.6	0.75	0.92	0.41
BCS, 1 to 5	3.47	3.48	3.45	3.45	0.024	0.32	0.83	0.86

[1]Treatments: R = raw soybeans; RHB = raw soybeans + HMBi; HT = heated soybeans; HHB = heated soybeans + HMBi. HMBi = 2-hydroxy-4-(methylthio) butanoic acid.

[2]Effects of soybean (SB), HMBi (HB), and interaction (SB × HB).

[3]Milk energy secretion.

[4]MES/Digestible OM intake.

[5]N in milk = CP/6.38.

3). The NE_L allowable milk was in excess of MP allowable milk in all diets. The difference between NE_L and MP allowable milk was higher for the 2 diets with raw soybeans than for the 2 diets with heated soybeans (7.7 vs. 5.6 kg/d) because MP allowable milk was lower in raw relative to heated soybeans (37.9 vs. 41.1 kg/d).

The replacement of raw with heated soybeans increased milk yield from 34.6 to 37.8 kg/d ($P < 0.01$), and consequently the daily yields of milk components (fat, protein, lactose, and total solids) and MES (Table 4). Calculated milk efficiency values [milk yield/DMI, MES/DMI, MES/digestible OM intake (**DOMI**), and N in milk/N intake] also showed positive responses to heated soybeans ($P < 0.01$; Table 4). Supplementation with HMBi did not improve lactation performance. The MES/DMI efficiency tended to increase when HMBi was added to raw soybeans, but decrease when it was added to heated soybeans ($P = 0.10$ for the interaction of soybean and Met).

Raw soybeans increased MUN ($P = 0.04$) and PUN ($P < 0.01$) concentrations (Table 4) relative to heated soybeans. Supplementation with HMBi decreased PUN in both diets ($P = 0.05$) without affecting MUN ($P = 0.43$). The decrease in PUN induced by HMBi and by heated soybeans was consistent over the day, although there was a marked sampling time effect (Figure 1).

The various Alla-based measures of relative rumen microbial yield were decreased by HMBi supplementation ($P \leq 0.05$), and trends of reduction were detected for UA/Crea × BW or $BW^{0.75}$ ($P = 0.10$). Type of soybeans had no detectable effect on the relative rumen microbial yield ($P \geq 0.12$; Table 5). Creatinine excretion did not differ, and the mean across treatments was 0.2425 mmol/kg of BW and 1.2085 mmol/kg of $BW^{0.75}$.

There was no evidence for treatment effects on total-tract apparent digestibility of nutrients ($P \geq 0.33$) (Table 6). Energy intake, estimated as DOMI, was similar across treatments ($P \geq 0.46$). Plasma glucose concentration did not differ (mean ± SEM: 53.8 ± 1.28 mg/dL; *P*-value: 0.76 for soybean, 0.76 for HMBi, and 0.55 for the interaction of soybean and HMBi).

Experiment 2

Supplementation of the on-farm diet with HMBi had no apparent effect on diet intake. There was similarity across treatments on feed availability and DMI, as well as for the analyzed composition of the offered TMR, feed refusals, and the consumed diet (Table 7). Daily variation was observed for some variables of diet composition and feed management; however, there was no indication of a treatment by day interaction ($P \geq 0.15$). The CP content of the consumed diet was in excess of 17% of DM. Soybean protein (from soybean meal and heat-treated soybeans) provided most of the

Table 5. Urinary metabolites indicative of changes in rumen microbial yield in experiment 1

	Treatment[1]					*P*-value[2]		
Item	R	RHB	HT	HHB	SEM	SB	HB	SB × HB
Urine, L/d	16.3	15.9	16.8	16.8	0.63	0.26	0.74	0.74
Alla,[3] mmol/d	297	248	281	252	16.9	0.73	0.02	0.56
UA,[4] mmol/d	45	36	39	33	2.8	0.12	0.01	0.74
Alla+UA, mmol/d	352	285	322	277	19.0	0.32	<0.01	0.57
Alla/Crea[5] × BW	1,231	1,041	1,223	1,082	73.4	0.82	0.03	0.74
UA/Crea × BW	182	155	171	151	13.7	0.59	0.10	0.83
(Alla+UA)/Crea × BW	1,441	1,226	1,392	1,224	92.3	0.78	0.04	0.80
Alla/Crea × $BW^{0.75}$	247	209	245	216	14.8	0.88	0.03	0.76
UA/Crea × $BW^{0.75}$	37	31	34	30	2.7	0.54	0.10	0.79
(Alla+UA)/Crea × $BW^{0.75}$	289	246	278	245	18.6	0.74	0.05	0.80
Alla/Crea	2.00	1.71	1.97	1.76	0.121	0.97	0.03	0.72
UA/Crea	0.30	0.25	0.27	0.24	0.022	0.45	0.04	0.68
(Alla+UA)/Crea	2.35	2.01	2.24	2.00	0.152	0.70	0.04	0.73
(Alla+UA)/DOMI,[6] mmol/kg	23.9	19.8	22.1	18.2	1.34	0.21	0.01	0.92
Crea, mmol/d	153	154	146	146	7.9	0.35	0.95	0.90
Crea/BW, mmol/kg	0.247	0.252	0.235	0.236	0.0132	0.27	0.91	0.85
Crea/$BW^{0.75}$, mmol/$kg^{0.75}$	1.232	1.253	1.174	1.175	0.0651	0.29	0.87	0.89

[1]Treatments: R = raw soybeans; RHB = raw soybeans + HMBi; HT = heated soybeans; HHB = heated soybeans + HMBi. HMBi = 2-hydroxy-4-(methylthio) butanoic acid.

[2]Effects of soybean (SB), HMBi (HB), and interaction (SB × HB).

[3]Alla = allantoin.

[4]UA = uric acid.

[5]Crea = creatinine.

[6]DOMI = digestible OM intake.

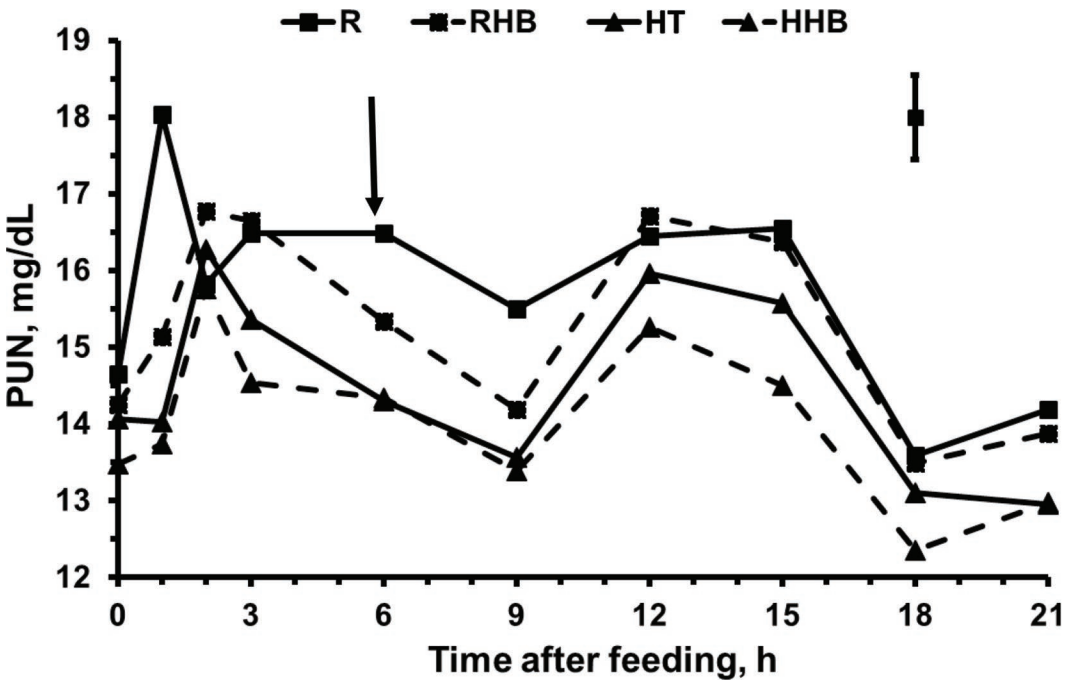

Figure 1. Plasma urea nitrogen (PUN) concentrations induced by raw soybeans (R), raw soybeans + HMBi (RHB), heated soybeans (HT), and heated soybeans + HMBi (HHB) in experiment 1. $P \leq 0.01$ for soybean, $P = 0.05$ for HMBi, $P = 0.74$ for soybean × HMBi, $P \leq 0.01$ for time, $P = 0.97$ for soybean × time, $P = 0.96$ for HMBi × time, $P = 0.56$ for soybean × HMBi × time. Arrow represents the second feeding of the day. HMBi = 2-hydroxy-4-(methylthio) butanoic acid.

Table 6. Digestible OM intake (DOMI) and total-tract apparent digestibility of nutrients (% of intake) in experiment 1

	Treatment[1]					*P*-value[2]		
Item	R	RHB	HT	HHB	SEM	SB	HB	SB × HB
DOMI, kg/d	14.8	14.8	14.7	15.2	0.32	0.52	0.46	0.48
D DM[3]	68.2	68.7	67.6	67.7	0.79	0.33	0.72	0.78
D OM[3]	69.9	70.4	69.7	69.6	0.76	0.53	0.75	0.71
D NDF[3]	53.3	54.3	53.9	53.9	1.07	0.90	0.62	0.62
D Non-NDF OM[3]	79.6	79.8	79.3	79.2	0.80	0.59	0.95	0.86

[1]Treatments: R = raw soybeans; RHB = raw soybeans + HMBi; HT = heated soybeans; HHB = heated soybeans + HMBi. HMBi = 2-hydroxy-4-(methylthio) butanoic acid.

[2]Effects of soybean (SB), HMBi (HB), and interaction (SB × HB).

[3]Digestibilities (D) of DM, OM, NDF, and non-NDF OM.

supplemental RDP and RUP; a source of supplemental NPN was not fed in this experiment.

Supplementation with HMBi increased milk protein concentration (+0.11 percentage units, $P = 0.03$) and yield (+47 g/d, $P = 0.05$), and had no detectable effect on content and yield of fat and lactose ($P \geq 0.12$; Table 8). Average DIM, cow size, and BCS did not differ ($P > 0.21$) across treatments, indicating that the blocking procedure was successful in achieving cow homogeneity across treatments. Dairy cows supplemented with HMBi had reduced ($P = 0.02$) PUN (13.9 vs. 15.6 mg/dL; Table 9), but no detectable difference in MUN was observed ($P = 0.20$). The HMBi increased rumen microbial yield assessed by the index Alla/Crea × GP or $GP^{0.75}$ in urine spot samples ($P = 0.02$; Table 9).

DISCUSSION

The objective of experiment 1 was to evaluate HMBi supplementation on lactation performance when the Met precursor was added to low protein diets that differed in the proportion of RDP and RUP from soybeans. It was desired, however, that RDP be adequate in all diets, and that seems to have been the case, as substituting heated soybeans for raw soybeans did not appear to affect differences in ruminal microbial yield (as assessed indirectly by the purine derivatives, Alla, and UA) or diet digestibility, but it decreased PUN and MUN, as expected. Therefore, this suggested that raw soybeans increased dietary RDP, and heated soybeans increased RUP and total dietary AA absorption. This is in accordance with the reduction in the instantaneously degradable protein fraction evaluated in situ of this heated soybean product relative to raw soybean (Dias Júnior et al., 2017). The PDI value of the batch of heated soybean used in the experiment (10.5% of CP) also suggests that the heat treatment was adequate to increase digestible RUP relative to raw soybeans (Hsu and Satter, 1995).

Substantial increases in the yields of milk, all solids, and milk lactose concentration were elicited by heated soybeans in experiment 1. Cows fed heated soybeans also had greater feed, N, and energy efficiencies than those fed raw soybeans. The increase in milk yield was around +3.1 kg/d with whole soybeans at 13% of the diet DM. Faldet and Satter (1991) observed a +4.5 kg/d response in milk yield when heated soybeans replaced raw soybeans for cows in early lactation, both

Table 7. Intake, diet, and refusals during 5 d of feed bunk sampling in experiment 2

	Treatment			*P*-value		
Item[1]	Control	HMBi[2]	SEM	Treatment	Day	Treatment × day
DMI, kg/d	19.5	19.1	0.46	0.59	0.11	0.99
Refusal, % of offered as-fed	4.9	5.6	0.54	0.16	<0.01	0.43
TMR DM, % of as-fed	43.4	43.9	0.65	0.64	0.02	0.80
Refusal DM, % of as-fed	38.7	41.2	2.02	0.48	0.07	0.28
Offered TMR CP, % of DM	17.0	17.2	0.30	0.67	0.29	0.45
Offered TMR NDF, % of DM	37.3	36.7	0.40	0.34	0.58	0.71
Refusal CP, % of DM	16.1	16.6	0.32	0.36	0.94	0.15
Refusal NDF, % of DM	39.3	41.5	2.13	0.54	0.01	0.15
Consumed diet CP, % of DM	17.1	17.3	0.41	0.71	0.28	0.46
Consumed diet NDF, % of DM	37.2	36.5	0.50	0.40	0.55	0.76

[1]Experimental units = 2 freestall groups per treatment.

[2]HMBi = 2-hydroxy-4-(methylthio) butanoic acid.

Table 8. Lactation performance of dairy cows in experiment 2

Item	Treatment		SEM	*P*-value
	Control	HMBi[1]		
Number of cows	114	120		
Days in lactation, d	189	196	5.5	0.40
Milk before,[2] kg/d	34.6	34.6	0.08	0.95
Milk, kg/d	34.6	34.8	0.53	0.83
Fat, kg/d	1.091	1.116	0.0278	0.56
Fat, %	3.18	3.23	0.074	0.69
CP, kg/d	1.049	1.096	0.0156	0.05
CP, %	3.07	3.18	0.031	0.03
Lactose, kg/d	1.589	1.609	0.0233	0.58
Lactose, %	4.57	4.63	0.022	0.12
Solids, kg/d	4.037	4.133	0.0582	0.28
Solids, %	11.72	11.93	0.088	0.11
BCS, 1 to 5	2.66	2.72	0.035	0.21
Girth perimeter, cm	203	204	0.6	0.52
MES,[3] Mcal/d	22.16	22.72	0.358	0.30

[1]HMBi = 2-hydroxy-4-(methylthio) butanoic acid.

[2]Milk yield before treatments allocation (covariate).

[3]Milk energy secretion.

at 13% of diet DM. However, the response of dairy cows to heat-treated soybeans seems to be inconsistent, probably reflecting differences in heat processing of the beans, control diet composition, or other experimental factors (Amanlou et al., 2012; Abdi et al., 2013). The efficiency of soybean MP conversion to milk protein was calculated. When heated replaced raw soybean in the diet, milk protein secretion increased 75 g/d, and predicted MP flow increased 122 g/d. The efficiency of converting the additional MP flow to milk protein synthesis was 61.5%, reasonably close to the 67% efficiency adopted by the NRC (2001) dairy model.

The proportional increase elicited by heated soybeans in daily yields of lactose (+10.6%) and fat (+9.7%) exceeded the increase in milk protein yield (+7.5%). Amino acids, in general, can regulate genes that code for protein, fat, and lactose secretion in the mammary gland (Osorio et al., 2016), probably explaining the positive response in all milk components to increased MP supply from soybeans. Dias Júnior et al. (2017) also observed an increase in milk lactose yield of dairy cows when heated soybeans replaced raw soybeans. The positive response in milk lactose was observed in 2 nonsimultaneous experiments evaluating the same heated soybean product of this experiment. The use of glucose-alternative energy sources by the mammary gland (Doepel and Lapierre, 2010) in response to increased MP flow from heated soybeans is consistent with the increase in lactose secretion at similar plasma glucose concentration. Other nutrient availability, probably glucose availability to the mammary gland, may have been the primary driver of milk synthesis (Lemosquet et al., 2009).

Table 9. Plasma urea N (PUN) and MUN concentrations and the ratio of allantoin (Alla) to creatinine (Crea) in urine in experiment 2

Item	Treatment		SEM	*P*-value
	Control	HMBi[1]		
PUN, mg/dL	15.6	13.9	0.45	0.02
MUN, mg/dL	16.4	15.7	0.37	0.20
Alla/Crea × GP,[2]	398	473	22.5	0.02
Alla/Crea × $GP^{0.75}$	105	125	5.9	0.02
Alla/Crea	1.85	2.20	0.095	0.03

[1]HMBi = 2-hydroxy-4-(methylthio) butanoic acid.

[2]Alla = allantoin; Crea = creatinine; GP = girth perimeter (cm).

The HMBi supplement had no detectable influence on cow lactation performance in experiment 1, regardless of the type of soybean fed (Table 4). Unexpectedly, the addition of HMBi to both soybean diets appeared to have reduced rumen microbial yield. Total Met supply may not have increased in response to HMBi, probably because of decreased supply and absorption of rumen microbial protein as indicated by the marked decreases in the urinary output of Alla and UA (Table 5). As DOMI was similar across treatments (Table 6), it appears that the decreased yield of rumen microbial cells was the result of a reduction in efficiency of microbial growth. However, in experiment 2, the addition of HMBi to an on-farm–formulated diet increased the concentration and the yield of milk protein of a large number of cows. The increased flow of microbial protein in response to HMBi in experiment 2 may have been the result of a greater proportion of ruminal carbon being used for the synthesis of AA rather than to generate VFA (Fowler et al., 2015). Gil et al. (1973) used mixed populations of rumen bacteria, using urea as N source and glucose or cellulose as carbohydrate substrates, to demonstrate that HMTBa or DL-Met addition can accelerate bacterial N incorporation and substrate digestion rates. Ruminal bacteria generally grow faster upon addition of AA, and hence more efficiently, because of a diluted maintenance requirement (Van Kessel and Russell, 1996; Kajikawa et al., 2002).

We observed major differences in how the cows responded to supplemental HMBi in the 2 experiments (Tables 4 and 8). The magnitude of the milk protein response to HMBi in experiment 2 paralleled the response to feeding rumen-protected DL-Met supplements (Patton, 2010). Both yield (+47 g/d) and concentration (+0.11 percentage units) of milk protein were increased by HMBi. Milk protein yield increased 5.28 g/g of MP Met supplemented, a high response compared with the 2.23 g of protein per gram of MP Met calculated in the meta-analysis of Zanton et al. (2014) as the mean of various RPMet sources.

The direction of change in ruminal microbial yield to RPMet supplementation agreed with the pattern of

milk protein response to HMBi. When HMBi decreased ruminal microbial yield, HMBi did not affect lactation performance, and it increased both milk protein yield and concentration when ruminal microbial yield was also stimulated. An NRC (2001) model prediction for the diet of experiment 2 (Table 10) was performed with the assumption that the proportion of each feed in the offered TMR DM was identical to the proportion in the formulated diet, and used nutrient composition of feeds from NRC (2001) tables. Exceptions were that corn silage composition was based on near-infrared spectroscopy analysis (3rLab/Rock River Laboratory, Lavras, Brazil), and heated soybean composition was the same as in experiment 1. The CP content of the manure-fertilized green chop Tifton (25% DM in as-fed, 65.2% NDF in DM) was set at 23% of DM; this assumption was made so that the diet predicted by the model had the same CP concentration of the consumed TMR (17.2% CP in DM. Table 7). Milk yield (34.8 kg/d), the concentrations of fat (3.21%) and CP (3.13%; Table 8), and DMI (19.3 kg/d; Table 7) were the means of the 2 treatments. Cow BW (624 kg) was estimated from the GP measured during the experiment (203 cm; Table 8) with a linear regression developed with 38 Holstein cows [639.7 ± 88.4 kg (466–867 kg)] simultaneously taped and weighed in our laboratory [BW (kg) = −1,000 + 8.0029 × GP (cm); $r^2 = 0.89$, $P < 0.01$]. The estimated diet RDP balance of experiment 2 (+189 g/d) was higher than the RDP balance predictions of experiment 1 (−47 to + 62 g/d. Table 3), in agreement with the MUN variation across experiments. The estimated MP allowable milk was 33.4 kg/d, and NE_L allowable milk was 33.9 kg/d in experiment 2, reasonably close to the observed milk yield (Table 9). The difference between experiments 1 and 2 in predicted RDP balance and MUN (12.8 vs. 16.1 mg/dL) suggested that HMBi stimulated rumen microbial yield only when RDP supply was in excess relative to the rumen requirement based on energy-sustainable microbial growth.

Phipps et al. (2008) evaluated the supplementation of diets varying in CP content (14.7% vs. 16.9% of DM) with HMBi. The high CP diet was formulated by the inclusion of formaldehyde-treated soybean meal and rapeseed meal, using the same types and content of forages and corn grain as the low CP diet. It was observed that HMBi decreased milk and total solids yield when added to the low CP diet, but increased milk protein yield when added to the high CP diet (Phipps et al., 2008). The meta-analysis of Leão et al. (2017), involving 32 published studies, suggested that diets with more than 16% CP were more responsive in milk protein yield to RPMet supplementation than diets with less than 16% CP, challenging the assumption that better AA profile of MP would allow for a reduction in diet CP content. This indicated that Met analogs may interact with dietary CP, presumably when RDP limiting diets are formulated.

Isopropanol is a plausible mediator for the negative action of HMBi on rumen microbial yield when the low protein diets were fed in experiment 1. The available evidence indicates that the HMBi that does not get absorbed is converted to HMTBa in the rumen (Graulet et al., 2005; Noftsger et al., 2005) and that virtually all of that HMBi generates isopropanol before or during absorption through the rumen wall (Graulet et al., 2005; Breves et al., 2010). The isopropanol generated during HMBi hydrolysis may recycle as acetone to the rumen, where it can again be reduced to isopropanol (Bruss and Lopez, 2000). The actual concentration of isopropanol in rumen fluid may be greater than would be predicted from the daily molar intake of HMBi. Alcohols can affect microbial cell wall fluidity and alter the transport of metabolites (Hui and Barton, 1973), which may limit AA incorporation into the cells, particularly when AA supply is scarce. Isopropanol also reduced methanogenesis from acetate in anaerobic bioreactors by reducing the transcript abundance of acetyl-CoA synthetase (Ince et al., 2011), suggestive of a potentially toxic effect on rumen function.

Table 10. Predicted protein and AA supplies (NRC, 2001) of the estimated basal diet of experiment 2[1]

Item[2]	Supply
MP allowable milk, kg/d	33.4
NE_L allowable milk, kg/d	33.9
CP, % of DM	17.2
RUP, % of DM	6.1
RDP, % of DM	11.2
RDP balance, g/d	189
MP required, g/d	2,210
MP supplied, g/d	2,151
MP balance, g/d	–60
MP–bacterial, g/d	1,073
MP–RUP, g/d	986
MP–endogenous, g/d	91
Lys, % of MP	6.48
Met, % of MP	1.80
Lys/Met	3.60
d Arg, g/d	105
d His, g/d	48
d Ile, g/d	104
d Leu, g/d	185
d Lys, g/d	139
d Met, g/d	39
d Phe, g/d	107
d Thr, g/d	103
d Val, g/d	115
Total d essential AA, g/d	945

[1]Milk yield = 34.8 kg/d, milk fat = 3.21%, milk CP = 3.13%, DMI = 19.3 kg/d, and BW = 624 kg [estimated from girth perimeter of 203 cm. BW (kg) = −1,000 + 8.0029 × girth perimeter (cm)].

[2]Designation d before amino acid indicates "digestible."

Another plausible mechanism for the reduction in microbial yield in response to HMBi would involve branched chain AA in ruminal fluid. A fraction of the released HMTBa in the rumen is aminated to L-Met for incorporation into microbial protein (Belasco, 1980). The steps in the synthesis of L-Met from DL-HMTBa involve the oxidation to 2-keto-4-(methylthio) butanoic acid, followed by transamination (Dibner and Knight, 1984). Branched chain AA transferase, present in many bacteria, is known to transaminate 2-keto-4-(methylthio) butanoic acid into Met, preferentially using Ile, Leu, and Val as amino donors (Sekowska et al., 2004). Limitation in the ruminal availability of branched chain AA could theoretically limit ruminal Met synthesis from HMTBa, and consequently its beneficial effect on rumen function (Gil et al., 1973). The interaction between ruminal protein supply and AA precursor supplementation may deserve further evaluation, especially when low protein diets are economically and environmentally more desirable than high protein diets, a trend in all animal production systems.

A common feature of experiments 1 and 2 was the decrease in PUN in response to HMBi. The conversion of DL-HMBi into L-Met, both at the tissue and rumen level, involves the incorporation of NH_3 from transaminated AA, a plausible explanation for part of the decreased PUN. A large decrease in PUN concentration 1 h postfeeding was observed when HMBi supplemented raw soybeans (Figure 1), suggesting that HMBi may have inhibited ruminal protein deamination. This is in accordance with the findings of Fowler et al. (2015), in which HMBi reduced the concentration and flow of NH_3-N in rumen fluid, increased the concentration of peptides, and increased the proportion of microbial N originating from NH_3-N. Results from these authors suggest that HMBi decreased deamination of feed AA or, more likely, increased the synthesis of AA from carbon skeletons and NH_3. Reduction in MUN in response to HMBi has been observed (St-Pierre and Sylvester, 2005), although this variable was not as sensitive as PUN to the variation imposed to the diets in our experiment.

CONCLUSIONS

In experiment 1, HMBi decreased rumen microbial yield and did not affect lactation performance, and it increased rumen microbial yield and milk protein yield and concentration in experiment 2. These results suggest that lactation response of dairy cows to HMBi may be partially mediated by ruminal events. Heated soybeans increased the efficiency of N utilization and the yields of milk, protein, fat, and lactose, but did not interact with HMBi supplementation.

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