The effects of 2-hydroxy-4-methylthio-butanoic acid supplementation on the rumen microbial population and duodenal flow of microbial nitrogen

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ABSTRACT

Four multiparous, lactating Holstein cows (average DIM 169.5 ± 20.5 d), fitted with ruminal and duodenal cannulas, were used in a 4×4 Latin square with a 2×2 factorial arrangement of treatments to investigate the effects of 2-hydroxy-4-methylthio-butanoic acid (HMTBA) when fed with diets differing in metabolizable protein (MP) supply and equal levels of crude protein on milk production and composition, rumen microbial activity, duodenal protein flow, and rumen bacterial community composition in vivo and in vitro. Experimental periods were 28 d in length. Cows were housed in individual tie stalls and were randomly assigned to 4 dietary treatments: low MP or high MP, supplemented with or without 25 g of HMTBA, which was top-dressed once daily at 0930 h. No interactions were observed between HMTBA and level of dietary MP, with the exception of ruminal acetate-to-propionate ratio. Milk yield was not affected by treatment and averaged 23.8 ± 2.06 kg/d. There was a tendency for increased milk protein percent in cows receiving low MP diets, averaging $3.30 \pm 0.09\%$ and $3.21 \pm 0.09\%$ for low MP and high MP, respectively. The total-tract apparent digestibility of organic matter, neutral detergent fiber, and nitrogen were greater in cows consuming the low MP diet. Rumen pH was lower in cows consuming high MP diets as well as in those consuming HMTBA. Rumen ammonia concentrations tended to be greater in cows consuming HMTBA, and volatile fatty acid concentrations were greater in cows consuming HMTBA. Duodenal dry

matter flow, nitrogen flow, and microbial nitrogen flow did not differ between treatments. The bacterial community structure of cows receiving HMTBA was not affected at the phylum level. The relative abundance of bacterial phyla in vivo differed when compared with in vitro conditions for *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, TM7, *Tenericutes*, *Spirochaetes*, SR1, and *Verrucomicrobia*.

Key words: HMTBA, microbial protein, rumen, rumen microbiome

INTRODUCTION

Metabolizable protein is the protein available for absorption in the small intestine (NRC, 2001). Deficiency in MP in dairy rations may harm productivity and milk and protein yields (Cabrita et al., 2011; Lee et al., 2012). One approach to supply sufficient MP is to formulate diets that support a high plane of microbial activity and growth in the rumen. This activity and growth results in increased microbial crude protein (MCP) available to the cow postruminally (NRC, 2001). Milk production is then benefited because the AA profile supplied by microorganisms is believed to be similar to that required by the cow (Korhonen et al., 2002). The practice of maximizing ruminal microbial activity is frequently achieved by supplying adequate energy and proteins in the diet (Hoover and Stokes, 1991). Additionally, increasing the supply of MP can be achieved by feeding sources of RUP, such as animal proteins and dried distillers grains and solubles (Hubbard et al., 2009). Because feeds high in RUP may be costly, promoting microbial protein activity with cheaper feeds is often a prudent strategy.

When a commercial 2-hydroxy-4-methylthio-butanoic acid (HMTBA) product (Alimet, Novus International, St. Charles, MO) is supplemented in dairy diets, rumen microbial activity, including MCP synthesis, fiber digestibility, and VFA production has been enhanced (Bull and Vandersall, 1973; Gil et al., 1973;

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Lundquist et al., 1983; Vázquez-Añón et al., 2001; Lee et al., 2015). In terms of animal performance, a recent meta-analysis (Feng et al., 2018) revealed that HMTBA supplementation increased milk fat yield. Despite these observations, little has been published regarding the mechanism of action in the rumen or its performance under different ruminal conditions, namely if the response is affected by the concentration of RDP and RUP supplied by the ration. Furthermore, much of the past research focusing on the effects of HMTBA on rumen microbial productivity has been conducted in vitro and does not discount the possibility of ruminal absorption of HMTBA (McCollum et al., 2000) and the effects of N recycling. We hypothesized that MCP flow, ruminal fiber digestibility, and VFA profile would change with HMTBA supplementation independent of dietary MP altered by adjusting RDP and RUP supply at the same level of crude protein. In addition, because prior in vitro work (Gil et al., 1973; Vázquez-Añón et al., 2001) has shown that HMTBA increases MCP synthesis, we hypothesized that specific bacterial populations would benefit from HMTBA supplementation and diet type resulting in the relative proportion of bacterial operational taxonomic units (OTU) in ruminal samples to be altered. Because much of the prior work evaluating HMTBA on microbial productivity have been conducted in vitro, a concurrent in vitro study was conducted to compare the effects of HMTBA on proportion of bacterial OTU in vitro versus in vivo.

MATERIALS AND METHODS

Animals, Experimental Design, and Treatments

The experimental cows were managed according to the guidelines stipulated by the University of Nebraska-Lincoln Animal Care and Use Committee. Four multiparous, lactating Holstein cows fitted with ruminal and closed T-shaped duodenal cannulas were used in this experiment, which was a 4 × 4 Latin square design (Kononoff and Hanford, 2006) with 28-d experimental periods. Cows were 169 ± 20.5 DIM at the start of the experiment and averaged 690 ± 98.5 kg of BW (as determined by weight tape). Cows were housed in a temperature-controlled barn at the Dairy Metabolism Facility in the Animal Science Complex of University of Nebraska-Lincoln (Lincoln, NE) in individual tie stalls equipped with rubber mats.

Dietary treatments (Table 1) were formulated to be either low or high in MP according to the dairy NRC (2001), assuming cows were 100 DIM, were a BW of 625 kg, and DMI was 22 kg and milk yield (MY) was 32 kg containing 3.5% fat and 3.1% true protein. Analyses of individual feed ingredients are listed in Table 2. Based

on this simulation, diets formulated to be low and high in MP were predicted to supply a concentration of 96.9 and 103 g/kg MP per unit of feed DM daily. A portion of ground corn was replaced with soybean meal in the high MP diet to achieve a concentration of MP in excess of requirement for milk production. The low MP diet included more urea so that an adequate amount of N was supplied, keeping CP content similar between diets and lowering MP down to a concentration considered to be deficient. Consequently, RDP and RUP supply were varied between treatments (Table 3). The low MP and high MP diets were predicted to supply 2,276 and 2,123 g/d of RDP and 1,115 and 1,261 g/d of RUP, respectively. Next, these low MP and high MP diets were top-dressed with 25 g of HMTBA with 225 g of ground corn as a carrier to target HMTBA inclusion at 0.1% of diet DMI, or with 250 g of ground corn to serve as negative control (low MP or high MP without HMTBA). The HMTBA and ground corn were top-dressed by direct application to the top of the TMR after it was deposited in feed bunks. The TMR were mixed daily and animals were fed once daily at 0930 h to target 5% refusals. Water was available for ad libitum consumption. Cows were milked twice daily at 0700 and 1800 h.

Table 1. Ingredient and chemical composition of TMR fed during the experiment formulated to supply concentrations of MP either deficient or in excess of requirements for lactating dairy cows¹

Ingredient, % of DM ²	Level of MP	
	Low MP	High MP
Corn silage	35.0	35.1
Corn grain	16.5	13.3
Alfalfa hay	13.7	13.7
Grass hay	9.10	9.12
Soybean hulls	7.96	7.98
Reduced fat DDGS	5.69	5.70
Molasses cane	4.09	4.10
Soybean meal	3.64	7.30
Limestone	1.09	1.09
Urea	0.75	0.15
Sodium bicarbonate	0.64	0.64
Dicalcium phosphate	0.58	0.58
Blood meal	0.45	0.46
Magnesium oxide	0.23	0.23
Salt	0.21	0.21
Calcium salts	0.17	0.17
Mineral premix	0.10	0.10
Vitamin premix	0.10	0.10

¹According to the CPM Dairy Ration Analyzer (v3.0.8.1; Boston et al., 2000).

²DDGS = dried distillers grains with solubles; calcium salts: Megalac (Church & Dwight Co. Inc., Princeton, NJ); mineral premix: formulated to contain 1.0% Ca, 0.50% P, 0.36% Mg, and 1.3% K; vitamin premix: formulated to supply approximately 120,000 IU of vitamin A/d, 24,000 IU of vitamin D/d, and 800 IU of vitamin E/d in the total ration.

Table 2. Chemical composition of corn silage, alfalfa hay, brome hay, low and high MP concentrates, and control and 2-hydroxy-4-methylthio-butanoic acid (HMTBA) top dress fed during the experiment¹ (n = 4)

Chemical ¹ % of DM ²	Corn silage		Alfalfa hay		Brome hay		Low MP concentrate		High MP concentrate		Control top dress		HMTBA top dress ³	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
DM	40.6	4.34	87.1	2.21	86.8	1.76	90.9	0.87	91.0	1.30	89.0	0.57	91.2	0.69
CP	7.80	0.40	21.5	0.61	15.5	0.48	22.3	0.64	22.2	1.33	10.0	0.31	8.75	0.72
Soluble protein	4.20	0.54	4.35	0.25	3.83	0.93	8.68	0.90	5.55	1.46	1.50	0.22	1.00	0.73
ADICP ³	0.95	0.06	2.69	1.17	1.26	0.11	1.03	0.52	0.78	0.07	0.65	0.04	0.51	0.09
NDICP ⁴	1.31	0.12	7.20	2.27	5.09	0.42	1.67	0.42	1.58	0.23	0.97	0.37	0.67	0.06
ADF	25.6	1.94	31.0	0.74	35.7	1.18	13.4	1.91	13.2	1.10	2.53	0.57	2.53	0.78
NDF	38.0	2.43	43.8	2.28	49.5	3.00	23.0	2.01	22.8	1.83	8.90	0.98	8.88	1.12
Lignin	3.20	0.27	7.10	0.80	4.28	0.26	1.73	0.84	1.78	0.81	1.06	0.68	1.77	0.50
Lignin, % NDF	8.43	0.73	16.2	1.31	6.67	0.47	7.58	3.71	7.64	2.96	11.9	7.81	20.4	6.64
NFC ⁵	45.3	2.08	22.7	4.82	23.7	29.3	42.6	1.55	42.9	1.27	77.2	1.66	76.0	0.75
Starch	35.2	2.11	2.90	0.72	0.95	0.25	29.5	2.24	25.7	0.98	69.3	1.77	60.2	1.42
Sugar	1.00	0.27	5.70	1.07	4.33	0.44	7.50	0.80	8.23	1.13	2.58	0.43	2.75	0.85
Ether extract	3.59	0.08	1.92	0.10	2.34	0.16	2.41	0.70	2.29	0.49	2.44	0.60	4.81	0.93
Ash	5.37	0.23	11.2	0.16	10.1	0.53	9.71	0.66	9.85	0.82	1.50	0.50	1.61	0.71
Ca, %	0.30	0.04	1.45	0.14	0.43	0.07	2.00	0.21	1.95	0.28	0.03	0.01	0.02	0.01
P, %	0.26	0.04	0.35	0.04	0.36	0.01	0.71	0.11	0.72	0.05	0.30	0.01	0.25	0.02
Mg, %	0.13	0.01	0.23	0.02	0.15	0.01	0.50	0.05	0.53	0.04	0.11	0.01	0.10	0.01
K, %	1.06	0.12	3.69	0.25	3.60	0.17	1.23	0.06	1.45	0.05	0.41	0.03	0.36	0.02
S, %	0.13	0.00	0.27	0.01	0.20	0.01	0.29	0.01	0.32	0.01	0.14	0.00	1.21	0.14
Na, %	0.02	0.01	0.02	0.01	0.02	0.01	0.86	0.10	0.90	0.14	0.01	0.00	0.01	0.00
Cl, %	0.17	0.01	0.34	0.03	1.30	0.11	0.42	0.05	0.41	0.06	0.07	0.01	0.17	0.03
Fe, mg/kg	310	195	252	51.6	192	29.0	595	85.7	600	60.6	66.8	10.1	98.8	12.2
Zn, mg/kg	29.5	1.91	31.5	1.91	29.5	1.73	186	25.8	214	22.9	36.3	2.87	31.5	1.73
Cu, mg/kg	6.50	0.58	12.3	7.85	14.5	10.3	52.3	3.40	55.0	5.89	3.00	0.82	3.00	0.00
Mn, mg/kg	24.8	1.89	31.5	17.3	26.8	12.7	170	40.1	173	40.3	10.5	1.29	8.75	0.96

¹Values determined by Cumberland Valley Analytical Services, Hagerstown, MD.²ADICP = acid detergent insoluble crude protein; NDICP = neutral detergent insoluble crude protein; NFC = nonfiber carbohydrate calculated by difference 100 - (% NDF + % CP + % Fat + % Ash).³Top dress consisted of 10% HMTBA.

Table 3. Chemical composition and particle size of low and high MP TMR fed during the experiment¹

Item ²	Level of MP			
	Low MP		High MP	
	Mean	SD	Mean	SD
Chemical, % of DM				
DM	61.3	1.80	61.9	1.82
CP	16.6	0.34	16.6	0.33
Soluble protein	6.00	0.35	4.53	0.59
RDP	10.9	—	10.2	—
RUP	5.30	—	6.00	—
ADICP	1.15	0.04	1.23	0.10
NDICP	2.54	0.25	2.71	0.24
ADF	22.2	1.02	22.7	0.13
NDF	34.7	1.10	35.1	1.60
Lignin	3.30	0.81	3.47	0.92
Starch	25.4	1.08	22.6	0.88
Sugar	4.00	0.40	4.70	1.41
Ether extract	2.32	0.55	2.88	0.27
Ash	8.13	0.23	8.43	0.76
Ca, %	1.09	0.05	1.12	0.08
P, %	0.45	0.01	0.46	0.03
Mg, %	0.29	0.02	0.30	0.01
K, %	1.73	0.08	1.84	0.13
S, %	0.23	0.01	0.25	0.01
Na, %	0.36	0.02	0.36	0.01
Cl, %	0.40	0.03	0.40	0.02
Particle size, %				
>19.0 mm	4.56	2.55	6.97	4.57
19.0–8.0 mm	29.4	3.39	29.3	3.28
8.0–1.18 mm	48.5	3.61	47.7	2.83
<1.18 mm	17.5	2.30	16.1	4.97

¹Values determined by Cumberland Valley Analytical Services, Hagerstown, MD.

²RDP and RUP values predicted by NRC (2001), therefore no SD is listed; ADICP = acid detergent insoluble crude protein; NDICP = neutral detergent insoluble crude protein; particle size determined using the Penn State Particle Separator on wet basis (Heinrichs and Kononoff, 2002).

Ruminal and Duodenal Digesta Sampling

Samples of whole rumen contents and duodenal digesta were collected every 4 h on d 23 through 26 of each period. Whole rumen contents were strained through 4 layers of cheesecloth, and pH of the filtrate was measured with a handheld pH probe (model M90, Corning Inc., Corning, NY) and placed in 50 mL conical tubes. The solid portion of the rumen contents were retained. Duodenal digesta contents (200 mL) were collected and placed in 250 mL Nalgene bottles (Thermo Scientific Inc., Waltham, MA). Collection time was advanced 1 h each collection d, so that every 60-min interval in a 24-h period was represented (6 samples/d, 4 d, and a total of 24 samples/cow per period). Samples were collected on d 23 at 0100, 0500, 0900, 1300, 1700, and 2100 h; d 24 at 0200, 0600, 1000, 1400, 1800, and 2200 h; d 25 at 0300, 0700, 1100, 1500, 1900, and 2300 h; and d 26 at 0400, 0800, 1200, 1600, 2000, and 0000 h. Samples

were then composited by cow, by day within period and immediately frozen at -20°C for later analysis.

Feed and Fecal Sampling

Approximately 2.5 kg of individual feed ingredients, as well as samples of the TMR for each treatment, were collected immediately after feeding on d 27 and 28. Samples of refusals were taken before feeding on d 28 and 29. Feed samples were frozen at -20°C for later analysis.

Fecal grab samples were collected at every other ruminal and duodenal digesta collection time. Specifically, fecal samples were collected on d 23 at 0100, 0900, and 1700 h; d 24 at 0200, 1000, and 1800 h; d 25 at 0300, 1100, and 1900 h; d 26 at 0400, 1200, and 2000 h. Approximately 0.50 kg of feces were placed in quart-sized plastic bags and were frozen at -20°C for later analysis.

Milk Sampling

Milk yields were recorded daily throughout each period. Milk samples were collected twice daily for 7 consecutive days on d 20 through 26, placed in 50-mL conical tube, and immediately frozen at -20°C for subsequent analysis. Additional samples were collected in 50-mL tubes containing 2-bromo-2-nitropropane-1,3 diol and shipped to DHIA (Heart of America DHIA, Manhattan, KS) where they were analyzed for fat, true protein, lactose, and SNF (AOAC, 2000) using a B2000 Infrared Analyzer (Bentley Instruments, Chaska, MN). Milk urea nitrogen was determined by the same laboratory in a ChemSpec 150 Analyzer (Bentley Instruments) using a modified Berthelot reaction. Yields of milk components were estimated according to milk weight at time of collection. During the last 7 d of each period, MY was averaged and used in statistical analysis.

Rumen Evacuation

Rumen contents were evacuated and weighed on d 27 approximately 4 h after feeding and on d 28 approximately 4 h before feeding to estimate pool sizes. Approximately 2.5 kg of ruminal digesta were taken during each evacuation and immediately placed in a 60°C forced-air oven and dried for 72 h for determination of DM.

Isolation of Ruminal Bacteria and Protozoa

Samples of whole rumen contents were collected for isolation of ruminal bacteria and protozoa on d 25 at 1000 and 1600 h (0.5 and 8.5 h after feeding) and on d

26 at 1200 and 1800 h (2.5 and 10.5 h after feeding). Ruminal bacteria were isolated according to the procedure described by Hristov et al. (2005). Briefly, ruminal contents were composited and squeezed through 4 layers of cheesecloth and the filtrate was retained. Solids remaining on the cheesecloth were added to a volume of cold buffer (McDougall, 1944) equal to the volume of the filtrate, and shaken manually in a screw-capped jar to dislodge the ruminal microorganisms loosely associated with feed particles. This suspension was then squeezed through 4 layers of cheesecloth and the 2 filtrates were combined (1:1, weight:weight). Using approximately 200 mL of the combined filtrates, bacteria were harvested immediately via differential centrifugation (Hristov and Broderick, 1996) with an initial low-speed centrifugation at $400 \times g$ for 5 min at 4°C and a subsequent high-speed centrifugation at $20,000 \times g$ for 15 min at 4°C. Samples were maintained on ice while being processed. The supernatant was then discarded and the isolated bacterial pellets were composited by cow and period and frozen at -20°C for later analysis.

Rumen protozoa were isolated using a separation funnel according to the procedure described by Shabi et al. (2000). The strained ruminal digesta were mixed with 1 volume of warm 0.9% saline and held in a separation funnel for 1.5 h at 39°C. The precipitate was then removed. The protozoal pellet was mixed with 500 mL of warm saline and kept in a separation funnel for another 1.5 h at 39°C. Protozoa were then collected and frozen at -20°C.

In Vitro Analysis

Whole rumen contents were collected via the rumen cannula of each cow approximately 4 h after feeding on d 23 of periods 1 and 2. Rumen contents were squeezed through 4 layers of cheesecloth into a pre-warmed thermos until approximately 2 L were collected. The fluid was then transferred into separation funnels, placed in a 39°C water bath and mixed with one volume of warmed McDougall's buffer (McDougall, 1948). The *in vitro* incubation procedure was carried out according to Tilley and Terry (1963). Fermentations were terminated at 24 h and a crude pellet was isolated from each fermentation tube by high-speed centrifugation ($30,000 \times g$ for 20 min at 4°C) according to Shabi et al. (2000). Then, DNA was extracted by repeat bead beating using a PowerMag Soil DNA Isolation Kit (Mo Bio, Carlsbad, CA). The concentration of DNA in each sample was measured by spectrophotometry (NanoDrop ND-1000 Spectrophotometer, NanoDrop Technologies Inc., Wilmington, DE) and stored at -20°C in aliquots of 25 µL for later analysis.

Laboratory Procedures

Analysis of Feed and Feces. Collected feed ingredients, Orts, TMR, and fecal samples were dried for 48 h at 60°C in a forced-air oven, ground to pass through a 1-mm screen (Wiley mill, Arthur H. Thomas Co., Philadelphia, PA). The feed samples were then composited by period, and a subsample of each was sent to an external laboratory (Cumberland Valley Analytical Services, Hagerstown, MD) for nutrient analysis of DM (method 930.15; AOAC International, 2000), N (Leco FP-528 N Combustion Analyzer, Leco Corp., St. Joseph, MI), soluble protein (borate-phosphate procedure; Krishnamoorthy et al., 1982), NDF with sodium sulfite (method 942.05; AOAC International, 2000; Van Soest et al., 1991) and with heat stable α -amylase (number A3306; Sigma Chemical Co., St. Louis, MO; 0.5 mL/sample) and no urea, ADF (method 973.18; AOAC International, 2000), ADIN and NDIN (Leco FP-528 N Combustion Analyzer; Leco Corp.), ADL (Goering and Van Soest, 1970), starch (Hall, 2009), sugar (DuBois et al., 1956), ether extract (method 2003.05; AOAC International, 2006), ash (method 942.05; AOAC International, 2000), and minerals (method 985.01; AOAC International, 2000). Samples of TMR were used to determine particle size on an as-fed basis according to Kononoff et al. (2003) using the Penn State Particle Separator.

For use in determination of apparent total-tract digestibility, feed samples, Orts, and fecal samples were analyzed at the University of Nebraska-Lincoln for N (Leco FP-528, Leco Corp.), NDF with sodium sulfite (without urea; Van Soest et al., 1991), starch (Megazyme, AOAC method 996.11 and AACC method 76.13), and ash (method 942.05; AOAC International, 2000). Heat stable α -amylase (number A3306; Sigma Chemical Co., St. Louis, MO) was included in the NDF procedure (0.5 mL/sample).

Ammonia and VFA in Rumen Digesta. Samples of rumen fluid collected from each cow in each period (over the course of the 4 d and representing a 24-h period) were analyzed for ammonia and VFA. Rumen fluid samples were analyzed for ammonia according to the methods of Chaney and Marbach (1962). The concentration of VFA were estimated according to Erwin et al. (1961).

Estimation of MCP Using Purines as a Microbial Marker. Collected duodenal contents were lyophilized and ground to pass through a 1-mm screen using a Wiley mill (Arthur H. Thomas Co.) Ground samples were analyzed for DM (100°C oven for 24 h). Subsamples of isolated ruminal bacterial pellets were ground with a mortar and pestle and analyzed for N

(method 990.03; AOAC, 2006; Leco FP-528 Nitrogen Combustion Analyzer, Leco Corp.). Purines (Zinn and Owens, 1986; Broderick and Merchen, 1992; Aharoni and Tagari, 1991) were used as a microbial marker to measure duodenal flow of total microbial N. Total purines were determined by optical density at 260 nm using a SpectraMax 250 (Molecular Devices, Sunnyvale, CA) spectrophotometer. Calculation of microbial N was based on the ratio of purine:N obtained from the isolated rumen bacterial pellet and on the concentration of purines in duodenal samples.

Rumen Microbial Community Analysis. Total rumen DNA was extracted as described above from samples of whole ruminal contents and was subjected to deep sequencing of 16S rDNA tags at 4 different time points (0, 4, 9, and 14 h after feeding, composited by period) to evaluate microbial community composition. Semiconductor-based sequencing was performed on the Ion Torrent Personal Genome Machine (Life Technologies, Carlsbad, CA) according to manufacturer protocols. Sequencing was performed using the 341F-518R (V3) segment of the 16S rRNA gene (Whiteley et al., 2012). Conditions for PCR were as follows: stage 1, 95°C for 2 min; stage 2, 25 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s; and stage 3, 72°C for 2 min. Negative control and positive control reactions were also performed to control for any PCR contamination. The methods used for emulsion PCR, bead deposition, and semiconductor-based sequencing on the Ion Torrent Personal Genome Machine were used as described by the manufacturer.

The raw data from Ion Torrent Personal Genome Machine was processed through a custom quality filter and analysis pipeline described in Lo Sasso, et al. (2014). The quality-filtered reads were clustered into OTU using USEARCH (Edgar, 2010; www.drive5.com/usearch/) at 97% sequence similarity (Castillo-Lopez et al., 2014) following the UPARSE pipeline (Edgar, 2013). Chimeric sequences were identified using UCHIME (Edgar et al., 2011) implemented in reference database mode using gold.fasta (available for download in UCHIME) as the reference database. Following chimera removal, the remaining OTU were classified taxonomically using the assign_taxonomy.py command within QIIME (Caporaso et al., 2010) trained using Greengenes database 12_10. The representative sequences of the OTU were aligned using the Ribosomal Database Project aligner tool (https://rdp.cme.msu.edu/tutorials/aligner/RDPTutorial_ALIGNER.html) to generate an alignment that was then imported into MOTHUR (Schloss et al., 2009), and a phylogenetic tree was generated using the dist.seqs and clearcut commands. The processed data were then used for

generating an OTU table, which was subsequently used for microbial community analysis. The OTU generated were analyzed as total OTU identified and as a core measurable microbiome (CMM) microbiota, which was defined as OTU present in 75% of samples in each treatment. Beta diversity analysis was performed in QIIME using weighted UniFrac distances and ordination plots were generated using principal coordinate analysis. In addition, the OTU distribution among treatments was used to obtain quantitative insight into the bacterial community changes as affected by dietary treatment.

Multivariate Association with Linear Models was performed using MaAsLin (<https://huttenhower.sph.harvard.edu/galaxy/>) to find associations between metadata and bacterial community abundance. The metadata used in the analysis included the observed concentrations of rumen ammonia, VFA, and pH, as well as estimates of nutrient digestibilities, lactation performance, duodenal flow, and microbial protein flow. Any association with a q-value ≤ 0.05 (the minimum false discovery rate) was identified as a significant association. BLAST similarity matches were made to the National Center for Biotechnology Information nonredundant database for those OTU that were significantly associated with a given metadata category to further confirm their taxonomy. In addition to the notched box plots or scatter plots, a complementary partial residual plot was also generated.

Calculation of Digesta Flow of DM. Indigestible NDF (iNDF; Ahvenjärvi et al., 2003) was determined for use as a digesta flow marker. The iNDF procedures for TMR, rumen solids, duodenal, and fecal samples were carried out to estimate measures of flow (Huhtanen et al., 1994). Approximately 1.25 g of 1-mm ground subsamples were weighed in triplicate into Dacron nylon bags (5 × 10 cm; Ankom Inc., Fairport, NY) with a pore size of 50 μ m. The bags were then heat-sealed using an Ankom heat sealer (Vanzant et al., 1998). Fifty Dacron bags were placed into larger nylon mesh bags (36 × 42 cm) that contained 2 secured 100-g weights. Nylon mesh bags were incubated for 12 d (Wu, 2005) in the ventral sac of the rumen of steers fitted with ruminal cannulas consuming a 70.5% grass hay and 29.5% grain (dried distillers grains, dry-rolled corn, salt, and vitamin and mineral premix) diet. After 12 d, the Dacron bags were removed and machine washed using 5, 3 min cycles consisting of a 1 min wash and a 2 min spin, rinsed in distilled water, and dried in a 60°C forced-air oven for 12 h. After drying, NDF was determined using an Ankom Fiber Analyzer (Ankom Technology, Fairport, NY). Fecal output was calculated by determining the intake of iNDF and dividing by the

concentration of iNDF in the feces. Duodenal flow was calculated by dividing the amount of iNDF intake by the concentration of iNDF in duodenal samples and expressed in g/d.

Statistical Analysis

Data collected on the effects of HMTBA supplementation and MP supplementation were analyzed using the GLIMMIX procedure of SAS (Version 9.2; SAS Institute, Inc., Cary, NC) as a 4×4 Latin square. Fixed model effects included treatment and period with cow as the random effect. Treatments were then partitioned into single degree of freedom contrasts for MP and inclusion of HMTBA and interaction as planned a priori.

Data obtained from ruminal fluid were analyzed as repeated measures using the simple diagonal covariance matrix in SAS. The effects of period, treatment, hour, and treatment $\times$ hour interaction were considered as fixed effects, and cow was considered as the random effect. As above, treatments were then partitioned into single degree of freedom contrasts for MP and inclusion of HMTBA and interaction as planned a priori.

The OTU distribution among treatments was analyzed using the GLIMMIX procedure of SAS (Version 9.2; SAS Institute, Inc., Cary, NC). Statistical differences were declared significant at $P \leq 0.05$ and tendencies were considered between $P > 0.05$ and $P \leq 0.10$.

RESULTS

Diet Composition

Ingredient composition of the experimental diets is listed in Table 1. By design, ingredient composition was

kept similar across experimental diets except for in the high MP diet, in which a portion of ground corn was replaced by soybean meal to achieve a concentration of MP determined as more than sufficient for milk production. The low MP diet included more urea than the high MP diet so that an adequate amount of N was supplied, keeping CP content similar between diets and lowering MP down to a concentration considered to be deficient. Assuming 22 kg of DMI, the dairy NRC (2001) model estimated that the low MP diet would supply 2,132 g of MP/d (94% of requirement), whereas the high MP diet would supply 2,267 g of MP/d (100% of requirement). Adjusting for actual DMI of the low and high MP diets, as well as DIM, MY, fat percentage, and protein %, the model estimated that the low MP diet supplied 2,335 g MP/d (106% of requirement), whereas the high MP diet supplied 2,543 g MP/d (114% of requirement). The model estimated that the low MP diet supplied 2,575 g/d of RDP (RDP balance: 278 g/d) and 1,258 g/d RUP (RUP balance: 180 g/d), whereas the high MP diet supplied 2,344 g/d of RDP (RDP balance: 120 g/d) and 1,457 g/d RUP (RUP balance: 381 g/d).

DMI, Milk Production, and Composition

The results of production performance are listed in Table 4. The DMI, MY, 3.5% FCM, milk fat percentage, milk fat yield, protein yield, and MUN were not affected by either level of MP or HMTBA supplementation, nor did we observe an interaction between the 2. Milk protein percent was not affected by HMTBA, but tended to be greater (3.30 vs. 3.21; $P = 0.06$) in cows receiving the low MP diet than those receiving the high MP diet.

Table 4. Effects of feeding 2-hydroxy-4-methylthio-butanoic acid (HMTBA) with low and high MP diets on milk production and composition

Item	Treatment				SEM ¹	P-value ²		
	Low MP		High MP			MP	HMTBA	MP × HMTBA
	Control	HMTBA	Control	HMTBA				
DMI, kg/d	23.5	23.7	24.1	24.1	1.15	0.48	0.96	0.89
Milk yield, kg/d	27.9	27.1	29.1	28.6	3.18	0.53	0.78	0.92
ECM ³	28.6	27.8	29.8	29.4	2.55	0.48	0.77	0.93
Fat, %	3.66	3.75	3.67	3.70	0.33	0.76	0.47	0.67
Fat yield, kg/d	1.00	0.97	1.06	1.04	0.09	0.35	0.79	0.97
Protein, %	3.26	3.33	3.19	3.22	0.09	0.06	0.27	0.67
Protein yield, kg/d	0.91	0.88	0.92	0.91	0.08	0.74	0.78	0.85
MUN, mg/DL	11.6	12.0	11.0	11.8	0.97	0.41	0.21	0.66

¹The highest SE of treatment means is shown.

²P-values for contrasts of level of MP and HMTBA supplementation and MP $\times$ HMTBA interaction.

³ECM = $0.327 \times \text{milk yield (kg)} + 12.95 \times \text{fat (kg)} + 7.20 \times \text{protein (kg)}$ adjusted for 3.5% fat and 3.2% total protein (Dairy Records Management System, 2014).

Table 5. Effects of feeding 2-hydroxy-4-methylthio-butanoic acid (HMTBA) with high or low MP diets on apparent total-tract nutrient digestibility estimated using iNDF as a digesta flow marker

Item	Treatment				SEM ¹	P-value ²		
	Low MP		High MP			MP	HMTBA	MP × HMTBA
	Control	HMTBA	Control	HMTBA				
N, %	69.8	69.3	64.9	66.0	1.99	0.01	0.80	0.50
NDF, %	55.3	53.1	50.1	51.7	2.45	0.01	0.77	0.08
OM, %	67.3	66.4	64.3	64.7	1.97	0.02	0.80	0.44

¹The highest SE of treatment means is shown.²P-values for contrasts of level of MP and HMTBA supplementation and MP × HMTBA interaction.

Nutrient Digestibility

Table 5 lists the results of apparent total-tract digestibility of nutrients estimated with the use of iNDF as a digesta flow marker. No effects of HMTBA supplementation or an interaction between HMTBA and level of MP on digestibility were observed. Digestibility of DM, N, NDF, and OM were increased ($P \leq 0.03$) in cows consuming the low MP diet.

Rumen pH, Pool Size, Ammonia, and VFA

Measurements of ruminal fermentation parameters are listed in Table 6. Rumen pH was affected by both level of MP and HMTBA supplementation. Rumen pH was lower (5.83 vs. 5.89; $P = 0.05$) in cows consuming the high MP diet than those consuming the low MP diet. Additionally, rumen pH was lower (5.82 vs. 5.90; $P < 0.01$) in cows supplemented with HMTBA than in cows receiving the control. Rumen pool of DM was not

affected by HMTBA supplementation but tended to be greater in cows receiving the high MP diet ($P = 0.08$).

Rumen ammonia tended to be greater (17.2 mg/dl vs. 16.5 mg/dl; $P = 0.06$) in cows receiving HMTBA than those receiving the control. The total VFA concentration was greater (126 mM vs. 119 mM; $P = 0.02$) in cows consuming HMTBA than in those receiving the control. The molar percentage of acetate in the rumen was greater ($P < 0.01$) in cows consuming control than those receiving HMTBA. Rumen propionate concentration was greater ($P < 0.01$) in cows receiving HMTBA than those receiving the control. Consequently, the ratio of acetate to propionate (A:P) was greater (2.78 vs. 2.58; $P < 0.01$) in cows receiving the control than those receiving HMTBA. Furthermore, an interaction ($P = 0.04$) of HMTBA and diet type was observed for A:P, which seemed to be less affected by HMTBA in cows receiving the high MP diet. Butyrate molar percentage was greater ($P = 0.05$) for HMTBA compared with control. Rumen isovalerate concentration was greater

Table 6. Effects of feeding 2-hydroxy-4-methylthio-butanoic acid (HMTBA) with low or high MP diets on rumen pH, pool, and concentration of ammonia and volatile fatty acids

Item	Treatment				SEM ¹	P-value ²		
	Low MP		High MP			MP	HMTBA	MP × HMTBA
	Control	HMTBA	Control	HMTBA				
pH	5.93	5.85	5.87	5.78	0.15	0.05	<0.01	0.90
Pool, kg DM	10.6	10.1	12.3	11.4	1.28	0.08	0.41	0.83
Pool, kg as is	73.4	71.6	75.6	72.9	7.60	0.47	0.35	0.85
Ammonia, mg/dL	17.0	17.3	16.0	17.2	0.88	0.14	0.06	0.30
Total VFA, mM VFA, mol/100 mol	120	126	118	126	6.36	0.77	0.02	0.65
Acetate	63.1	61.4	63.0	62.2	1.34	0.33	<0.01	0.21
Propionate	23.0	25.1	23.4	24.5	1.23	0.83	<0.01	0.08
Isobutyrate	0.32	0.52	0.43	0.40	0.07	0.90	0.23	0.12
Butyrate	11.1	10.6	11.0	10.8	0.40	0.97	0.05	0.40
Isovalerate	0.95	1.07	1.04	1.20	0.13	0.09	0.03	0.71
Valerate	1.45	1.45	1.59	1.67	0.09	<0.01	0.33	0.33
Acetate:propionate	2.82	2.52	2.75	2.64	0.20	0.60	<0.01	0.04

¹The highest SE of treatment means is shown.²P-values for contrasts of level of MP and HMTBA supplementation and MP × HMTBA interaction.

Table 7. Effects of feeding 2-hydroxy-4-methylthio-butanoic acid (HMTBA) with low and high MP diets on duodenal digesta and N flows estimated using purines and indigestible NDF as a digesta flow marker

Item	Treatment				SEM ¹	P-value ²		
	Low MP		High MP			MP	HMTBA	MP × HMTBA
	Control	HMTBA	Control	HMTBA				
Duodenal flow, kg DM/d	15.7	15.7	15.0	16.9	1.18	0.79	0.38	0.39
Duodenal N flow, g N/d	687	703	670	773	62.5	0.60	0.27	0.41
Microbial N flow, g N/d	343	270	319	328	44.3	0.71	0.47	0.37
Microbial N of total N flow, %	49.8	39.7	49.2	43.8	7.06	0.72	0.15	0.64

¹The highest SE of treatment means is shown.²P-values for contrasts of level of MP and HMTBA supplementation and MP × HMTBA interaction.

($P = 0.03$) in cows consuming HMTBA than in those consuming the control. Valerate was affected by level of MP; cows consuming the high MP diet had greater ($P < 0.01$) valerate concentrations than those consuming the low MP diet. All other rumen VFA concentrations were not affected by level of MP.

Duodenal Flow of DM, Nitrogen, and Microbial Nitrogen

Estimates of duodenal flow of DM, N, and microbial N, which were measured using purines and iNDF as a flow marker, are listed in Table 7. No effects of HMTBA supplementation, level of MP, or an interaction between the 2 were observed on duodenal flow of DM, N, and microbial N. The concentration of purines in isolated microbial samples averaged 17.7 ± 4.36 mg/g DM.

Community Composition of Bacteria in the Rumen

Normalized bacterial phyla relative to total number of reads recovered from rumen digesta are listed in Table 8. In the total OTU analysis, proportion of bacterial phyla did not differ between concentration of MP or HMTBA supplementation. The analysis of core OTU is listed in Supplemental Tables S1 and S2 (<http://doi.org/10.3168/jds.2019-17664>).

Community Composition of Bacteria Isolated from In Vitro Fermentation

Normalized bacterial phyla relative to total number of reads recovered from in vitro fermentation are listed in Table 9. The analysis of core OTU is listed in Supplemental Table S3 (<http://doi.org/10.3168/jds.2019-17664>). The OTU based analysis of the total OTU revealed that the relative abundance of *Firmicutes* de-

Table 8. Effects of feeding 2-hydroxy-4-methylthio-butanoic acid (HMTBA) with low and high MP diets on proportion of phyla of rumen bacterial populations relative to total number of reads recovered from ruminal digesta attained from bioinformatics analysis of total OTU

Item	Treatment				SEM ¹	P-value ²		
	Low MP		High MP			MP	HMTBA	MP × HMTBA
	Control	HMTBA	Control	HMTBA				
Total analysis								
<i>Firmicutes</i>	59.4	56.8	56	57.1	2.92	0.51	0.74	0.43
<i>Bacteroidetes</i>	27.7	29.8	29.8	29.9	2.33	0.61	0.59	0.62
Other	4.38	4.58	4.20	3.60	0.74	0.92	0.38	0.90
<i>Proteobacteria</i>	1.63	2.73	2.83	2.15	0.75	0.63	0.74	0.20
TM7	1.68	1.90	1.85	1.98	0.35	0.35	0.21	0.70
<i>Tenericutes</i>	1.10	1.03	1.25	1.18	0.14	0.34	0.62	1.00
<i>Actinobacteria</i>	0.68	0.05	0.45	0.08	0.36	0.79	0.22	0.74
SR1	0.70	0.73	0.73	0.98	0.22	0.43	0.43	0.51
<i>Spirochaetes</i>	0.50	0.58	0.60	0.68	0.13	0.20	0.33	1.00
<i>Fibrobacteres</i>	0.31	0.46	0.39	0.44	0.06	1.00	0.11	0.21
<i>Verrucomicrobia</i>	0.10	0.05	0.15	0.10	0.03	0.17	0.17	0.62

¹The highest standard error of treatment means is shown.²P-values for contrasts of level of MP and HMTBA supplementation and MP × HMTBA interaction.

Table 9. Effects of feeding 2-hydroxy-4-methylthio-butanoic acid (HMTBA) with low and high MP diets on proportion of phyla of rumen bacterial populations relative to total number of reads recovered from in vitro fermentation attained from bioinformatics analysis of total OTU

Item	Treatment				SEM ¹	P-value ²		
	Low MP		High MP			MP	HMTBA	MP × HMTBA
	Control	HMTBA	Control	HMTBA				
Total analysis								
<i>Firmicutes</i>	35.0	45.4	31.1	27.4	6.38	<0.01	0.36	0.06
<i>Bacteroidetes</i>	48.9	40.6	47.8	60.2	7.85	0.06	0.67	0.04
Other	1.60	1.29	1.44	2.14	0.30	0.19	0.44	0.06
<i>Proteobacteria</i>	10.2	9.32	14.6	5.40	4.09	0.94	0.14	0.22
TM7	0.20	0.25	0.20	0.25	0.25	0.59	0.46	0.80
<i>Tenericutes</i>	0.56	0.45	0.58	0.90	0.23	0.09	0.42	0.12
<i>Actinobacteria</i>	0.01	0.01	0.10	0.00	0.08	0.54	0.46	0.47
SR1	0.46	0.57	0.69	0.65	0.27	0.34	0.85	0.63
<i>Spirochaetes</i>	1.02	0.68	1.53	0.97	0.55	0.40	0.34	0.82
<i>Fibrobacteres</i>	0.72	0.18	0.20	0.64	0.31	0.89	0.82	0.03
<i>Verrucomicrobia</i>	0.33	0.23	0.16	0.33	0.07	0.52	0.46	0.01

¹The highest standard error of treatment means is shown.²P-values for contrasts of level of MP and HMTBA supplementation and MP × HMTBA interaction.

creased ($P = 0.01$) from the low MP diet compared with the high MP diet. In contrast, there was a trend for the proportion of phyla *Tenericutes* ($P = 0.09$) to be greater in the high MP diet than in low MP diet. An interaction between MP and HMTBA was observed ($P \leq 0.05$) in *Bacteroidetes*, *Fibrobacteres*, and *Verrucomicrobia*. The relative abundance of phyla also differed ($P \leq 0.01$) by method (in vitro vs. in vivo; Table 10) in *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, TM7, *Tenericutes*, *Spirochaetes*, SR1, and *Verrucomicrobia* as listed in Table 10.

Associations of OTU and Metadata

Results generated by the MaAsLin analysis identified significant ($P \leq 0.05$) associations between bacterial

OTU and HMTBA, level of dietary MP, DMI, microbial N flow, percent microbial N flow of total N flow, MY, percent milk fat, percent milk lactose, percent milk protein, milk fat yield, ammonia, N digestibility, and NDF digestibility. The association scatter grams and complementary partial residual plots, along with the top microorganism identified by the National Center for Biotechnology Information are represented in Supplemental Figures S1 through S18 (<http://doi.org/10.3168/jds.2019-17664>).

DISCUSSION

This study employed the use of a replicated 4×4 Latin square design (Kononoff and Hanford, 2006). Because ruminal and duodenal digesta sampling as performed in this study is laborious, a 4×4 Latin square design was logistically beneficial, as only 4 cows were required. However, several assumptions are made with this type of design. First, we assumed there is no carryover of treatment effects into subsequent periods, and that the length of the adaptation was sufficient. Using a single 4×4 Latin square, Castillo-Lopez et al. (2014) were successful in observing differences in rumen characteristics similar to those observed in this study. Second, the 4×4 Latin square may have limited statistical power, in that the chance of type II errors are increased with fewer observations. This becomes especially relevant to this experiment in terms of estimating responses in milk production and components, as this study was underpowered to determine these differences; rather, our focus was directed to the ruminal effects of HMTBA supplementation, where this type of experimental design is more appropriate. St-Pierre

Table 10. Comparison of the proportion of phyla of rumen bacterial populations relative to total number of reads recovered from ruminal digesta using in vivo or in vitro techniques in the total microbiomes

Item	Method		SEM ¹	P-value
	In vivo	In vitro		
<i>Firmicutes</i>	57.3	34.7	2.77	<0.01
<i>Bacteroidetes</i>	29.3	49.4	2.85	<0.01
Other	4.19	1.62	0.19	<0.01
<i>Proteobacteria</i>	2.34	9.88	1.45	<0.01
TM7	1.85	0.23	0.05	<0.01
<i>Tenericutes</i>	1.14	0.62	0.07	<0.01
<i>Actinobacteria</i>	0.32	0.03	0.12	0.12
SR1	0.79	0.59	0.06	<0.01
<i>Spirochaetes</i>	0.59	1.05	0.10	0.01
<i>Fibrobacteres</i>	0.40	0.44	0.10	0.96
<i>Verrucomicrobia</i>	0.10	0.26	0.03	<0.01

¹The highest SE of treatment means is shown.

and Sylvester (2005) determined that significant milk protein yield responses to feeding different methionine sources (including HMTBA) were not observed until after 5 wk of supplementation ($P < 0.05$). This suggests that larger and longer-term studies may be required to determine differences in milk production variables in response to HMTBA.

Assumed DIM, DMI, MY, fat percentage, and protein percentage used to design dietary treatments low or high in MP supply using NRC (2001) differed from actual observations. Although the original low and high MP diets were designed to supply 94.4% and 100.4% of MP requirements, respectively, adjusting the model for observed animal inputs and chemical composition of feedstuffs resulted in a supply of 106.3% and 113.5% of MP requirements, respectively. Unfortunately, actual level of MP supplied was not deficient in the low MP diet as originally intended to test our hypothesis that rumen microbial activity, specifically MCP flow, ruminal fiber digestibility, and VFA profile would change with HMTBA supplementation independent of deficient or adequate supply of MP. Therefore, our focus shifted in interpretation of our observations to variation in RDP and RUP supply attained by exchanging soybean meal with urea in dietary treatments.

With the exception of the ruminal acetate-to-propionate ratio, no interactions were observed between HMTBA and level of MP supply in the diet, and accordingly, RDP and RUP concentrations. This is noteworthy in terms of our hypothesis, in that the effects of HMTBA were seemingly unaltered under conditions in which dietary MP supply differed, albeit a deficiency in dietary MP was not attained as originally designed due to actual DMI and production.

Supplementation of HMTBA did not affect nutrient digestibility, however an effect of the level of dietary MP was observed. The digestibilities of DM, OM, N, and NDF were all increased in cows receiving diets which were lower in MP (higher in RDP). In our attempt to attain a concentration of MP that was deficient in the low MP diet, soybean meal was reduced by 3.20% of the diet DM and replaced with 2.60% corn grain and 0.60% urea (relative to the high MP diet). Consequently, it is not a surprise that nutrient digestibility estimates were greater for the low MP diet, as corn grain tends to be more digestible than soybean meal, being lower in fiber and higher in starch (Macgregor, 2000). This was reflected in the nutrient analyses of the TMR, as the low MP diet was higher in starch. Additionally, the increase of urea in the low MP diet contributed to a 1.47% increase in soluble protein relative to the high MP diet, which may explain elevated estimates of N digestibility in cows consuming the low MP diet, as

urea is a highly digestible source of N (Griswold et al., 2003).

Several unexpected effects of HMTBA supplementation were observed in rumen measurements. First, acetate concentration was decreased as propionate concentration was increased. This is contrary to what Rosser et al. (1971) and Lundquist et al. (1983) observed *in vivo*. In 2 more recent studies (Vázquez-Añón et al., 2001; Noftsker et al., 2005), an effect of HMTBA on VFA concentration was not observed, although our observed increase in total VFA concentration was in agreement with Martin et al. (2013), who also observed increased VFA. The only interaction of HMTBA and diet type observed in this study was the acetate-to-propionate ratio, which decreased when HMTBA was supplemented in the low MP diet, but less so than with the high MP diet. The interaction was likely driven by the increase in the concentration of propionate we observed when HMTBA was supplemented with the low MP diet. As no others have observed an increase in the concentration of propionate in response to HMTBA, we have no explanation for this interaction. Second, rumen pH was decreased with HMTBA supplementation, whereas no effect on rumen pH was observed in *in vitro* work by Vázquez-Añón et al. (2001) and Noftsker et al. (2005). Our observations of increased rumen VFA concentration were the likely driver of the reduction in pH. Third, a trend for increased ammonia concentration was observed. This observation was contrary to previous research (Vázquez-Añón et al., 2001) where a decrease in ammonia concentration was observed; however, Blake et al. (1986) suggested that HMTBA supplementation may stimulate protein digestion, releasing more ammonia in the rumen. With mixed results, the effects of HMTBA on VFA concentration, ammonia, and pH in the rumen remains unclear and warrants further investigation.

An effect of HMTBA supplementation on MCP flow was not observed in this study, similar to the results of Noftsker et al. (2005) but in contrast to those of Lee et al. (2015). Rumen protozoa were isolated using the separatory funnel method described by Shabi et al. (2000), which may have led to and overestimation of protozoal N in calculation of MCP flow (Sylvester et al., 2005).

In the analysis of total OTU at the phylum level, the rumen bacterial community was unaffected by treatment (Table 8). However, an analysis of the CMM was conducted to identify OTU present in 75% of samples to elucidate potential changes otherwise lost to PCR or sequencing errors. These data are presented in the supplement (Supplemental Table S1, <http://doi.org/10.3168/jds.2019-17664>). Although not significant in the

total analysis ($P < 0.11$), *Fibrobacteres* appeared to increase with HMTBA supplementation in the analysis of the CMM. Ramirez Ramirez et al. (2012) observed that relative abundance of *Fibrobacteres* tended to be sensitive to dietary changes. Increased proportions of *Fibrobacteres* may contribute to the enhanced fiber digestibilities observed during HMTBA supplementation in previous studies (Bull and Vandersall, 1973; Gil et al., 1973; Noftsker et al., 2005), although we did not observe increased fiber digestibility in this study. Martin et al. (2013) observed an increase in *Fibrobacteres* without effects on fiber digestibility in response to HMTBA; the authors suggested that HMTBA may indirectly affect the growth of cellulolytic bacteria by concurrently stimulating noncellulolytic bacteria, enhancing their growth via supply of branched-chain volatile fatty acids. Firkins (2010) similarly suggests that cellulolytics work in a consortium with proteolytics, benefiting indirectly from preformed amino acids, which could explain an increase in *Fibrobacteres* in response to supplementation of HMTBA. The bacterial community structure was not affected by low or high MP diets; although adjustments in RDP and available carbohydrates were made to alter concentration of dietary MP between diets, these modifications had no effect on the rumen microbial community structure. Analysis of the bacterial communities isolated from in vitro fermentation revealed significant differences from those observed in vivo in most phyla. This finding suggests that in vitro fermentation after 24 h was not completely representative of that which was observed in vivo. These differences may be a result of the differences in VFA absorption, buffering of pH from saliva, substrate composition after 24 h, and many other factors that are different between the in vivo and in vitro systems.

In conclusion, rumen fermentation characteristics changed with inclusion of HMTBA, whereas MCP flow was unchanged. A better understanding of the effects of HMTBA in the rumen, including potential influences on the microbial community structure, would help nutritionists know how to best use it as a supplement in dairy rations.

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