Assessing the ruminal action of the isopropyl ester of 2-hydroxy-4-(methylthio) butanoic acid in continuous and batch cultures of mixed ruminal microbes

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ABSTRACT

In dairy rations, Met is often a limiting amino acid that is provided by rumen-undegradable protein and rumen-protected sources of Met. A Met precursor, 2-hydroxy-4-(methylthio) butanoic acid (HMB) has undergone considerable study for ruminal and postruminal metabolism, whereas its isopropyl ester (HMBi) has been evaluated primarily with respect to its supply of metabolizable Met rather than as a preformed source of Met for microbial metabolism. A control and 3 isomolar Met treatments—0.097% DL-Met, 0.048% DL-Met plus 0.055% HMBi (Met + HMBi treatment), and 0.11% HMBi—were pulse-dosed every 8 h into continuous cultures simultaneously with feeding. Treatment had no effect on digestibilities of acid-detergent fiber or true organic matter. Digestibilities of neutral detergent fiber and hemicellulose were linearly decreased with increasing HMBi inclusion. Concentration of $\text{NH}_3\text{-N}$ tended to decrease linearly and quadratically, and $\text{NH}_3\text{-N}$ flow tended to decrease linearly, with increasing HMBi inclusion; in contrast, the proportion of bacterial N derived from $\text{NH}_3\text{-N}$ increased linearly. Peptide N increased linearly and tended to be affected quadratically (highest for the HMBi treatment). Acetate and propionate production both decreased with increasing HMBi, but acetate declined more such that acetate:propionate increased linearly. Isobutyrate production decreased, but isovalerate and valerate increased with increasing HMBi inclusion. Relative changes in population abundance were not detected by denaturing gradient gel electrophoresis. In the second study, which was done in batch culture, Met treatments consisted of control, 0.097% L-Met, 0.097% L-Met, 0.125% DL-HMBi, 0.098% DL-HMB, 0.250% DL-HMBi (2× HMBi), 0.049% DL-Met + 0.063% DL-HMBi (Met + HMBi), and 0.098%

DL-HMB + 0.039% isopropanol. All of these Met treatments were unlabeled (i.e., at natural abundance of ^{13}C) but simultaneously dosed with equivalent dosages of $[1\text{-}^{13}\text{C}]\text{-L-Met}$. All 8 treatments were inoculated with faunated or partially defaunated inocula. Protozoal abundance had minor effect on measurements. The unlabeled L-Met treatment had the lowest ^{13}C enrichment of Met in the microbial pellet followed by Met + HMBi and then D-Met or DL-HMB, which were lower than remaining treatments. The percentage of the $[1\text{-}^{13}\text{C}]\text{-L-Met}$ dose recovered in microbial Met was lowest for the L-Met treatment; intermediate for D-Met, DL-HMB (with or without isopropanol), and Met + HMBi treatments; and highest for HMBi, 2× HMBi, and control. Results suggest that racemization of D-Met lags behind L-Met. The similar conversions of the HMBi and 2× HMBi treatments compared with the control suggests a low degradation of HMBi to provide unlabeled Met to dilute the $[1\text{-}^{13}\text{C}]\text{-L-Met}$ dose for protein synthesis. The lack of treatment by time interaction suggests that these initial responses carried through during the 24 h of incubation. The proportion of HMBi available to ruminal microbes can influence microbial metabolism, potentially through formation of L-Met.

Key words: methionine, hydroxymethylthiobutanoic acid, microbial protein synthesis

INTRODUCTION

The isopropyl ester (HMBi) of 2-hydroxy-4-(methylthio) butanoic acid (HMB) has increased total milk production, FCM production, and true milk protein relative to HMB (St-Pierre and Sylvester, 2005). Despite increasing the escape and postruminal supply of this Met precursor, a proportion of HMBi is hydrolyzed to HMB for ruminal degradability or possibly absorption from the rumen or omasum (Noftsker et al., 2005). Increasing HMB decreased the proportion of bacterial N derived from $\text{NH}_3\text{-N}$ in continuous culture while increasing concentration of isobutyrate and decreasing propionate, and HMB increased ADF di-

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gestibility relative to isomolar DL-Met (Noftsker et al., 2003). Because of the continuous infusion of the HMB in that study, we reasoned in the current study that infrequent pulse-dosing of the more slowly available form, HMBi, would sustain ruminally available Met and therefore increase efficiency of microbial protein synthesis. In addition to the recognized role of AA to stimulate growth of amylolytic bacteria, AA also might stimulate fibrolytic bacteria (Firkins et al., 2007). Both HMB and HMBi increased 16S rRNA gene copies of the cellulolytic bacteria, *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*, presumably through increased availability of Met (Martin et al., 2013). Our first objective was to assess the dose response of HMBi when replacing DL-Met in continuous culture to assess the amount and efficiency of bacterial protein synthesis, fermentation profile, and fiber digestibility.

A second study investigated factors influencing HMBi metabolism by ruminal microbes in batch culture. Alcohols such as isopropanol (from ester hydrolysis of HMBi) can promote leakage of cellular contents through bacterial membranes (Ezeji et al., 2010) or can detach bacteria from feed particles (Trabalza-Marínucci et al., 2006). Although the effect of the isopropanol released from HMBi is unknown (Martin et al., 2013), alcohols are common in silages (Kristensen et al., 2007) and isopropanol might even be produced by some rumen microbes (Sato and Shiogama, 2010). Little is known about rumen microbial metabolism of D-Met and D-HMB relative to their more biologically active L-stereoisomers. Because total DL-Met degradation is so extensive (Hegedüs et al., 1995; Bach and Stern, 1999), D-Met presumably would be extensively rapidly racemized. Similarly, HMB is an approximately racemic mixture of stereoisomers (Lobley et al., 2006). Transport of L-Met into the cytoplasm in *Escherichia coli* is much more efficient because of its greater affinity compared with that of D-Met (Zhang et al., 2003). Slower D-Met transport into the cytoplasm could localize it in the periplasm for racemization. However, both D-Met and D-Leu, which are not traditionally thought of as components of peptidoglycan (as is D-Ala, most notably), are produced in periplasm and have important sensing and signaling roles in cell wall synthesis and remodeling of various bacterial strains tested (Lam et al., 2009; Cava et al., 2011). Although nonspecific transport and conversion of D-HMB has been characterized for animal tissues (Martín-Venegas et al., 2011), mitochondria (the main cellular compartment for D-HMB metabolism) typically are not expressed in ruminal microbes. Ruminal protozoa express hydrogenosomes, which are likely adaptations from mitochondria by anaerobic eukaryotes (Hackstein and Tielens, 2010), so HMB metabolism might be prioritized in protozoa

(Noftsker et al., 2005). Whereas D-stereoisomers are detected in rumen bacteria (Schieber et al., 1999), little is known specifically about D-Met or D-HMB metabolism for rumen microbes. Consequently, the aim of the second experiment was to directly assess the role of isopropanol and the D-Met stereoisomer compared with DL-HMB and DL-HMBi through controlled additions to batch cultures of mixed ruminal microbes from 2 inocula sources that have low or higher concentrations of protozoa.

MATERIALS AND METHODS

Experiment 1: Experimental Design, Sampling, and Analyses

A dual-flow continuous culture fermentation system (Noftsker et al., 2003) was used in a 4×4 Latin square. The 4 periods lasted 12 d, with the first 8 d for adaptation. Ruminal contents were collected from 2 multiparous lactating Holstein cows receiving a normal lactation diet, squeezed through 8 layers of cheesecloth, sealed tight to minimize access to air, and transported to the laboratory in an insulated container previously warmed to 39°C. After inoculation into fermenters, artificial saliva was infused to maintain liquid and solids dilution rates of 12.5 and 5.5%/h, respectively. The pH was maintained between 6.0 and 7.0, and temperature was kept constant at $39 \pm 1^\circ\text{C}$. The fermentation vessels varied in measured working volume from approximately 1,600 to 1,835 mL, so DM input was varied proportionately (averaging 100.0 g/d, ranging from 94.3 to 108.2 g/d) to maintain a constant volume-to-DM ratio. The diets were 50:50 forage:concentrate and fed in 3 equal meals per day at 0000, 0800, and 1600 h. The diet composition is shown in Table 1 and was formulated without soybean meal to complement RDP in alfalfa to prevent excessive RDP from potential responses to Met supplementation. Although the CP and RDP were lower than in typical diets, the buffer provided urea at 0.4 g/L. With an average of 5.1 L/d of buffer outflow, the urea-N provided an equivalent of >5% unit CP and RDP to the diets, which averaged 100 g/d of DMI input (data not shown). The treatments contained no supplement, 0.097% DL-methionine (DL-Met treatment designation), 0.048% DL-Met plus 0.055% HMBi (**Met + HMBi**), or 0.11% HMBi (HMBi treatment). The amounts of HMBi and DL-Met were chosen from the previous continuous culture study of Noftsker et al. (2003). The supplements and control were provided at 3.6 mL of liquid directly into the fermentation vessels at each feeding with overflow halted to allow mixing.

On d 10, 11, and 12, a 15% sample of the total effluent (liquid and solid) was taken and composited. The

Table 1. Ingredient and chemical composition in Experiments 1 and 2

Ingredient composition	% DM
Alfalfa pellets	50.00
Pelleted concentrate	
Corn grain, ground	31.00
Dry distillers grain	6.50
Blood meal, ring-dried	0.50
Soybean hulls	10.80
CaPO ₄	0.33
Limestone	0.065
MgO	0.13
Trace mineralized salt ¹	0.50
Vitamins A, D, and E ²	0.104
Chemical composition	
NDF	34.9
ADF	23.6
CP	14.2
RDP ³	7.9

¹Contained 0.10% Mg; 38.08% Na; 58.0% Cl; 0.04% S; 5,000 mg/kg of Fe; 7,500 mg/kg of Zn; 2,500 mg/kg of Cu; 6,000 mg/kg of Mn; 100 mg/kg of I; 60 mg/kg of Se; and 50 mg/kg of Co.

²Vitamin premixes: 0.012% of vitamin A (30,000 IU/g), 0.033% of vitamin D (3,000 IU/g), and 0.059% of vitamin E (20,000 IU/454 g).

³Rumen-degraded protein from NRC (2001) library values.

3-d composite sample was lyophilized and analyzed for ADF (AOAC, 1990) and for NDF in the presence of heat-stable α -amylase and sodium sulfite (Van Soest et al., 1991). Hemicellulose was calculated as NDF – ADF. For each of the 3 d, a 47-mL aliquot of the effluent sample was acidified with 3 mL of 6 N HCl to stop fermentation, and all the samples were composited. The composited sample was analyzed for VFA using gas chromatography (Harvatine et al., 2002) and for ammonia-N by the colorimetric method of Chaney and Marbach (1962). Bacteria were pelleted from a total effluent (liquid and solid) sample of the 3-d composite (Karnati et al., 2009a). Nitrogen and OM concentration were determined for effluent and lyophilized bacteria by micro-Kjeldahl (AOAC, 1990). We infused (NH₄)₂SO₄ that was 10% enriched with ¹⁵N, samples were collected and analyzed for ¹⁵N enrichment for NH₃-N and bacterial N, and bacterial N flow and bacterial N derived from NH₃-N were calculated as described by Noftsker et al. (2003).

Denaturing Gradient Gel Electrophoresis Analysis of Bacteria (Experiment 1)

Three 15-mL samples of total effluent were taken between 31.5 and 41 h as described in another manuscript from our laboratory (Firkins et al., 2015). These samples were stored at –80°C. After thawing, 1.5 mL was aliquoted for DNA extraction, with minor changes compared with the procedures described by Yu and Morrison (2004) and Karnati et al. (2009b). Briefly, 2 rounds of bead-beating were done to break the cells, then nucleic acid precipitation, treatment with RNase

A and proteinase K, and column purification were also carried out. The V3 region of 16S rRNA gene was amplified using primers 357f-GC (synthesized with a 40-bp GC clamp at its 5' end) and 519r. The amplification reactions were comprised of 100 pmol of each primer, 250 μ M dNTP, 2 mM magnesium chloride, 0.05% bovine serum albumin, 1 $\times$ PCR buffer, and 5.0 units of Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) in 50 μ L. A hot-start thermocycling program was used as follows: initial denaturation at 94°C for 5 min; 10 touchdown cycles at 94°C for 30 s, then the annealing temperature decreased from 61°C to 56°C by 0.5°C/cycle, followed by an extension step at 72°C for 30 s; then 25 cycles with denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min; and the program ended with a final 72°C extension for 30 min. The PCR reaction included negative and positive controls. The PCR product was approximately 200 bp, as verified by electrophoresis in a 1% agarose gel. The PCR products were resolved on a 7.5% polyacrylamide gel (37.5:1) with a 40 to 70% denaturing gradient for 1,600 V·h. The gel was stained with SYBR green and visualized with a Gel Logic 1500 imager (Kodak, New Haven, CT). The TIFF images were imported, and cluster analysis was performed as per Karnati et al. (2009b).

Experiment 2: Batch Culture Incubation of Met and HMBi Derivatives

The second experiment apportioned the potential sources of variation associated with HMBi metabolism by ruminal microbes in batch culture, which prevented passage of undegraded HMBi or HMB. Treatments consisted of (1) an unsupplemented control, (2) 0.097% L-Met, (3) 0.097% D-Met, (4) 0.125% DL-HMBi, (5) 0.098% DL-HMB, (6) 0.250% DL-HMBi (2 $\times$ HMBi), (7) 0.049% DL-Met + 0.063% DL-HMBi, and (8) 0.098% DL-HMB + 0.039% isopropanol. The CON, HMBi, and Met + HMBi treatments were designed to be similar to those in experiment 1. With the exception of the control and 2 $\times$ HMBi treatments, the inclusion rate for other treatments was isomolar as a percentage of the diet DM. Inclusion rates for Met + HMBi combined are isomolar with single treatments of Met or Met analogs. The 2 $\times$ HMBi inclusion was chosen assuming 50% escape of HMBi relative to L-Met (i.e., predicted to be isomolar on a metabolized basis as in Martin et al., 2013).

Inoculating Fermenters for Usage as Inoculum in Batch Culture (Experiment 2)

Ruminal fluid was maintained in continuous culture for adaptation and subsequent inoculation in batch

culture to better mimic results from experiment 1 and, second, to wash protozoa out of faunated fermenters to prepare partially defaunated inoculum with minimal effect on the prokaryotic communities. Approximately 2.5 L of rumen fluid were collected from each of 2 Holstein cows (5 L total) and processed as described for experiment 1. The fermenters were fed 40 g/d of a 50:50 mixture of alfalfa and concentrate pellets as described by Karnati et al. (2009a), except that fluid and solids dilution rates were set to 7 and 5%/h, respectively. In the first fermenter, the agitation paddles were set to 50 RPM, and the protozoa were retained using a compound filter that consisted of a 50- μ m filter wrapped in layers of nylon cotton. In the second fermenter, the agitation paddles were set to 160 RPM, and the protozoa were primarily effluxed using a wire mesh filter with a pore size of 300 μ m. These preincubations were repeated the same for the second replication of the batch culture experiment except that an extra day (8 vs. 7 d) was used to preincubate fermenter contents before inoculation of 50-mL batch culture tubes. On the day of the inoculation (corresponding with 0 h of incubation in batch culture), samples of fermenter vessel liquids were taken, fixed in formalin, and counted (Dehority, 1984).

A total of 0.5 g of feed (0.25 g of each crushed alfalfa pellets and crushed concentrate pellets that were used in experiment 1) were weighed into 50-mL centrifuge tubes. On the morning of the inoculation, treatments were dissolved in distilled water and dosed into the appropriate tubes. In addition to the various treatments, all tubes were also dosed with isomolar concentrations of treatments that were based on doses in experiment 1. To all tubes were added 0.33 μ mol of [1- 13 C]-L-Met [98% purity, 99 atom percent excess (APE), Cambridge Isotopes, Andover, MA] and 0.118 mg/tube of 10% enriched (15 NH $_4$) $_2$ SO $_4$ (Isotec Inc., Miamisburg, OH). For comparison purposes, the total dosage of the unlabeled L-Met treatment was predicted to dilute the [1- 13 C]-L-Met to 10.2 APE, whereas the 15 NH $_3$ was diluted by NH $_3$ in the inocula to approximately 0.08 APE. The large difference in APE minimized interference in analysis of 13 C-enriched Met. Fermenter vessels were sampled before inoculation to record the density of protozoa in each inoculum. These steps were repeated in the second replication, which served as a block.

Fermenter liquid from each vessel was mixed with an equal volume of fresh media solution (no additional cysteine was added to media solution), passed through 2 layers of cheesecloth into 2 separate vessels and warmed in a hot water bath to 39°C while bubbling with CO $_2$. Based on results from a pilot study, this dilution was needed to provide enough buffering capacity without over-diluting the microbes. While continually mixing by hand, 30 mL of the proper faunated or defaunated

inoculum was added to the assigned batch culture tubes. The tubes were gassed with CO $_2$, immediately capped with rubber stoppers with one-way valves, and swirled with continuous shaking or swirling. At appropriate times, tubes were uncapped, 1 mL of 6 N HCl added to stop fermentation, and vortexed. Tubes were refrigerated until further processing.

Measurements and Laboratory Analysis (Experiment 2)

Two replicate tubes from each treatment/time point/inocula were processed for 13 C enrichment of bacterial Met. Following low-speed centrifugation (500 $\times$ g at 4°C for 15 min) to remove particulate matter and protozoa, the remaining bacterial pellet was precipitated (20,000 $\times$ g , 4°C, 15 min) and washed 3 times with 0.9% (wt/vol) saline solution, lyophilized, and stored at -10°C. Samples were shipped on ice to the University of Maryland for determination of bacterial 13 C-Met enrichment (Firkins et al., 2015).

Unfortunately, free Met in the supernatant was destroyed more than expected by the addition of 6 N HCl that was added to each tube to halt the fermentation during processing. Therefore, we could not use product-to-precursor 13 C enrichment ratios to calculate bacterial Met originating from preformed Met. Further, the microbial pellet was contaminated by an unknown, but presumably minor, quantity of feed from particulate matter that could not be removed using standard filtering and differential centrifugation techniques. Consequently, we estimated microbial N and Met kinetics using the following approach:

$$\text{total NAN} - \text{feed N} = \text{total microbial N (mg)} \quad [1]$$

$$[1] - \text{blank total NAN} = \text{incremental microbial N (mg)} \quad [2]$$

Feed N was estimated by incubating each feed for 0, 2, 8, and 24 h in situ (the same length of time of incubation in vitro in the current study) and determining the percentage of original feed N remaining at each time point. This percentage was multiplied by the amount of feed N added to the batch cultures to estimate feed N at respective times. The total NAN in the tubes minus the estimated undegraded feed N was used to estimate the amount of microbial N in the tubes in equation [1]. We recognize that [1] has some N from microbial and small particulate matter from the inocula. Small undegraded feed sources are needed for preparation of robust inoculum (Firkins, 1997). Incremental microbial N (equation [2]) was calculated as microbial N at 2, 8,

and 24 h minus the microbial N in blank tubes from the corresponding time points. We note that these calculations subtract microbial N at zero time but also any particulate N from the inocula. Therefore, results from equation [2] are probably more accurate than those from equation [1]; however, results from [2] are less precise because errors accumulated from both samples (substrate added) and blanks (substrate not added). We recognize a greater potential for inaccuracy and higher error introduced in this approach, but resultant sample availability prevented chemical analysis, and microbial AA profile is relatively constant among treatments (Clark et al., 1992) even though N and nucleic acid concentration do vary (Firkins et al., 2006).

Total and incremental microbial (bacteria + protozoa) Met was calculated from equations [1] and [2], respectively, using published estimates. Protozoal and bacterial N (17.7 and 82.3% of microbial N) were taken from a fermenter study maintained similarly to our inocula (Karnati et al., 2009a). The total AA from protozoal and bacterial samples (80.7 and 70.9% of total CP; CP = N $\times$ 6.25) and percentage of Met in the total AA for protozoal and bacterial samples (2.2 and 2.0% of total recovered AA) were averaged from different treatments within 3 published sources (Volden et al., 1999; Korhonen et al., 2002; Jensen et al., 2006) for protozoa and bacteria, respectively. Microbial Met in the incubation tubes could then be calculated using equations [1] for total or [2] for incremental microbial N as

$$[1] \times (\text{protozoal N/microbial N}) \times (\text{protozoal total AA/protozoal N}) \times (\text{protozoal Met/protozoal total AA}) = \text{protozoal Met (mg)}; \quad [3]$$

$$[2] \times (\text{protozoal N/microbial N}) \times (\text{protozoal total AA/protozoal N}) \times (\text{protozoal Met/protozoal total AA}) = \text{incremental protozoal Met (mg)}; \quad [4]$$

$$[1] \times (\text{bacterial N/microbial N}) \times (\text{bacterial total AA/bacterial N}) \times (\text{bacterial Met/bacterial total AA}) = \text{bacterial Met (mg)}; \quad [5]$$

$$[2] \times (\text{bacterial N/microbial N}) \times (\text{bacterial total AA/bacterial N}) \times (\text{bacterial Met/bacterial total AA}) = \text{incremental bacterial Met (mg)}; \quad [6]$$

$$\text{protozoal Met [3] + bacterial Met [5] = total microbial Met (mg)}; \quad [7]$$

incremental protozoal Met [4] + incremental bacterial

$$\text{Met [6] = incremental microbial Met}; \quad [8]$$

$$[7] \times \text{APE of } ^{13}\text{C Met in pellet} = \text{microbial } ^{13}\text{C-Met (mg)}; \quad [9]$$

$$[8] \times \text{APE of } ^{13}\text{C Met in pellet} = \text{incremental microbial } ^{13}\text{C-Met (mg)}; \quad [10]$$

$$[9]/^{13}\text{C-Met dosed (mg)} \times 100 = \text{microbial Met derived from dose (\%)}; \text{ and } [11]$$

$$[10]/^{13}\text{C-Met dosed (mg)} \times 10 = \text{incremental microbial Met derived from dose (\%)}; \quad [12]$$

Statistical Analysis (Experiments 1 and 2)

Data from the first experiment (continuous culture) were analyzed using Proc MIXED of SAS (Cary, NC) for a 4 $\times$ 4 Latin square design. The effect of fermenter was a random effect, and period was considered a fixed effect to allow convergence of the mixed model. The first preplanned orthogonal contrast compared the average of all the Met treatments versus the control. Two additional contrasts were used to determine linear or quadratic response to DL-Met, Met + HMBi, and HMBi.

Data from the second experiment were analyzed as a randomized complete block with 2 $\times$ 8 factorial arrangement of treatments using the Proc Mixed procedure of SAS, according to the following model:

$$Y_{ijkl} = \mu + b_i + I_j + T_k + H_l + IT_{ik} + IH_{jl} + TH_{kl} + ITH_{jkl} + e_{ijkl},$$

where Y_{ijkl} is the dependent, continuous variable; μ is the overall population mean; b_i is the random effect of the i th block (1 df); I_j is the fixed effect of the j th inoculum (1 df); T_k is the fixed effect of the k th treatment (7 df); H_l is the fixed effect of the l th hour (3 df); IT_{jk} is the fixed effect of the j th inoculum $\times$ k th treatment (7 df); IH_{jl} is the fixed effect of the j th inoculum $\times$ l th hour (3 df); TH_{kl} is the fixed effect of the k th treatment $\times$ l th hour (21 df); ITH_{jkl} is the fixed effect of the j th inoculum $\times$ k th treatment $\times$ l th hour (21 df), e_{ijkl} is the residual error, assumed independent and $\sim N(0, \sigma_e^2)$, (63 df).

Trichloroacetic acid-soluble N, peptides, and NH_3 data were analyzed with a covariate so that the dependent variables could be adjusted for the presence of the substances in blank tubes. When interactions were significant, differences among simple means were determined using Fisher's Protected LSD; when interactions were $P > 0.10$, main effect means were compared with a protected LSD.

RESULTS AND DISCUSSION

Experiment 1: Continuous Culture of DL-Met Replaced by DL-HMBi

Nutrient Digestibility. Treatment did not affect ($P > 0.10$) ADF or true OM digestibilities (Table 2). Both NDF ($P = 0.04$) and hemicellulose ($P = 0.01$) digestibilities decreased linearly with increasing HMBi. Methionine and HMB supplementation have yielded inconsistent effects on nutrient digestibilities. Supplementation of HMB at 0.055 and 0.110% in continuous culture had no significant effect on digestibilities of hemicellulose, NDF, or true OM (Noftsker et al., 2003). Vázquez-Añón et al. (2001) noted no changes in true OM, NDF, ADF, or NSC digestibilities associated with HMB, but HMB at 0.20 and 0.77% of DM (which are much higher than our HMBi dosage at 0.11%) increased efficiency of microbial protein synthesis in continuous

culture compared with their control or highest dose (1.43%). Bach and Stern (1999) noted that high availability of Met from protein sources was associated with decreased degradability of some other AA. In our companion report that used $[1-^{13}\text{C}]\text{-L-Met}$ kinetics, Firkins et al. (2015) noted that increasing HMBi apparently decreased Met uptake and degradation. Continuous culture systems such as ours have rapid stirring, which probably effluxes soluble protein faster than in the rumen. The extent of NDF digestibility is decreased (as is OM digestibility), but the variability is increased in continuous culture compared with total tract NDF or OM digestibilities (Hristov et al., 2012). Even removal of urea from the buffer decreased the degraded protein (without any change in diet) and N uptake of dietary N because of N limitation in continuous culture (Griswold et al., 2003). Those authors noted that hemicellulose-degrading bacteria were particularly stimulated by pre-formed AA (8 vs. 11% RDP) when $\text{NH}_3\text{-N}$ was limiting. Thus, HMBi might have decreased availability of AA for hemicellulose-degrading bacteria in our study because of the low amino N provided in the RDP (Table 1). Martin et al. (2013) noted that supplying HMB or HMBi increased abundance of cellulolytic bacteria in the rumen but not indices of NDF digestibility (enzyme activities and in situ disappearance). Thus, we consider it highly unlikely for NDF digestibility to be depressed by HMBi in vivo.

Table 2. Nutrient digestibilities, nitrogen fluxes, and nitrogen partitioning for rumen bacteria in continuous cultures supplemented with DL-Met, HMBi¹, or both (Experiment 1)

Item	Treatment				SEM	Contrast ²		
	Con	Met	Met + HMBi	HMBi		Control vs. all	Linear	Quadratic
Digestibility (%)								
NDF	42.0	46.3	46.0	38.8	2.6	NS ³	0.04	NS
ADF	48.4	46.6	51.8	47.3	3.6	NS	NS	NS
Hemicellulose	28.9	44.4	33.8	20.7	5.0	NS	0.01	NS
True OM	50.4	51.3	50.6	49.2	2.0	NS	NS	NS
Nitrogen flows (g/d)								
Total N	3.10	3.04	3.14	2.93	0.15	NS	NS	NS
$\text{NH}_3\text{-N}$	0.30	0.33	0.35	0.27	0.02	NS	0.06	NS
NAN	2.80	2.71	2.80	2.66	0.15	NS	NS	NS
Bacterial N	1.57	1.62	1.57	1.50	0.08	NS	NS	NS
NANBN ⁴	1.23	1.10	1.22	1.16	0.09	NS	NS	NS
Nitrogen partitioning								
Ammonia N (mg/dL)	6.22	6.80	7.20	5.65	0.37	NS	0.07	0.08
TCA-soluble N (mg/dL)	11.4	12.1	12.3	11.8	0.37	0.06	NS	NS
Peptide N ⁵ (mg/dL)	5.17	5.28	5.14	6.13	0.27	NS	0.04	0.09
Bacterial N derived from $\text{NH}_3\text{-N}$ (%)	86.2	81.8	88.5	89.1	1.80	NS	0.02	NS
Efficiency of bacterial N synthesis ⁶	28.9	32.0	31.7	30.9	1.95	NS	NS	NS

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

²All supplementation (HMBi, Met + HMBi, MET) vs. control, and linear and quadratic responses to the 3 supplements.

³NS = not significant; $P > 0.10$.

⁴NANBN = nonammonia nonbacterial nitrogen = NAN – bacterial N.

⁵Nitrogen soluble in 10% (vol/vol) TCA.

⁶Grams of microbial N produced/kilograms of OM truly digested.

Partitioning of N. The flows of NAN, bacterial N, total N, and nonammonia nonbacterial N in the effluent were not different by treatment (Table 2). Flow of $\text{NH}_3\text{-N}$ tended ($P = 0.06$) to decrease linearly with increasing HMBi replacement of Met, although the quadratic contrast approached significance ($P = 0.11$). The concentration of $\text{NH}_3\text{-N}$ tended to be affected linearly ($P = 0.07$) and quadratically ($P = 0.08$), mostly because HMBi numerically decreased $\text{NH}_3\text{-N}$ concentration compared with DL-Met or Met + HMBi. The $\text{NH}_3\text{-N}$ approached minimal optimum levels of 2 to 5 mg/dL (Hoover, 1986). Methionine supplementation increased $\text{NH}_3\text{-N}$ flow in continuous culture in another study (Bach and Stern, 1999). Ammonia concentration was increased by HMB replacement of DL-Met in continuous culture (Noftsker et al., 2003) but was not affected by increased HMB addition when no Met was substituted (Vázquez-Añón et al., 2001). For the concentration of TCA-soluble N, which includes $\text{NH}_3\text{-N}$ and amino-N (including small peptides and free AA), the control tended ($P = 0.06$) to be less than the average of the other 3 treatments. However, peptide-N (which also contains free AA) increased linearly ($P = 0.04$) and tended to be affected quadratically ($P = 0.09$), this time with HMBi numerically having the highest peptide concentration (opposite of $\text{NH}_3\text{-N}$). The percentage of bacterial N derived from $\text{NH}_3\text{-N}$ increased ($P = 0.02$) as HMBi replaced Met. Thus, HMBi supplementation seems to decrease peptide degradation or decrease preference for preformed amino-N, which is contrary to our original hypothesis. The concentrations of peptide N for all treatments were greater than those reported by Griswold et al. (2003), who used similar procedures and had higher percent of RDP in their diets. In our companion report (Firkins et al., 2015), we provide supportive evidence for accumulation of Met and potentially other AA in the fermenter medium with increasing HMBi inclusion.

Efficiency of bacterial protein synthesis was not affected by treatment (Table 2). Bacterial N flow and the efficiency of microbial protein synthesis were not affected by supplementation of Met (Bach and Stern, 1999) or HMB (Noftsker et al., 2003). In contrast, increasing dosages of HMB quadratically affected bacterial efficiency (Vázquez-Añón et al., 2001), with increasing efficiency at middle HMB doses (higher than typically fed).

VFA Production. Total VFA production was decreased linearly ($P = 0.02$) with increasing HMBi replacement of Met, primarily resulting from decreased acetate ($P = 0.06$) and propionate ($P < 0.01$; Table 3). Propionate also was affected quadratically ($P = 0.05$). Because propionate was affected proportionately greater, the acetate-to-propionate ratio tended to be af-

ected linearly ($P = 0.08$) and quadratically ($P = 0.06$), indicating increasing ratios for both Met + HMBi and for HMBi treatments than for the DL-Met treatment. Butyrate and branched-chain VFA were not affected by treatment. Isobutyrate decreased linearly ($P = 0.05$) with increasing HMBi, although isobutyrate was numerically greater for DL-Met and Met + HMBi than the control treatment. Isovalerate tended ($P = 0.08$) to increase linearly with increasing HMBi. The control was lower ($P = 0.01$) than the average of the other 3 treatments for valerate, which increased linearly ($P < 0.01$) but plateaued (quadratic response, $P = 0.05$) with increasing HMBi substitution for DL-Met. Part of the decreased acetate and propionate can be explained by increasing valerate (condensation of acetate and propionate) with increasing HMBi. Glucose fermentation to valerate consumes 1 net pair of electrons and protons (1 set of reducing equivalents) versus propionate's consumption of 2 sets of reducing equivalents per mole of glucose, whereas acetate is a net producer of 2 sets of reducing equivalents (Russell and Wallace, 1988). In addition, reducing equivalents can be consumed during NH_3 assimilation into AA (e.g., glutamate dehydrogenase or alanine dehydrogenase), integrating VFA profile with the increasing bacterial N being derived from $\text{NH}_3\text{-N}$ (Table 2).

The decrease in total VFA production without a concurrent decrease in OM digestibility indicates a shift in the flux of the carbon skeletons. Balancing of carbon for catabolic and anabolic reactions is well described for anaerobic microbes (Russell and Wallace, 1988). For example, Karnati et al. (2009a) discussed a shift in flux of glucose carbon toward de novo synthesis of FA and diverted from VFA production after defaunating continuous cultures. In another study from our laboratory (Firkins et al., 2015), we provide evidence that increasing HMBi supplementation increasingly accumulated free Met in fermenter fluid, even though DL-Met dosage decreased. Because the free Met was not incorporated into bacterial protein, we surmised that Met degradation decreased (as supported by the increasing peptide concentration in Table 2, which includes free AA) or else more Met and other AA were fermented to products other than VFA (and not measured in the current study). All of these responses can explain decreased VFA production without concomitant decrease in true OM digestibility. The decreased NDF digestibility without a decrease in OM digestibility for increasing HMBi might be a result of a competition by amylolytic bacteria (NSC was not measured but is the missing component of OM) versus hemicellulolytic bacteria for preformed AA in our low-RDP diets (Table 1). We also note that OM digestibility is peculiarly low in continuous culture (Hristov et al., 2012), perhaps because of

Table 3. Volatile FA production per day for rumen bacteria in continuous culture supplemented with DL-Met, HMBi¹, or both (Experiment 1)

Production (mmol/d)	Treatment				SEM	Contrast ²		
	Control	DL-Met	Met + HMBi	HMBi		Control vs. all	Linear	Quadratic
Total VFA	183	183	177	172	7	NS ³	0.02	NS
Acetate	119	118	116	111	5.11	NS	0.06	NS
Propionate	35.7	36.1	32.4	31.9	1.24	0.01	<0.01	0.05
Butyrate	19.9	20.6	18.4	18.5	0.84	NS	NS	NS
Isobutyrate	0.56	0.66	0.66	0.52	0.05	NS	0.05	NS
Isovalerate	3.33	3.37	4.90	4.80	0.57	NS	0.08	NS
Valerate	4.06	3.97	4.81	4.90	0.25	0.01	<0.01	0.05
BCVFA ⁴	3.88	4.02	5.56	5.32	0.60	NS	NS	NS
Acetate:Propionate	3.33	3.28	3.58	3.48	0.09	NS	0.08	0.06

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

²All supplementation (DL-Met, Met + HMBi, HMBi) vs. control.

³NS = not significant; $P > 0.10$.

⁴BCVFA = branched-chain VFA, which includes isobutyrate and isovalerate.

the large amount of buffer infusion to compensate for no VFA absorption.

Increased Met supplementation through protein sources in continuous culture has had no effect on total or individual VFA but increased AA outflow (Bach and Stern, 1999). When comparing HMB, HMBi, and DL-Met in vivo, no differences in VFA concentration were observed (Noftsker et al., 2005), but supplementing HMB in continuous culture increased isobutyrate and decreased propionate production (Noftsker et al., 2003). Continuous culture allows measurement of VFA production, whereas VFA absorption rates could vary among VFA in vivo.

Bacterial Population as Assessed by Denaturing Gradient Gel Electrophoresis Banding. The dendrogram in Figure 1 shows that bands cluster primarily by period, and no relative changes were noted in the abundance of the present populations associated with treatment. These lack of banding patterns contrasts with banding by Met source (including HMB) in the studies of Noftsker et al. (2003) in continuous culture and Karnati et al. (2007) in dairy cows. Although denaturing gradient gel electrophoresis only assesses abundant populations (Karnati et al., 2009b) and would presumably preclude the detection of the low-abundance/high-activity deaminating populations of bacteria, the current results suggest that differences among treatments were a result of shifts in metabolic activity, not major shifts in bacterial population structure.

Experiment 2: Batch Culture of Met and Met Analogs

Protozoal Counts. Samples of inocula collected the morning of batch culture inoculation contained an average of 23,740 protozoal cells/mL in faunated

inocula, which was about 9 times greater than those in the defaunated inocula (average of 2,670 protozoal/mL). The predominant protozoa in the defaunated inoculum were small *Entodinium* species, which are more difficult to remove from continuous culture fermenters but also represent less activity because of their lower biomass compared with the larger protozoa. The highly abundant *Entodinium caudatum* is particularly bacterivorous and proteolytic compared with the larger protozoa, although differences probably are magnified in studies that used protozoal cells that previously had been starved (Diaz et al., 2014).

Enrichment of [1^{13}C]-L-Met in Microbial Protein. There were no interactions ($P > 0.10$) among faunation status and Met treatment or among time; thus, main effect means are presented in Table 4. Methionine enrichment of the pellet and associated calculations documented main effects of inoculum ($P < 0.07$), treatment ($P < 0.07$), and time ($P < 0.05$). Compared with the faunated inoculum, the partially defaunated inoculum (averaged over other effects in the model) increased the proportion of ^{13}C -Met dose incorporated into microbial Met, and this 10% difference increased to 21% for the incremental microbial Met derived from the dose (correcting for blanks). Protozoa can incorporate direct AA, but their preferred source is bacterial protein (Williams and Coleman, 1992). The quadratic responses to time documented peaks at 8 h as microbial protein synthesis increased uptake of preformed L-Met dose followed by decreases at 24 h as recycling of microbial Met diluted the label such that more Met was synthesized de novo (perhaps by microbes growing on the more slowly degraded fiber).

The control treatment had the numerically highest APE and percentage of label inclusion in total microbial Met because there was no other supplemental Met

Figure 1. Comparison of the bacterial community structure from continuous culture samples; dendrogram shows cluster analysis that was performed using the Jaccard method; P = period 1 to 4; HMBi = 2-hydroxy-4-(methylthio) butanoic acid isopropyl ester; CON = control; MET = DL-Met; and HMBi+MET = 50:50 mixture of HMBi and MET. The length of scale depicts the percentage similarity between different lanes.

source to dilute the $[1-^{13}\text{C}]$ -L-Met label that was included in equal doses in all treatments (Table 4). The L-Met treatment decreased ($P < 0.05$) the APE of ^{13}C -Met in the pellet through dilution compared with the control treatment because the unlabeled L-Met provided about 90% of the total dosed L-Met (including both the unlabeled L-Met treatment plus the $[1-^{13}\text{C}]$ -L-Met label). The D-Met treatment was lower ($P < 0.05$) than the control but higher than the L-Met treatment. Thus, the D-Met treatment slowed bacterial incorporation of the $[1-^{13}\text{C}]$ -L-Met dose into bacterial protein while the D-Met was undergoing stereoisomerization, but the lack of treatment by time interaction suggests a residual response (lack of compensation) compared with control. Firkins et al. (2015) discussed how D-Met might accumulate in periplasmic contents and decrease uptake of L-Met into cytoplasm. The L-Met versus D-Met comparison lost significance for incremental microbial Met because of this calculation's higher variability relative to the means.

The HMB and HMBi treatments had no effect on enrichment of bacterial Met (Table 4). That their results more closely resemble those of D-Met than those of L-Met suggests a more similar action between D-Met and the DL-HMB and DL-HMBi analogs compared with L-Met. However, no way of distinguishing the response exists because the precursor free Met could not be recovered (degraded during storage). The HMBi did increase ($P < 0.05$) the APE of bacterial Met compared with HMB, suggesting greater breakdown of HMB into the free Met precursor to dilute the APE of microbial Met compared with HMBi.

Assuming HMB and HMBi were one-half D-stereoisomers (Lobley et al., 2006), those treatments' responses are expected to be halfway between the L-Met and D-Met treatments (Table 4). When DL-Met was added to HMBi (half dosages each to make a combined equimolar dose), the APE was decreased intermediate between the L-Met and D-Met treatments. Numerical interpolation of the APE results suggests that HMB was about

Table 4. Main effect means for ¹³C-L-Met enrichment and transfer to microbial methionine in precipitated residue from batch cultures from 2 inocula administered to 8 Met treatments incubated for 3 time points¹

Item	Inocula ²				Treatment ³				Hour ⁴			
	F	D	SE	C	L-Met	D-Met	DL-HMB	DL-Met + HMBi	DL-Met + HMBi	2× HMBi	HMB + IPProp	SE
Met enrichment in pellet (APE)	1.13	1.19	0.13*	1.31 ^a	0.77 ^d	1.18 ^b	1.03 ^c	1.29 ^a	1.29 ^a	1.26 ^{ab}	1.26 ^{ab}	0.12*
¹³ C-Met in total microbial Met (% of dose)	2.74	3.07	0.14 [#]	3.69 ^a	1.75 ^c	2.77 ^b	2.86 ^b	3.00 ^{ab}	3.30 ^{ab}	2.97 ^b	2.86	0.27*
¹³ C-Met in incremental microbial Met (% of dose)	1.78	2.16	0.14*	2.68 ^a	1.26 ^c	1.85 ^{bc}	1.91 ^{bc}	2.07 ^{ab}	2.16 ^{ab}	1.90 ^{bc}	1.64	0.27 [#]

^{a-c}Means with different superscripts differ by $P \leq 0.05$ (superscript * associated with SE for respective treatments) or by $P \leq 0.07$ if the main effect is $P = 0.07$ (superscript #).
¹No 2- or 3-way interactions ($P > 0.10$) were observed. APE = atom percentage excess. Incremental microbial ¹³C-Met was calculated after subtracting microbial ¹³C-Met from corresponding blank tubes (without substrate added) and calculated as a percentage of the [1-¹³C]-L-Met dose.
²Main effect means of faunated (F) and partially faunated (D) inocula.
³All tubes had equivalent ¹³C-labeled L-Met tracer but had 8 unlabeled treatments: (1) control (C; no methionine source), (2) L-Met, (3) D-Met, (4) DL-HMB [2-hydroxy-4-(methylthio)-butanoic acid], (5) DL-Met + HMBi (isopropyl ester of HMB), (6) HMBi, (7) 2× the dose of HMBi, or (8) HMB + unmethylated isopropanol (IPProp). All methionine treatments were isomolar except for 2× HMBi, which had twice the total amount of methionine equivalent per tube.
⁴Linear and quadratic effects of time, weighted for unequal spacing, are depicted with L ($P < 0.01$) and Q ($P < 0.01$) superscripts.

as inert as D-Met, but less inert than HMBi. Doubling the HMBi had no response, suggesting no saturation effect on [1-¹³C]-L-Met metabolism.

Our hypotheses that HMBi conversion to Met could be offset by including a greater quantity of HMBi or that adding isopropanol would inhibit incorporation of Met into protein were not supported. However, the above responses do support HMB being more extensively degraded in the rumen than HMBi (Noftsker et al., 2005; Martin et al., 2013). Our responses also suggest that D-stereoisomers of Met and Met analogs do decrease the incorporation of preformed L-Met into microbial protein.

CONCLUSIONS

Preformed Met supply (directly or through HMB conversion) appeared to influence the activity of hemicellulose-degrading bacteria in continuous culture. Increased supplementation of HMBi increased the flow of free peptides and AA but decreased the net production of total VFA, indicating that carbon was being used for synthesis of AA rather than using preformed AA. Batch culture dosage of [1-¹³C]-L-Met documented a decreased uptake of the label into bacterial protein, as influenced by supplying D-Met directly or through its precursor, HMB (or HMBi that is partially hydrolyzed to HMB). Isopropanol and doubling the HMBi had little effect on the rate of L-Met incorporation into microbial N, suggesting that differences were a result of HMBi being less degraded than HMB or D-Met.

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