Effect of 2-hydroxy-4-methylthio-butanoic acid on ruminal fermentation, bacterial distribution, digestibility, and performance of lactating dairy cows

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ABSTRACT

The objective of this experiment was to test the effect of a Met analog, 2-hydroxy-4-methylthio-butanoic acid (HMTBa), on ruminal fermentation and microbial protein synthesis, nutrient digestibility, urinary N losses, and performance of dairy cows. Eight multiparous lactating Holstein dairy cows were assigned to 4 levels of HMTBa [0 (control), 0.05, 0.10, and 0.15% (dry matter basis)] in a replicated 4 × 4 Latin square trial. Experimental periods were 28 d, including 21 d for adaptation. Ruminal ammonia and microbial N were labeled through a 6-d intraruminal infusion of ¹⁵NH₄Cl, and microbial protein synthesis in the rumen was estimated using the reticular sampling technique. Treatment had no effect on dry matter intake (28.4 to 29.8 kg/d), milk yield (44.1 to 45.3 kg/d), feed efficiency, and milk composition. Total-tract apparent digestibility of nutrients was generally not affected by treatment, except digestibility of crude protein and starch decreased quadratically with HMTBa supplementation. Fecal, but not urinary, and total excreta N losses were increased quadratically by HMTBa. Ruminal pH, ammonia concentration, protozoal counts, and the major volatile fatty acids were not affected by treatment. Microbial N outflow from the rumen was linearly increased by HMTBa. 2-Hydroxy-4-methylthio-butanoic acid linearly increased the proportion of *Fecalibacterium* and quadratically decreased the proportion of *Eubacterium* in ruminal contents. Of the individual bacterial species, HMTBa increased or tended to increase *Prevotella loescheii* and *Prevotella oralis*. 2-Hydroxy-4-methylthio-butanoic acid linearly increased the concentration (and yield) of 15:0 in milk fat. In the conditions of this cross-over experiment, HMTBa had no effect on feed intake and performance of dairy cows, decreased dietary crude protein digestibility, and increased microbial N outflow from the rumen.

Key words: 2-hydroxy-4-methylthio-butanoic acid, rumen fermentation, rumen bacteria, dairy cow

INTRODUCTION

Since early work in the 1960s and 1970s, Met has been considered a limiting or first limiting AA for lactating dairy cows in the United States (Griel et al., 1968; Broderick et al., 1970, 1974; Schwab et al., 1976). Various forms of the Met analog, 2-hydroxy-4-(methylthio)-butanoic acid (HMTBa), are commercially available and have been used primarily as sources of Met with variable rumen fermentation and production responses in dairy cows (Noftsker et al., 2005; Chen et al., 2011). Reportedly, HMTBa is rapidly absorbed and efficiently converted to L-Met in the tissue of nonruminant species (Knight and Dibner, 1984; Dibner, 2003; Jendza et al., 2011) and ruminants (McCollum et al., 2000; Lobley et al., 2006) and serves as a source of methyl groups or Cys synthesis. The bioavailability of HMTBa relative to that of DL-Met has been extensively studied with reports in the literature of 60 to 80% (depending on the function: BW gain, feed conversion, breast meat yield) in broiler chicken (Zelenka et al., 2013) and several meta-analyses have reported bioavailability of 81 to 100% in poultry (Vazquez-Añon et al., 2006; Vedenov and Pesti, 2010). A recent study with ¹³C-labeled HMTBa infused intravenously demonstrated that the compound can serve as a source of Met and also has a Met-sparing effect in dairy cows, with 15% of the milk protein Met originating from direct conversion of HMTBa to Met (Lapierre et al., 2011).

Limited data have suggested a possible effect of HMTBa on rumen fermentation, fiber degradability, and microbial protein synthesis. With a reported ruminal degradability of 50% or more (Koenig et al., 1999, 2002; Noftsker et al., 2005), HMTBa could affect microbial fermentation by a direct stimulatory effect of Met on certain cellulolytic bacteria or through an indirect effect on the noncellulolytic species in the rumen (Martin et al., 2013). Vazquez-Añon et al. (2001) reported, for example, a quadratic increase in bacterial N outflow and the efficiency of bacterial N synthesis in continuous

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culture fermenters. In vivo data on the effect of HMTBa on ruminal fermentation and specifically microbial protein synthesis are scarce or lacking. Although in vitro systems are often used to study rumen fermentation, in vitro data do not always correspond to in vivo responses and need verification in live animal experiments (Hristov et al., 2012).

Thus, the main objective of this study was to investigate the effect of HMTBa on ruminal fermentation, bacterial genus and species composition, and microbial protein synthesis in dairy cows. Our hypothesis was that HMTBa may stimulate microbial protein synthesis and specific cellulolytic bacteria in the rumen. Production effects, total-tract apparent digestibility of nutrients, urinary N losses, and milk FA composition effects of HMTBa were also studied.

MATERIALS AND METHODS

Animals involved in these experiments were cared for according to the guidelines of the Pennsylvania State University Animal Care and Use Committee. The committee reviewed and approved the experiment and all procedures carried out in the study.

Animals and Experimental Design

This experiment used 8 lactating multiparous Holstein cows in a 4×4 Latin square design, balanced for residual effects, with 2 concurrent squares. Cows were 51 (SD = 3.9) DIM at the beginning of the experiment and had an average BW during the trial of 660 (SD = 67.3) kg. Cows were fitted with soft plastic ruminal cannulas (10.2 cm internal diameter; Bar Diamond Inc., Parma, ID). The trial had 4 experimental periods, each 28 d in duration. Within period, the first 21 d served as adaptation and the remaining 7 d were used for sample and production data collection. Animals were managed according to the current standard practices at the dairy facility; recombinant bST was administered at the beginning (i.e., d 1) and in the middle (d 14) of each experimental period. The following 4 treatments were tested in this trial: control, 0% HMTBa (dietary DM basis); low, 0.05% HMTBa (as 0.057% Alimet, Novus International Inc., St. Charles, MO); medium, 0.10% HMTBa (0.114% Alimet); and high, 0.15% HMTBa (0.171% Alimet). The basal diet was formulated to meet or exceed the NE_L and MP requirements of a Holstein cow with 660 kg of BW producing 45 kg of milk/d with 3.60% milk fat and 3.05% true milk protein (NRC, 2001). Diets were fed as TMR once daily at 0800 h targeting 5 to 10% refusals. The HMTBa was fed through a premix that contained 100% fine ground corn (control) or 3.33% HMTBa, 6.67% HMTBa, or 10%

HMTBa replacing fine ground corn for low, medium, or high HMTBa, respectively. The premix was top-dressed once daily mixed with a small portion of the TMR, replacing an equivalent amount of corn grain. Cows consume most feed after new feed delivery and after milking (DeVries et al., 2003), and although we did not measure HMTBa consumption, it is safe to assume that the HMTBa premix was completely consumed. Cows had continuous access to a fresh water source.

Sampling, Markers, and Measurements

During the entire experiment, TMR offered and refusals were recorded daily. Samples of the forages were collected once weekly and samples of the TMR and refusals were collected twice weekly. Samples of the concentrate feeds were collected once per experimental period. Samples were dried for 48 h at 65°C in a forced-air oven and ground in a Wiley Mill (A. H. Thomas Co., Philadelphia, PA) through a 1-mm sieve for analysis. Composite (equal DM weight basis) samples of the TMR were submitted to Cumberland Valley Analytical Services (Maugansville, MD) for wet chemistry analyses of CP, NDF, ADF, ether extract, Ca, P, and estimated NE_L (details at <http://www.foragelab.com/Resources/Lab-Procedures>, accessed June 26, 2014). Organic matter was determined following the procedure of AOAC International (2000). Composite TMR samples were also analyzed for starch and indigestible NDF (iNDF). Indigestible NDF (used as an intrinsic digestibility and large particulate matter marker) was analyzed according to Huhtanen et al. (1994), except 25- μ m pore size filter bags (Ankom Technology, Macedon, NY) were used for the ruminal incubation. Starch was analyzed using an enzymatic-colorimetric procedure (Knudsen, 1997). Feed refusals were only analyzed for DM, and intake of nutrients was not corrected for composition of the refusals.

The reticular sampling (Hristov, 2007) and triple-marker (France and Siddons, 1986) techniques were used to estimate microbial protein outflow from the rumen. Markers, sampling, sample processing, and calculations were as described in Hristov (2007). Briefly, iNDF, Yb-acetate·4H₂O (Chemos GmbH, Regenstauf, Germany), and Cr-EDTA (Binnerts et al., 1968) were used as markers for the large and small particles and fluid phases of ruminal contents, respectively. Microbial protein was labeled with ¹⁵NH₄Cl (Cambridge Isotope Laboratories Inc., Andover, MA). All markers, except iNDF, were continuously infused into the rumen, through the rumen cannula, using peristaltic pumps. Markers were diluted in distilled water and infused for a total of 6 d (d 22 through 27 of each experimental period). Marker solution infusion rate was 1,759 (SD

= 81) for Cr and Yb and 1,750 (SD = 80) mL/cow per d for ^{15}N . Chromium and Yb markers were infused through one line and ^{15}N through another. Average infusion rates were 2.7 for Cr, 2.1 for Yb, and 250 mg/cow per day for ^{15}N . Actual Cr and Yb doses were estimated based on Cr and Yb output in fecal DM. Samples of reticular contents were collected during the last 2 d of the infusion; at 0800, 1400, and 2000 h (d 26); and at 0200, 0500, 1100, 1700, and 2200 h (d 27) and processed as in Hristov (2007). One reticular contents sample was collected before initiation of the marker infusion and analyzed for background concentrations of Cr, Yb, and ^{15}N . Chromium and Yb were analyzed at the University of Missouri–Columbia's Agricultural Experiment Station Chemical Laboratory (method 968.08D[b], AOAC International, 2002 and Combs and Satter, 1992, respectively) and ^{15}N enrichment was analyzed on a Delta V Advantage Isotope-Ratio Mass Spectrometer (Thermo Finnigan MAT GmbH, Bremen, Germany) interfaced with a Costech ECS 4010 C/N/S elemental analyzer (Costech Analytical Technologies Inc., Valencia, CA). Samples were pulverized using a Mixer Mill MM 200 (Retsch GmbH, Haan, Germany) before the ^{15}N analysis. Bacterial pellets were isolated from composite samples (volume based, per cow and experimental period) of reticular contents by differential centrifugation (Hristov and Ropp, 2003) and analyzed for total purines (Hristov et al., 2005a) and ^{15}N enrichment. The proportion of microbial N in the NAN of reticular contents was estimated based on $\delta^{15}\text{N}$ of the bacterial standard isolated from reticular samples as follows: background-corrected $\delta^{15}\text{N}$ (‰) of NAN in reticular contents (large particles, small particles, or fluid) $\div$ background-corrected $\delta^{15}\text{N}$ (‰) of reticular bacterial standard. Microbial N outflow from the rumen was calculated based on the concentration of microbial N in NAN of the reticular large and small particulate matter and fluid phase and the outflow rates of these phases calculated using the triple marker technique of France and Siddons (1986) as described in Hristov (2007).

Milk samples were collected before (background) and on the last 2 d of the marker infusion (d 26 and 27 of each experimental period) and processed to isolate milk protein (Hristov and Ropp, 2003), which was freeze-dried and analyzed for $\delta^{15}\text{N}$. The contribution of rumen microbial N to milk protein N was estimated as background-corrected $\delta^{15}\text{N}$ (‰) of milk protein $\div$ background-corrected $\delta^{15}\text{N}$ (‰) of reticular bacterial standard. The basis for this approach is explained in Hristov et al. (2005a).

Spot fecal (about 500 g) and urine (about 300 mL per sampling) samples were collected during each experimental period at the same time points as the reticular

samples. Fecal samples were dried at 65°C in a forced-air oven for 48 h and then composited (equal dry weight basis) by cow and period. Composite samples were analyzed for OM, CP, NDF, ADF, and starch. Neutral-detergent fiber and ADF were analyzed using Ankom's A200 fiber analyzer (Ankom Technology, Macedon, NY) based on Van Soest et al. (1991), using amylase and sodium sulfide in the NDF analysis. An aliquot of each composite fecal sample was analyzed for N on a Costech ECS 4010 C/N/S elemental analyzer (Costech Analytical Technologies Inc.); CP was calculated as $\text{N} \times 6.25$. Composite fecal samples were also analyzed for iNDF, Cr, and Yb as described above. Total-tract apparent digestibility was calculated based on iNDF concentration in TMR and feces (Schneider and Flatt, 1975). Aliquots of the urine samples were immediately acidified with 2M H_2SO_4 to a pH <3.0, diluted 1:10 with distilled water and stored frozen at -20°C . These samples were composited (equal volume based) by cow and period and analyzed for allantoin (Chen et al., 1992), uric acid (Stanbio Uric Acid Kit 1045, Stanbio Laboratory Inc., San Antonio, TX), urea-N (Stanbio Urea Nitrogen Kit 580, Stanbio Laboratory Inc.), and creatinine (Stanbio Creatinine Kit 0400; Stanbio Laboratory Inc.). Another aliquot of the composited urine samples was freeze-dried and analyzed for N using a Costech ECS 4010 C/N/S elemental analyzer. Daily volume of excreted urine and excretion of N, urinary urea-N, and purine derivatives were estimated as described in Lee et al. (2012). Urinary excretion of purine derivatives was used to estimate duodenal microbial N flow as described in Hristov et al. (2009). The ratio of purine N to total N in bacterial pellets isolated from reticular contents was used in the calculations.

Whole rumen contents samples were collected before and at 2, 4, 6, 8, 10, 14, 18, and 24 h after the morning feeding on d 27 and 28 of each experimental period. Samples were collected and processed as described elsewhere (Hristov et al., 2011). Rumen fluid pH was analyzed immediately (pH meter 59000–60 pH Tester, Cole-Parmer Instrument Company, Vernon Hills, IL) and the samples were further processed for analyses of ammonia, VFA, and protozoal counts (Hristov et al., 2011). Aliquots of the whole rumen contents were frozen immediately at -20°C . These samples were composited (on an equal wet weight basis) per cow and period and analyzed for bacterial genera and species distribution. The 16S rRNA gene V4 variable region PCR primers 515/806 (Caporaso et al., 2011) were used in a single-step 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, Germantown, MD) under the following conditions: 94°C for 3 min, followed by 28 cycles (5 cycle used on PCR products) of 94°C for 30 s, 53°C for 40 s and 72°C for 1 min, after which a

final elongation step at 72°C for 5 min was performed. Sequencing was performed at Molecular Research DNA (<http://www.mrdnalab.com/>, Shallowater, TX) on an Ion Torrent PGM following the manufacturer's guidelines. Sequence data were processed using a proprietary analysis pipeline (MR DNA). In summary, sequences were depleted of barcodes and primers, then sequences <150 bp were removed, and sequences with ambiguous base calls and with homopolymer runs exceeding 6 bp were also removed. Sequences were denoised, operational taxonomic units generated, and chimeras removed. Operational taxonomic units were defined by clustering at 3% divergence (97% similarity). Final operational taxonomic units were taxonomically classified using BLASTn against a curated database derived from GreenGenes, RDP II and NCBI (<http://www.ncbi.nlm.nih.gov/>; DeSantis et al., 2006, <http://rdp.cme.msu.edu>).

Blood samples were collected from the tail vein or artery into heparinized tubes at 6, 10, and 14 h after feeding on d 24 of each experimental period. Blood samples were immediately centrifuged ($1,500 \times g$ for 15 min at 4°C) and plasma was stored at -20°C until analyzed. Individual plasma samples were analyzed for Met after extraction with acetonitrile precipitation by HPLC (Shimadzu Prominence, Kyoto, Japan)/MS (AB Sciex API 4000, Concord, Canada) with reference to an internal standard (L-methionine $^{13}\text{C}_5$, ^{15}N). Aliquots of the plasma samples were composited per cow and period, and the composite samples were analyzed for plasma urea-N (Stanbio Urea Nitrogen Kit 0580, Stanbio Laboratory Inc.).

Milk samples were collected at 2 consecutive milkings (p.m. and a.m.) and 3 consecutive days (d 22, 23, and 24) during each experimental period. Samples were placed in tubes containing 2-bromo-2-nitropropane-1,3-diol for analysis of milk fat, true protein, lactose, and MUN (Pennsylvania DHIA, University Park, PA) using infrared spectroscopy (MilkoScan 4000, Foss Electric, Hillerød, Denmark). Morning and evening samples were analyzed separately so milk component concentration and yield could be weighed for a.m. and p.m. milk yields. Aliquots of the milk samples were composited on an equal volume basis per cow and period and processed for analysis of milk FA according to Rico and Harvatiné (2013). Briefly, fat was extracted using hexane isopropanol, base transmethylated with sodium methoxide, and FA-methyl esters separated by GC-FID (SP-2560; 100 m $\times$ 0.25 mm i.d. with 0.2- μm film thickness; Supelco Inc., Bellefonte, PA).

Cow BW was recorded throughout the trial using AfiFarm 3.04E scale system (S.A.E. Afikim, Rehovot, Israel) while cows exited the milk parlor.

Statistical Analysis

All data were analyzed using PROC MIXED of SAS (2003, SAS Inst. Inc., Cary, NC). Data, except as stated below, were analyzed by ANOVA Latin square with treatment and period in the model. Square and cow within square were random effects, whereas all others were fixed. Milk production, DMI, feed efficiency, milk composition, FCM, rumen fermentation, and plasma Met data were analyzed as repeated measures assuming an autoregressive(1) covariance structure with treatment, period, sampling time point, and treatment $\times$ sampling time point interaction in the model. In both models the error term was assumed to be normally distributed with mean = 0 and constant variance. Square and cow within square were the random effects, whereas all others were fixed. Orthogonal and polynomial contrasts were used to evaluate the effects of HMTBa supplementation and application rate (C vs. Tr = control vs. HMTBa treatments, L = linear effect of HMTBa, and Q = quadratic effect of HMTBa). Significant differences were declared at $P \leq 0.05$. Differences at $0.05 < P \leq 0.10$ were considered as a trend toward significance. Means are presented, unless indicated otherwise, as least squares means.

RESULTS AND DISCUSSION

On average, all treatment diets provided NE_L and MP in excess of the cows' requirements (Table 1). Feed intake and milk production and composition were not affected by HMTBa in this experiment (Table 2). It is noted that DMI was numerically similar (except perhaps for low HMTBa) among treatment, but milk production for the HMTBa treatments decreased numerically by about 1 kg/d versus the control. Due to variability in the data, however (and the low number of experimental units), this difference was not statistically significant. Production responses to HMTBa reported in the literature vary significantly among studies. Early data reported positive effects of HMTBa on milk production (Griel et al., 1968) and milk fat concentration and yield (Holter et al., 1972; Lundquist et al., 1983; Huber et al., 1984) in dairy cows. More recent data by Uchida et al. (2003) and Noftsker et al. (2005), however, indicated no effect of HMTBa on milk production and composition. Similarly, Rulquin et al. (2006), Johnson-VanWieringen et al., (2007), and Lapierre et al. (2011) did not find any production effects of HMTBa or a combination of HMTBa and Lys-HCl in cows fed basal diets with 15.3 to 19.5% CP. Similar lack of effect of HMTBa on DMI and milk yield was recently reported by Baldin

et al. (2014). On the other hand, a rumen-protected form of HMTBa increased milk production and milk protein yield, without an effect on DMI, in dairy cows fed a diet, for which Met was estimated to be the first limiting AA (Sklan and Tinsky, 1996). Piepenbrink et al. (2004) also observed increased milk yield postpartum with HMTBa supplementation (probably due to a numerical increase in DMI) and a recent study with cows producing about 28 kg of milk/d reported a 7.5% increase in milk yield (with no corresponding effect on DMI), 9.7% increase in milk fat percent, and 63% increase in serum Met concentration with HMTBa (Wang et al., 2010).

Intake of nutrients during the fecal and urine collection periods was not affected by treatment, but subtle trends were present in total-tract apparent digestibility; HMTBa tended to linearly decrease ($P = 0.08$) ADF and quadratically decreased CP and starch digestibilities ($P = 0.01$ and 0.02 , respectively; Table 3). Digestibility of ADF tended to decrease by about 2% by the highest level of HMTBa and that of CP and starch decreased 5 and 1%, respectively, by the medium HMTBa. Treatment had no effect on ruminal (true) degradability of DM (59.6 ± 0.97 ; $P > 0.18$), OM (62.5 ± 0.91 ; $P > 0.22$), CP, and NDF (data not shown in tables). In contrast, Noftsger et al. (2005)

Table 1. Composition of the basal diet fed in the trial and balance of key nutrients (all diets had the same ingredient and chemical composition)

Item	Diet ¹			
	Control	Low	Medium	High
Ingredient, % of DM				
Corn silage ²		46.1		
Grass hay ³		8.4		
Corn grain, ground		10.7		
Soybean meal, 48% CP		7.0		
Canola meal		6.9		
Soybean seeds, whole, heated		6.1		
Cottonseed hulls		5.4		
Molasses ⁴		4.3		
Mineral and vitamin premix ⁵		2.83		
HMTBa premix ⁶		1.5		
Limestone		0.35		
Urea		0.25		
Salt		0.17		
Analyzed composition, ⁷ % of DM				
OM		93.6 $\pm$ 0.10		
CP		15.9 $\pm$ 0.34		
NDF		33.0 $\pm$ 0.83		
ADF		20.4 $\pm$ 0.38		
Ether extract		2.9 $\pm$ 0.05		
Ca		0.79 $\pm$ 0.08		
P		0.39 $\pm$ 0.01		
NE _L , Mcal/kg		1.63 $\pm$ 0.01		
Nutrient balance ⁸				
NE _L , Mcal/d	2.5	1.5	4.2	2.7
RDP, g/d	40	40	40	41
MP, g/d	106	78	192	146

¹Control = 0% (diet, DM basis) 2-hydroxy-4-methylthio-butanoic acid (HMTBa; as Alimet, Novus International Inc., St. Charles, MO); low = 0.05% HMTBa; medium = 0.10% HMTBa; high = 0.15% HMTBa.

²Corn silage was 39.0% DM and (DM basis): 7.6% CP and 36.8% NDF.

³Grass hay was 82.6% DM and (DM basis): 10.8% CP and 70.1% NDF.

⁴Molasses (Westway Feed Products, Tomball, TX) contained (DM basis): 3.9% CP and 66% total sugar.

⁵The premix contained (%; as-is basis): trace mineral mix, 0.86; MgO (56% Mg), 8.0; NaCl, 6.4; vitamin ADE premix, 0.48; limestone, 37.2; selenium premix, 0.07; and dry corn distillers grains with solubles, 46.7. Ca, 14.1%; P, 0.39%; Mg, 4.59%; K, 0.44%; S, 0.39%; Se, 6.91 mg/kg; Cu, 362 mg/kg; Zn, 1,085 mg/kg; Fe, 186 mg/kg; vitamin A, 276,717 IU/kg; vitamin D, 75,000 IU/kg; and vitamin E, 1,983 IU/kg.

⁶Top-dressed and contained: 100% fine ground corn (control) or 3.33% HMTBa, 6.67% HMTBa, or 10% HMTBa replacing fine ground corn for low, medium, or high, respectively.

⁷Four ($\pm$ SE) composite samples were analyzed (Cumberland Valley Analytical Services, Maugansville, MD). NE_L was calculated based on the chemical composition of the diets (<http://www.foragelab.com/Resources/Report-Calculations>; accessed June 23, 2014).

⁸Averaged estimates based on NRC (2001) using actual DMI, milk yield, milk composition, and BW of the cows (last week of each experimental period).

Table 2. Effect of 2-hydroxy-4-methylthio-butanoic acid (HMTBa) on DMI, milk yield and composition, and BW in dairy cows

Item	Diet ^{1,2}				SEM	Contrast ³		
	Control	Low	Medium	High		C vs. Tr	L	Q
DMI, kg/d	29.3	28.4	29.8	29.2	1.84	0.69	0.56	0.68
Milk yield, kg/d	45.3	44.3	44.3	44.1	6.00	0.19	0.27	0.54
Feed efficiency, ⁴ kg/kg	1.55	1.56	1.48	1.49	0.141	0.51	0.23	0.93
Milk fat, %	3.59	3.77	3.48	3.70	0.259	0.43	0.86	0.76
Milk fat, kg/d	1.66	1.68	1.55	1.66	0.109	0.60	0.56	0.35
Milk true protein, %	3.04	3.04	3.04	3.05	0.099	0.63	0.49	0.86
Milk true protein, kg/d	1.41	1.35	1.36	1.37	0.140	0.13	0.40	0.18
3.5% FCM, kg/d	47.1	46.7	44.5	46.6	4.34	0.39	0.48	0.29
FCM feed efficiency, kg/kg	1.61	1.64	1.52	1.62	0.062	0.70	0.60	0.39
Lactose, %	4.80	4.78	4.79	4.79	0.036	0.67	0.84	0.80
Lactose, kg/d	2.23	2.15	2.15	2.17	0.290	0.12	0.34	0.21
MUN, mg/dL	13.0	12.7	13.3	13.1	1.52	0.85	0.49	0.93
NE _L intake, Mcal/d	47.7	48.7	48.0	47.2	2.86	0.55	0.94	0.83
Milk NE _L , Mcal/d	32.1	31.7	30.5	31.8	2.91	0.34	0.47	0.26
Milk NE _L ÷ NE _L intake, %	67.3	68.1	63.8	67.5	2.49	0.65	0.61	0.36
BW, kg	654	674	655	649	24.6	0.54	0.34	0.10

¹Control = 0% (diet DM basis) HMTBa (as Alimet, Novus International Inc., St. Charles, MO); low = 0.05% HMTBa; medium = 0.10% HMTBa; high = 0.15% HMTBa.

²Least squares means; production data, n = 216 to 224; milk composition, n = 96; BW, n = 32 (n represents number of observations used in the statistical analysis).

³C vs. Tr = control vs. HMTBa treatments; L = linear effect of HMTBa; Q = quadratic effect of HMTBa.

⁴Milk yield/DMI.

reported increased ruminal OM and NDF digestibility as a result of HMTBa supplementation of the diet. In an earlier study, however, HMTBa had no effect on nutrient degradability in continuous culture fermenters (Vazquez-Añon et al., 2001). The effects observed in the current experiment were likely due to a combination of numerically increased DMI and increased fecal N excretion with the medium and high HMTBa treat-

ments (compared with the control) as well as natural variability in the data.

Nitrogen secretion and excretion, urinary purine derivatives excretion, and rumen microbial protein synthesis estimates are shown in Table 4. Most variables were not affected by HMTBa. As % of N intake, urinary N excretion was increased ($P = 0.04$; a quadratic response) by HMTBa and so was absolute and relative

Table 3. Effect of 2-hydroxy-4-methylthio-butanoic acid (HMTBa) on intake and total-tract apparent digestibility of nutrients in dairy cows

Item	Diet ^{1,2}				SEM	Contrast ³		
	Control	Low	Medium	High		C vs. Tr	L	Q
Intake, ⁴ kg/d								
DM	29.0	28.4	29.7	29.2	1.68	0.91	0.21	0.69
OM	27.2	26.6	27.7	27.4	1.58	0.91	0.21	0.69
NDF	9.6	9.4	9.8	9.6	0.55	0.99	0.25	0.69
ADF	5.9	7.8	6.1	6.0	0.34	0.99	0.25	0.68
CP	4.6	4.5	4.7	4.6	0.27	0.74	0.17	0.76
Starch	6.9	6.7	7.0	6.9	0.39	0.91	0.21	0.69
Apparent total-tract digestibility, %								
DM	68.0	68.1	66.9	67.7	0.38	0.23	0.17	0.24
OM	69.2	69.2	68.2	68.9	0.39	0.26	0.20	0.29
NDF	48.6	49.0	47.7	49.1	0.56	0.98	0.92	0.39
ADF	41.2	41.1	40.8	40.3	0.64	0.31	0.08	0.52
CP	69.6	68.4	66.1	68.6	0.85	0.02	0.09	0.01
Starch	96.4	95.9	95.5	96.5	0.39	0.19	0.99	0.02

¹Control = 0% (diet DM basis) HMTBa (as Alimet, Novus International Inc., St. Charles, MO); low = 0.05% HMTBa; medium = 0.10% HMTBa; high = 0.15% HMTBa.

²Least squares means; n = 32 (n represents number of observations used in the statistical analysis).

³C vs. Tr = control vs. HMTBa treatments; L = linear effect of HMTBa; Q = quadratic effect of HMTBa.

⁴Intake during the digestibility data collection periods.

Table 4. Effect of 2-hydroxy-4-methylthio-butanoic acid (HMTBa) on N intake, N secretion in milk, urinary and fecal N excretion, urinary purine derivative (PD) excretion, and blood plasma urea N (PUN) concentration in dairy cows

Item	Diet ¹				SEM	Contrast ²		
	Control	Low	Medium	High		C vs. Tr	L	Q
N intake, kg/d	0.735	0.722	0.751	0.743	0.0425	0.74	0.17	0.76
Urine N, kg/d	0.163	0.168	0.175	0.156	0.0138	0.69	0.67	0.11
As % of N intake	22.3	23.5	23.6	20.7	0.93	0.80	0.26	0.04
Urinary urea N, kg/d	0.104	0.103	0.106	0.101	0.0074	0.93	0.80	0.62
As % of N intake	14.3	14.5	14.2	13.5	0.43	0.72	0.25	0.36
Fecal N, kg/d	0.224	0.227	0.254	0.232	0.0099	0.04	0.05	0.03
As % of N intake	30.4	31.6	33.9	31.4	0.85	0.02	0.09	0.01
Fecal and urine N, kg/d	0.386	0.394	0.429	0.387	0.0227	0.13	0.38	0.02
As % of N intake	52.7	55.1	57.5	52.1	1.06	0.13	0.92	0.004
Milk protein N, ⁴ kg/d	0.220	0.212	0.213	0.215	0.0218	0.12	0.38	0.17
As % N intake	29.9	29.5	28.3	29.2	1.32	0.14	0.16	0.24
N secreted or excreted in milk protein and excreta, kg/d	0.607	0.607	0.642	0.602	0.0435	0.37	0.60	0.06
As % of N intake	82.6	84.6	85.9	81.2	1.45	0.33	0.59	0.009
Excretion of PD, mmol/d								
Allantoin	490	497	508	495	62.9	0.59	0.72	0.53
Uric acid	67	64	65	62	2.2	0.15	0.09	0.88
Total PD	558	562	574	557	64.3	0.75	0.91	0.55
Purine-N:bacterial-N	0.108	0.106	0.108	0.106	0.0022	0.61	0.63	0.96
Microbial N production, ⁵ g/d	473	478	487	481	57.2	0.72	0.75	0.79
PUN, mg/dL	11.8	10.5	11.3	11.5	0.61	0.10	0.90	0.05

¹Control = 0% (diet, DM basis) HMTBa (as Alimet, Novus International Inc., St. Charles, MO); low = 0.05% HMTBa; medium = 0.10% HMTBa; high = 0.15% HMTBa.

²Least squares means; n = 32 (n represents number of observations used in the statistical analysis).

³C vs. Tr = control vs. HMTBa treatments; L = linear effect of HMTBa; Q = quadratic effect of HMTBa.

⁴Milk true protein $\times$ 6.38.

⁵Based on excretion of urinary purine derivatives.

fecal N excretion ($P \leq 0.03$). This resulted in increased ($P \leq 0.02$) total fecal and urinary N excretion with the low and medium HMTBa levels compared with the control. Except for an inconsequential trend of a linear decrease ($P = 0.09$) in urinary uric acid excretion, treatment had no effect on urinary purine derivative

excretion and the estimated rumen microbial protein synthesis. Plasma urea N was quadratically decreased ($P = 0.05$) by the low HMTBa application level, which was in disagreement with the urine and fecal N excretion data. Previous studies have not detected differences in estimated excretion of urinary or fecal N

Table 5. Effect of 2-hydroxy-4-methylthio-butanoic acid (HMTBa) on rumen fermentation in dairy cows

Item	Diet ^{1,2}				SEM	Contrast ³		
	Control	Low	Medium	High		C vs. Tr	L	Q
pH	6.00	5.97	5.94	5.93	0.050	0.36	0.31	0.86
Ammonia, mM	5.2	5.3	5.1	5.8	0.42	0.45	0.21	0.44
Protozoa, ⁴ $\times 10^4$ /mL	144	144	145	143	19.7	0.79	0.91	0.86
Total VFA, mM	128.3	125.8	124.1	125.8	1.75	0.15	0.25	0.26
Acetate	79.1	77.1	77.4	78.9	1.24	0.31	0.96	0.11
Propionate	28.2	27.5	27.3	26.7	0.81	0.15	0.08	0.97
Isobutyrate	0.87	0.88	0.82	0.84	0.051	0.75	0.61	0.89
Butyrate	16.0	16.4	14.8	15.2	0.43	0.22	0.03	0.85
Isovalerate	1.80	1.79	1.72	1.85	0.058	0.77	0.75	0.14
Valerate	2.25	2.17	2.07	2.12	0.050	0.01	0.02	0.16
Ac:Pr ratio	2.86	2.87	2.90	3.00	0.082	0.27	0.05	0.42

¹Control = 0% (diet DM basis) HMTBa (as Alimet, Novus International Inc., St. Charles, MO); low = 0.05% HMTBa; medium = 0.10% HMTBa; high = 0.15% HMTBa.

²Least squares means; n = 288 (n represents number of observations used in the statistical analysis).

³C vs. Tr = control vs. HMTBa treatments; L = linear effect of HMTBa; Q = quadratic effect of HMTBa.

⁴Log-transformed data were used for the statistical analysis.

Figure 1. Effect of 2-hydroxy-4-methylthio-butanoic acid (HMTBa) on rumen pH in dairy cows (mean $\pm$ SE); control = 0% (diet DM basis) HMTBa (as Alimet, Novus International Inc., St. Charles, MO); low = 0.05% HMTBa; medium = 0.10% HMTBa; high = 0.15% HMTBa. For statistical analysis of the data, see Table 5.

when HMTBa was fed to cows in crossover (Chen et al., 2011) or continuous design studies (St-Pierre and Sylvester, 2005).

Rumen fermentation data, concentration of microbial N in reticular contents, and microbial N production data are shown in Tables 5 and 6. Treatment had no effect of rumen fermentation, except that butyrate and valerate concentrations decreased ($P \leq 0.03$) and propionate tended to decrease ($P = 0.08$) linearly, which

resulted in increased ($P = 0.05$) acetate:propionate ratio with HMTBa supplementation. Rumen fluid pH was not affected by treatment at any sampling point during the feeding cycle (Figure 1). The largest numerical difference in pH among treatments was observed at 18 h (5.97, 6.12, 5.93, and 5.82 for control, low, medium, and high, respectively; $P \geq 0.33$). Based on the ^{15}N data, no difference was observed in the proportion of microbial N in the fractions of reticular content due to treatment (Table 6). Numerical differences ($P = 0.14$ to 0.17) were found in the concentration of microbial N in large particles and whole reticular contents, which did not reach statistical significance. Microbial N production in the rumen increased linearly ($P = 0.04$) with HMTBa supplementation. Microbial N outflow from the rumen was on average about 27% higher when estimated based on excretion of urinary purine derivatives compared with the estimates based on reticular sampling. The urinary purine derivative method produced a larger variability in the estimates ($\text{CV} = 0.22$) than the reticular sampling method ($\text{CV} = 0.16$), which contributed to the lack of statistically significant effect with the former method. Similar to the current study, the urinary purine derivatives method produced about 21% higher estimates for microbial protein synthesis compared with a duodenal sampling method in a previous study (Hristov et al., 2005b). In a continuous culture study, Vazquez-Añon et al. (2001) reported no effect of HMTBa applied at up to 1.43% (feed DM basis) on VFA concentrations and medium pH but observed a quadratic increase in bacterial N

Table 6. Effect of 2-hydroxy-4-methylthio-butanoic acid (HMTBa) on $\delta^{15}\text{N}$ of bacterial and reticular contents N, concentration of microbial N in reticular contents, and estimated microbial N production in the rumen and contribution of microbial N to milk protein in dairy cows

Item	Diet ^{1,2}				SEM	Contrast ³		
	Control	Low	Medium	High		C vs. Tr	L	Q
$\delta^{15}\text{N}$, ‰								
Rumen bacterial protein	122	119	119	121	6.6	0.59	0.81	0.54
Reticular contents								
Fluid	116	113	112	115	6.08	0.64	0.84	0.58
Small particles	81.0	79.3	80.2	82.1	3.62	0.85	0.68	0.42
Large particles	47.2	48.8	49.9	50.7	2.40	0.33	0.26	0.87
Whole contents	74.8	75.2	74.9	77.6	3.22	0.67	0.42	0.61
Milk protein	49.4	49.4	48.2	49.2	2.44	0.76	0.76	0.72
Microbial N, mg/g of DM								
Ruminal fluid	19.7	19.9	19.5	20.0	1.07	0.97	0.91	0.76
Small particles	38.5	39.8	39.9	39.4	0.95	0.17	0.40	0.20
Large particles	14.3	16.0	15.7	16.7	1.11	0.14	0.14	0.72
Whole contents	21.5	22.3	22.1	22.9	0.88	0.21	0.17	0.95
Microbial N production, g/d	365	365	390	391	22.3	0.19	0.04	0.95
Milk protein N from microbial protein, ⁴ %	41.3	42.3	40.1	40.7	1.00	0.79	0.31	0.83

¹Control = 0% (diet DM basis) HMTBa (as Alimet, Novus International Inc., St. Charles, MO); low = 0.05% HMTBa; medium = 0.10% HMTBa; high = 0.15% HMTBa.

²Least squares means; n = 32 (n represents number of observations used in the statistical analysis).

³C vs. Tr = control vs. HMTBa treatments; L = linear effect of HMTBa; Q = quadratic effect of HMTBa.

⁴Found as: $\delta^{15}\text{N}$ of milk protein $\div$ $\delta^{15}\text{N}$ of rumen bacterial protein.

Table 7. Effect of 2-hydroxy-4-methylthio-butanoic acid (HMTBa) on rumen bacterial genus composition (as percentage¹ of total isolates) in dairy cows

Item	Diet ^{2,3}				SEM	Contrast ⁴		
	Control	Low	Medium	High		C vs. Tr	L	Q
<i>Prevotella</i>	34.3	33.5	35.0	33.6	1.71	0.89	0.91	0.86
<i>Phascolarctobacterium</i>	8.40	7.59	6.92	6.54	1.000	0.19	0.13	0.81
<i>Tannerella</i>	6.33	6.84	6.49	7.19	1.054	0.29	0.23	0.81
<i>Oscillospira</i>	5.66	6.24	6.04	7.29	0.732	0.27	0.13	0.61
<i>Parabacteroides</i>	5.43	5.51	5.05	5.98	0.953	0.87	0.57	0.37
<i>Ruminococcus</i>	3.99	4.09	4.24	3.21	0.569	0.80	0.34	0.27
<i>Coprococcus</i>	3.27	3.58	3.70	3.38	0.358	0.44	0.75	0.33
<i>Bacteroides</i>	3.09	3.38	3.26	3.18	0.187	0.45	0.88	0.38
<i>Clostridium</i>	3.04	3.26	2.96	3.29	0.300	0.72	0.75	0.87
<i>Acidaminococcus</i>	2.96	3.06	2.75	2.30	0.759	0.46	0.09	0.35
<i>Butyrivibrio</i>	1.86	1.95	2.08	1.75	0.160	0.74	0.79	0.24
<i>Alistipes</i>	1.88	1.84	1.74	1.94	0.316	0.77	0.90	0.34
<i>Roseburia</i>	1.83	1.72	1.69	1.74	0.170	0.56	0.69	0.63
<i>Fibrobacter</i>	1.59	1.42	1.41	1.55	0.259	0.70	0.91	0.60
<i>Succinivibrio</i>	1.79	0.64	1.88	1.36	0.407	0.38	0.99	0.45
<i>Treponema</i>	1.72	1.30	1.45	1.08	0.328	0.20	0.18	0.93
<i>Lachnospira</i>	1.09	1.45	0.86	1.29	0.305	0.73	0.99	0.90
<i>Pseudobutyrvibrio</i>	0.83	0.79	1.04	0.83	0.074	0.51	0.45	0.25
<i>Eubacterium</i>	0.82	0.70	0.73	0.86	0.146	0.42	0.59	0.05
<i>Selenomonas</i>	0.63	0.82	0.55	0.70	0.167	0.67	0.89	0.85
<i>Blautia</i>	0.56	0.63	0.68	0.58	0.071	0.49	0.78	0.34
<i>Aggregatibacter</i>	0.16	0.52	0.59	0.75	0.491	0.21	0.19	0.75
<i>Fecalibacterium</i>	0.35	0.50	0.45	0.57	0.068	0.05	0.04	0.85
<i>Hemophilus</i>	0.47	0.18	0.62	0.38	0.135	0.69	0.79	0.88
<i>Anaerotruncus</i>	0.34	0.42	0.40	0.44	0.049	0.14	0.17	0.64
<i>Streptococcus</i>	0.32	0.39	0.40	0.37	0.044	0.19	0.38	0.27

¹The percentage represents the percentage of the total sequences analyzed within the sample.

²Control = 0% (diet DM basis) HMTBa (as Alimet, Novus International Inc., St. Charles, MO); low = 0.05% HMTBa; medium = 0.10% HMTBa; high = 0.15% HMTBa.

³Least squares means; n = 32 (n represents number of observations used in the statistical analysis).

⁴C vs. Tr = control vs. HMTBa treatments; L = linear effect of HMTBa; Q = quadratic effect of HMTBa.

outflow and efficiency of N synthesis. Although in vitro and continuous culture data should be interpreted with caution (Hristov et al., 2012), results from the current study are in support of those reported by Vazquez-Añon et al. (2001) and suggest a subtle, stimulatory effect of HMTBa on the ruminal microbes. To act on the rumen microbes, HMTBa has to be metabolized, at least partially, in the rumen. In vivo and in vitro studies have shown 40 to 50% ruminal escape of HMTBa (Koenig et al., 1999; Vazquez-Añon et al., 2001; Koenig et al., 2002), which means a significant amount of HMTBa is metabolized in the rumen. A much lower escape rate was reported by Noftsger et al. (2005), although potential absorption through the rumen wall was not accounted for in that study. The exact mode of action of HMTBa in the rumen is not clear, but is probably related to the specific stimulatory effect of AA and peptides on microbial growth, particularly of some cellulolytic bacteria (Cotta and Russell, 1982; Russell, 1993; Van Kessel and Russell, 1996). Although the latter data are from in vitro studies, they are in line with some reports for increased fiber degradation with

HMTBa in vivo (Noftsger et al., 2005). The effect of HMTBa on some VFA in the current study is difficult to explain. Concentration of butyrate, for example, was decreased, but the prevalence of *Butyrivibrio* spp. (or *Butyrivibrio fibrisolvens*; see later discussions) was not affected by HMTBa. Metabolites of Met-analogs may alter rumen VFA molar proportions, as suggested for HMBi by Martin et al. (2013), but most studies showed no effect of HMTBa (or HMBi) on rumen VFA concentrations (Vazquez-Añon et al., 2001; Noftsger et al., 2005; Martin et al., 2013).

In the current study, treatment had no effect on ruminal bacterial genus and species distribution (Tables 7 and 8, respectively). The percentage of genus *Fecalibacterium* of the total sequences analyzed within a sample was linearly increased ($P = 0.04$), whereas the percentage of *Acidaminococcus* tended to be decreased ($P = 0.09$) and that of *Eubacterium* was decreased quadratically ($P = 0.04$) by HMTBa. Of the individual bacterial species, *Prevotella loescheii* and *Prevotella oralis* were increased or tended to be increased ($P \leq 0.08$) by HMTBa, compared with the control. No specific effect of

Table 8. Effect of 2-hydroxy-4-methylthio-butanoic acid (HMTBa) on rumen bacterial species composition (as percentage¹ of total isolates) in dairy cows

Item	Diet ^{2,3}				SEM	Contrast ⁴		
	Control	Low	Medium	High		C vs. Tr	L	Q
<i>Prevotella ruminicola</i>	23.2	22.2	22.6	22.2	1.51	0.65	0.72	0.87
<i>Tannerella forsythia</i>	6.33	6.84	6.49	7.19	1.054	0.29	0.23	0.81
<i>Oscillospira</i> spp.	5.64	6.21	6.00	7.26	0.724	0.27	0.13	0.60
<i>Parabacteroides distasonis</i>	4.78	4.80	4.31	4.16	0.809	0.96	0.72	0.32
<i>Prevotella brevis</i>	2.82	2.72	2.79	2.72	0.227	0.73	0.79	0.94
<i>Coprococcus eutactus</i>	2.49	2.76	2.82	2.52	0.287	0.50	0.90	0.30
<i>Prevotella loeschei</i>	2.11	2.30	3.01	2.61	0.321	0.04	0.03	0.17
<i>Acidaminococcus intestini</i>	2.54	2.65	2.36	2.06	0.715	0.53	0.14	0.44
<i>Ruminococcus bromii</i>	2.17	2.24	2.06	1.43	0.450	0.63	0.26	0.46
<i>Fibrobacter succinogenes</i>	1.59	1.41	1.40	1.53	0.257	0.68	0.89	0.60
<i>Succinivibrio</i> spp.	1.79	0.64	1.88	1.36	0.407	0.38	0.99	0.51
<i>Lachnospira pectinoschiza</i>	1.09	1.45	0.86	1.29	0.305	0.73	0.99	0.90
<i>Alistipes finegoldii</i>	1.07	1.15	0.98	1.06	0.168	0.95	0.56	0.99
<i>Ruminococcus flavefaciens</i>	1.04	0.94	1.21	1.01	0.205	0.93	0.77	0.71
<i>Roseburia eubacterium recta</i>	1.04	0.95	1.04	1.01	0.176	0.76	0.99	0.80
<i>Bacteroides uniformis</i>	0.90	1.12	0.96	1.04	0.074	0.11	0.43	0.34
<i>Prevotella oralis</i>	0.76	0.77	0.95	0.93	0.073	0.21	0.08	0.87
<i>Prevotella shahii</i>	0.82	0.88	0.87	0.82	0.056	0.59	1.00	0.38
<i>Roseburia faecis</i>	0.78	0.78	0.64	0.71	0.082	0.44	0.34	0.63
<i>Butyrivibrio fibrisolvens</i>	0.64	0.67	0.82	0.67	0.069	0.27	0.42	0.14
<i>Prevotella oris</i>	0.74	0.62	0.66	0.61	0.076	0.23	0.33	0.65
<i>Bacteroides barnesi</i>	0.63	0.66	0.69	0.63	0.093	0.75	0.95	0.59
<i>Prevotella copri</i>	0.64	0.72	0.64	0.56	0.114	0.98	0.52	0.50
<i>Alistipes putredinis</i>	0.61	0.54	0.57	0.70	0.129	0.95	0.42	0.23

¹The percentage represents the percentage of the total sequences analyzed within the sample.

²Control = 0% (diet DM basis) HMTBa (as Alimet, Novus International Inc., St. Charles, MO); low = 0.05% HMTBa; medium = 0.10% HMTBa; high = 0.15% HMTBa.

³Least squares means; n = 32 (n represents number of observations used in the statistical analysis).

⁴C vs. Tr = control vs. HMTBa treatments; L = linear effect of HMTBa; Q = quadratic effect of HMTBa.

HMTBa on the common rumen cellulolytic species was observed in this experiment. Similar lack of effect of HMTBa on the structure of the rumen bacterial community was reported by Noftsker et al. (2003) in continuous culture. These authors suggested that perhaps protozoa are affected by HMTBa supplementation, but a latter study from the same group found no effect of HMTBa (or its isopropyl ester, HMBi) on ruminal protozoal genera distribution (Karnati et al., 2007). A more recent study, however, reported increased abundance of 2 important cellulolytic bacteria (*Fibrobacter succinogenes* and *Ruminococcus flavefaciens*) in dairy cows receiving HMTBa (and HMBi), but this had no consequential effects on ruminal fermentation, cellulolytic activities, or corn silage in situ NDF degradability in dry cows (Martin et al., 2013).

With a few notable exceptions, HMTBa had no effect on milk FA composition in the current study (Table 9). Supplementation of the basal diet with HMTBa tended to decrease or decreased linearly ($P = 0.06$ and 0.04 , respectively) the concentration of 4:0 and 6:0 and increased ($P \leq 0.01$) concentrations of 15:0 and 17:0 in milk fat. Treatment also slightly decreased *trans*-6,8

18:1 ($P = 0.02$) and *trans*-10 18:1 ($P = 0.04$). The yield of 15:0 was quadratically increased ($P = 0.009$) by HMTBa. A similar increase in 15:0 concentration in milk fat was reported for HMTBa by Rulquin et al. (2006). The so-called odd- and branched-chain FA in milk fat, which include 15:0 and 17:0, are found in microbial lipids (Keeney et al., 1962) and are also synthesized from propionate in the adipose and mammary tissues (Vlaeminck et al., 2006). Considered predominantly of microbial origin, these FA have been proposed as biomarkers of microbial synthesis or methane production in the rumen (Vlaeminck et al., 2006; Dijkstra et al., 2011; Fievez et al., 2012). Although the concept has been questioned and needs verification under various dietary regimens (Dewhurst et al., 2007; Mohammed et al., 2011; French et al., 2012), the trends observed for milk FA in the current study are in agreement with the trends in microbial N concentration in reticular contents and microbial N outflow from the rumen. The decrease in *trans*-10, 18:1 is consistent with beneficial effects on milk fat yield as this isomer is associated with the alternate biohydrogenation pathway and milk fat depression. However, the basal diet in the current

Table 9. Effect of 2-hydroxy-4-methylthio-butanoic acid (HMTBa) on milk fatty acid concentration in total milk fat or yield

Item	Diet ^{1,2}				SEM	Contrast ³		
	Control	Low	Medium	High		C vs. Tr	L	Q
Fatty acid concentration, g/100 g								
4:0	4.58	4.44	4.51	4.42	0.053	0.033	0.06	0.56
6:0	2.58	2.51	2.56	2.49	0.053	0.029	0.04	0.91
8:0	1.49	1.45	1.49	1.44	0.056	0.20	0.20	0.76
10:0	3.53	3.42	3.50	3.37	0.197	0.22	0.20	0.82
11:0	0.08	0.07	0.08	0.09	0.006	0.58	0.51	0.13
12:0	3.89	3.77	3.86	3.72	0.204	0.28	0.25	0.82
13:0	0.13	0.12	0.13	0.14	0.005	0.83	0.22	0.11
14:0	11.7	11.3	11.7	11.5	0.24	0.34	0.97	0.42
14:0 <i>iso</i>	0.10	0.09	0.11	0.12	0.009	0.80	0.26	0.37
14:1	1.10	1.07	1.12	1.14	0.055	0.83	0.24	0.42
15:0	0.99	0.95	1.08	1.18	0.037	0.08	<0.001	0.11
15:0 <i>anteiso</i>	0.19	0.20	0.20	0.21	0.007	0.19	0.08	0.85
16:0	28.2	28.5	28.2	28.0	0.50	0.99	0.59	0.59
16:0 <i>iso</i>	0.23	0.22	0.24	0.26	0.013	0.54	0.08	0.26
16:1	1.11	1.05	1.11	1.13	0.103	0.85	0.71	0.56
17:0	0.48	0.48	0.50	0.51	0.008	0.12	0.01	0.47
17:0 <i>iso</i>	0.05	0.05	0.05	0.05	0.003	0.26	0.18	0.81
17:0 <i>anteiso</i>	0.26	0.26	0.28	0.28	0.008	0.17	0.06	0.90
17:1	0.15	0.18	0.18	0.19	0.014	0.01	0.01	0.35
18:0	11.1	11.4	11.4	11.4	0.45	0.39	0.60	0.50
<i>trans</i> -4 18:1	0.02	0.02	0.02	0.02	0.002	0.23	0.78	0.16
<i>trans</i> -5 18:1	0.02	0.01	0.01	0.01	0.003	0.15	0.39	0.11
<i>trans</i> -6,8 18:1	0.27	0.27	0.26	0.26	0.004	0.06	0.02	0.95
<i>trans</i> -9 18:1	0.20	0.20	0.20	0.20	0.010	0.22	0.25	0.52
<i>trans</i> -10 18:1	0.41	0.39	0.38	0.38	0.022	0.06	0.04	0.56
<i>trans</i> -11 18:1	0.96	0.98	0.95	0.95	0.024	0.98	0.64	0.64
<i>trans</i> -12 18:1	0.34	0.32	0.33	0.33	0.007	0.08	0.19	0.30
<i>cis</i> -9 18:1	17.7	18.1	17.4	17.9	1.11	0.71	0.99	0.98
<i>cis</i> -11 18:1	0.83	0.82	0.87	0.91	0.031	0.31	0.03	0.32
<i>cis</i> -12 18:1	0.38	0.37	0.38	0.40	0.013	0.76	0.22	0.35
18:2n-6	2.99	2.91	3.06	3.16	0.048	0.42	0.02	0.13
18:3	0.40	0.40	0.41	0.42	0.008	0.44	0.07	0.27
20:0	0.14	0.14	0.14	0.14	0.006	0.13	0.21	0.35
<i>cis</i> -9, <i>trans</i> -11 CLA	0.44	0.44	0.43	0.44	0.033	0.66	0.50	0.94
Other	2.77	2.76	2.83	2.99	0.072	0.12	0.003	0.10
Σ <16 (de novo)	30.3	29.4	30.3	29.8	0.69	0.23	0.69	0.52
Σ >16 (preformed)	37.1	37.8	37.3	37.9	0.91	0.43	0.48	0.96
Σ 16	29.6	29.8	29.5	29.4	0.46	0.99	0.71	0.72
Σ <i>trans</i> 18:1	2.23	2.19	2.15	2.14	0.041	0.22	0.15	0.78
Fatty acid yield, g/d								
4:0	71.2	69.6	65.3	68.5	4.67	0.20	0.22	0.30
6:0	40.0	39.4	37.0	38.5	1.68	0.23	0.20	0.41
8:0	23.1	22.9	21.5	22.3	0.74	0.31	0.24	0.51
10:0	54.3	53.8	50.4	52.1	1.53	0.29	0.22	0.53
11:0	1.27	1.13	1.14	1.36	0.078	0.57	0.49	0.06
12:0	59.7	59.0	55.5	57.5	1.63	0.27	0.23	0.46
13:0	1.96	1.82	1.83	2.13	0.138	0.74	0.28	0.04
14:0	179.7	176.3	168.1	178.7	7.95	0.33	0.59	0.15
14:0 <i>iso</i>	1.61	1.48	1.56	1.83	0.185	0.97	0.36	0.25
14:1	17.2	16.7	16.2	17.7	1.78	0.56	0.58	0.04
15:0	15.0	14.7	15.5	18.2	0.86	0.08	<0.001	0.009
15:0 <i>anteiso</i>	2.98	3.10	2.91	3.31	0.251	0.46	0.22	0.31
16:0	436.2	441.9	407.0	434.7	20.27	0.49	0.40	0.30
16:0 <i>iso</i>	3.57	3.41	3.50	4.11	0.288	0.74	0.16	0.16
16:1	17.0	16.4	15.8	17.3	2.57	0.62	0.95	0.25
17:0	7.35	7.54	7.17	7.91	0.531	0.58	0.33	0.36
17:0 <i>iso</i>	0.75	0.77	0.76	0.82	0.042	0.45	0.24	0.58
17:0 <i>anteiso</i>	3.96	4.05	3.96	4.24	0.339	0.47	0.27	0.53
17:1	2.34	2.83	2.57	2.89	0.402	0.04	0.07	0.62
18:0	17.0	182.3	164.8	175.4	8.38	0.91	0.80	0.94
<i>trans</i> -4 18:1	0.35	0.24	0.27	0.32	0.034	0.14	0.76	0.06
<i>trans</i> -5 18:1	0.24	0.19	0.12	0.20	0.048	0.13	0.31	0.10
<i>trans</i> -6,8 18:1	4.25	4.18	3.79	4.01	0.268	0.13	0.09	0.33

Continued

Table 9 (Continued). Effect of 2-hydroxy-4-methylthio-butanoic acid (HMTBa) on milk fatty acid concentration in total milk fat or yield

Item	Diet ^{1,2}				SEM	Contrast ³		
	Control	Low	Medium	High		C vs. Tr	L	Q
<i>trans</i> -9 18:1	3.17	3.15	2.87	3.10	0.354	0.30	0.31	0.26
<i>trans</i> -10 18:1	6.35	6.08	5.51	5.84	0.708	0.03	0.03	0.13
<i>trans</i> -11 18:1	14.8	15.2	13.8	14.7	0.85	0.77	0.52	0.75
<i>trans</i> -12 18:1	5.28	5.04	4.71	5.06	0.278	0.08	0.18	0.09
<i>cis</i> -9 18:1	273.1	285.0	249.8	275.4	36.48	0.83	0.59	0.56
<i>cis</i> -11 18:1	12.7	12.8	12.4	14.0	1.20	0.60	0.16	0.14
<i>cis</i> -12 18:1	5.88	5.66	5.55	6.17	0.494	0.78	0.53	0.13
18:2n-6	46.1	45.5	43.6	48.6	3.69	0.88	0.47	0.10
18:3	6.21	6.11	5.83	6.52	0.500	0.82	0.48	0.07
20:0	2.09	2.20	2.02	2.15	0.091	0.75	0.97	0.91
<i>cis</i> -9, <i>trans</i> -11 CLA	6.85	6.82	6.25	6.75	0.965	0.31	0.34	0.20
Other	42.6	42.9	40.6	46.1	3.83	0.67	0.13	0.04
Σ <16 (de novo)	468.1	459.9	437.0	462.0	19.43	0.31	0.47	0.20
Σ >16 (preformed)	574.8	595.7	535.7	584.0	52.20	0.91	0.76	0.57
Σ 16	456.8	461.7	426.3	456.1	22.81	0.49	0.45	0.26
Σ <i>trans</i> 18:1	34.4	34.1	31.1	33.2	2.41	0.22	0.19	0.29

¹Control = 0% (diet DM basis) HMTBa (as Alimet, Novus International Inc., St. Charles, MO); low = 0.05% HMTBa; medium = 0.10% HMTBa; high = 0.15% HMTBa.

²Least squares means; n = 32 (n represents number of observations used in the statistical analysis).

³C vs. Tr = control vs. HMTBa treatments; L = linear effect of HMTBa; Q = quadratic effect of HMTBa.

experiment resulted in low levels of *trans*-10, 18:1 and no evidence of diet induced milk fat depression, limiting the ability to observe a larger treatment effect.

Plasma Met concentration was not affected by treatment (21.2, 21.1, 20.2, and 20.6 μmol/L for control, low, medium, and high HMTBa, respectively; SEM = 1.31; n = 95; $P_L = 0.64$, $P_Q = 0.81$) in the current experiment. Koenig et al. (1999, 2002) and Zanton et al. (2014) reported increased serum Met concentrations with pulse-dosed intraruminal administration of HMTBa. Similar to the results from the current experiment, however, plasma Met was not affected by feeding HMTBa in the study by Rulquin et al. (2006).

CONCLUSIONS

Feed intake, milk production, and milk composition were not affected by HMTBa in this crossover experiment. The observed decrease in apparent, total-tract digestibility of CP (and the associated increase in fecal N losses) and starch are difficult to explain. There were no well-defined effects of HMTBa on ruminal fermentation and bacterial genus and species composition, but the concentration of microbial N in reticular contents tended to numerically increase and the estimated microbial N production in the rumen linearly increased with increasing HMTBa application rate. These effects were in line with the observed increase in 15:0 (and to a lesser extent 17:0) FA concentration and yield in milk fat. Overall, the rumen and milk FA data from this experiment seem to suggest a positive effect of HMTBa on microbial protein synthesis in the rumen.

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