Effects of 2-Hydroxy-4-(methylthio) Butanoic Acid (HMB) on Microbial Growth in Continuous Culture¹

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ABSTRACT

2-Hydroxy-4-(methylthio) butanoic acid (HMB) positively affects milk composition and yield, potentially through ruminal actions. Four continuous culture fermenters were used to determine the optimal concentration of HMB for digestibility of organic matter (OM), neutral detergent fiber (NDF), acid detergent fiber (ADF), and hemicellulose and synthesis of microbial N. A highly degradable mix of hay and grain was used as a basal diet to simulate a typical lactation diet. Three concentrations of HMB (0, 0.055, and 0.110%) and one concentration of dl-Met (0.097%) were infused into the fermenters according to a 4 × 4 Latin square design. Digesta samples were collected during the last 3 d of each of the four 10-d experimental periods. Digestibility of OM, hemicellulose, and NDF was largely insensitive to treatment. Digestibility of ADF showed a quadratic effect to supplementation of HMB, with 0.055% having lower digestibility than 0 or 0.110%. Total production of VFA was not influenced by HMB supplementation, but differences in concentration and production of individual VFA were seen. Isobutyrate increased linearly with increasing HMB supplementation. Propionate concentration decreased linearly with increased HMB supplementation, but propionate production showed a quadratic trend ($P = 0.13$). A higher concentration of acetate was detected for dl-Met compared with the highest HMB concentration. There were trends ($P < 0.15$) for dl-Met to decrease the production of isobutyrate and to lower the concentration of butyrate when compared with HMB. Microbial efficiency was not different among treatments. The proportion of bacterial N produced from $\text{NH}_3\text{-N}$ decreased linearly with increasing HMB, and bacteria receiving dl-Met synthesized more N from $\text{NH}_3\text{-N}$ than those receiving HMB. These data suggest that supplementation of HMB may have a sparing effect

on branched chain volatile fatty acids because the fatty acids are not needed to provide carbon for synthesis of valine, isoleucine and leucine with ammonia. Comparisons of bacterial community structure in the fermenter effluent samples using PCR amplicons containing the ribosomal intergenic spacer region and its flanking partial 16S ribosomal RNA gene showed no distinct banding patterns, though treatments tended to group together. Both Met and HMB affect the rumen microbial population, but Met supplied as dl-Met does not act identically to that supplied as HMB.

(Key words: continuous culture fermenters, 2-Hydroxy-4-(methylthio) butanoic acid, methionine, bacterial community analysis, rumen)

Abbreviation key: BCFVA = isobutyrate, isovalerate, and valerate; HMB = 2-hydroxy-4-(methylthio)-butanoic acid, NANBN = nonammonia-nonbacterial nitrogen, RIS = ribosomal intergenic spacer, rDNA = ribosomal DNA, RIS-LP = RIS-length polymorphism.

INTRODUCTION

Methionine and Lys have been identified as two of the first limiting AA in dairy rations (Schwab, 1992). Lysine is required for maximum milk protein synthesis (King et al., 1991), whereas Met affects both milk fat (Hansen et al., 1991) and milk protein synthesis (Rulquin and Delaby, 1997). Recently there has been a renewed interest in Met supplied as 2-hydroxy-4 (methylthio)-butanoic acid (HMB). Research in the 1960s and 1970s documented increases in milk fat and milk yield (Patton et al., 1970b), whereas others (Wallenius and Whitchurch, 1975) found no changes. 2-Hydroxy-4 (methylthio)-butanoic acid is at least partially rumen degradable (Vazquez-Anon et al., 2001) and will affect the rumen microbial population.

Microbial protein reaching the duodenum represents the largest contribution of protein for ruminant animals (Firkins, 1996), and has an AA profile more similar to that of both milk and lean tissue than that of most protein sources used as animal feeds (NRC, 2001). Peptides and AA have stimulated microbial protein synthesis when substituted for ammonia in vitro (Russell and

Received October 9, 2002.

Accepted February 11, 2003.

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¹Salaries and research support were provided by state and federal funds appropriated by The Ohio Agricultural Research and Development Center, The Ohio State University. Manuscript No. 2-02AS.

Strobel, 1993) and in vivo (Rooke and Armstrong, 1989). *Streptococcus bovis*, an active proteolytic bacterium, spilled less energy as heat when AA were included in the medium, suggesting more efficient use of carbon and energy when AA were provided (Russell and Strobel, 1993). Patterson and Kung (1988) determined that bacteria incorporate Met into cellular material, regardless of precursor (dl-Met, HMB, or HMB esters, salts or amides). Gil et al. (1973a, 1973b) found that supplying HMB in the urea-containing medium greatly enhanced rate of growth of rumen bacteria on glucose or cellulose and was not being used as an energy source. *Prevotella ruminicola* 23, *Butyrivibrio fibrisolvens* and *Selenomonas ruminantium* are stimulated by cysteine (Cotta and Russell, 1982), which can be produced from Met (Stipanuk, 2000). The rumen bacterium *B. fibrisolvens* degrades cellulose, hemicellulose, and protein in the rumen and can make up a large portion of the rumen microorganisms (Van Soest, 1994). *P. ruminicola* is a predominant hemicellolytic organism (Dehority, 1991). If these organisms are provided with supplemental HMB, causing an increase in overall numbers of fibrolytic organisms, fiber digestibility could be improved.

Yu and Mohn (2001) developed a composite method for investigating bacterial community structure in an aerated lagoon. The method is based on analyses of PCR amplicons containing the ribosomal intergenic spacer (RIS) region and its flanking partial 16S rRNA gene. Ribosomal DNA (rDNA) is the region of DNA that encodes the rRNA genes. The 16S-23S ribosomal DNA-RIS region has a highly variable length, and can hence be used as a marker to distinguish different bacterial species.

The hypotheses for this trial were that HMB supplementation would increase microbial growth in continuous culture over an unsupplemented control by either sparing Met precursors for more efficient protein synthesis or by shifting bacterial species. The objective of this study was to quantify the effects on microbial populations supplemented with HMB by measuring flow of N, digestibility of OM, ADF, NDF, and hemicellulose, and VFA production in continuous culture. Changes in the microbial community profile were analyzed by RIS length polymorphisms (RIS-LP).

MATERIALS AND METHODS

Experimental Design

Four continuous culture fermenters were used in this experiment (Hannah et al., 1986, Hoover et al., 1976). The design was a 4 × 4 Latin square with four continuous culture fermenters over four periods of 10 d each. The four experimental treatments (as a percentage of diet DM) were provided through the buffer input: 0%

Table 1. Ingredients and nutrient composition of control diet.

Ingredient	% of DM
Alfalfa hay	50.0
Ground, shelled corn	30.9
Barley	10.8
Soybean meal, 48% CP	4.4
Distillers dried grains	2.7
Trace mineralized salt	0.49
Dicalcium phosphate	0.31
Magnesium sulfate	0.25
Magnesium oxide	0.12
Vitamin A (30,000 IU/g)	0.05
Vitamin D (3000 IU/g)	0.06
Vitamin E (20,000 IU/lb)	0.01
Selenium 90 (90 ppm)	0.02
Nutrients	
NDF ¹	23.4
Forage NDF	15.5
ADF	11.7
CP	16.5
RDP	12.8
RUP	4.9
NE _L (Mcal/kg)	1.54

¹NDF, ADF, and CP are averages of seven assays in our laboratory. RDP, RUP, and NE_L were estimated using the NRC (2001) assuming a BW of 650 kg, 45 kg/d milk, and 25.9 kg/d DMI.

HMB (control), 0.055% HMB, 0.110% HMB, or 0.097% of dl-Met; the latter two provided equivalent moles per day of Met. Alfalfa hay and a grain mix were ground in a Wiley Mill (Arthur H. Thomas, Philadelphia, PA) through a 2-mm mesh screen and dried in a 55°C oven. Diets fed consisted of 50% forage and 50% grain mix and were fed at rates of 100 g DM/d (Table 1). The alfalfa hay was 32.2% NDF, 20.8% ADF, and 20.2% CP. The control diet was formulated using the CPM Model, v.1, (1998) to contain more than the recommended concentration of RDP, so that inadequate degradable protein would not be a factor in the potential response to methionine supplements.

Continuous Culture Operation

The experiment was conducted in four 10-d periods, with each period consisting of 7 d for adaptation followed by 3 d of sample collection. The dual flow continuous culture system used in this study was similar to that described by Hoover et al. (1976) and modified by Hannah et al. (1986). Diets were fed automatically throughout the day to provide steady state conditions. Volumes of the four fermenters were between 1700 and 1800 ml. The liquid and solids dilution rate were maintained at 12% and 5.5%/h, respectively, by regulation of filtrate removal rates and buffer input. The pH was maintained at 6.2 ± 0.1 by automatic administration of 5 N NaOH or 3 N HCl. Agitation was set at 160 rpm, and temperature was maintained at 39°C. The fermenters were continuously purged with N₂ to maintain anaero-

biosis. Ruminal inoculum was obtained from two dry, ruminally fistulated Holstein cows receiving the control diet (no additional Met). Inoculum from both cows was pooled and then divided among the four fermenters. Ruminal contents were squeezed through two layers of cheesecloth and placed in fermenters within 20 min of collection. Acid and alkali use, temperature, filtrate flow, agitation, and pH were recorded and recalibrated, if needed, every 6 h. The solid and liquid effluents were weighed once daily to determine flow rates.

Sample Collection and Analysis

On d 5, 10% enriched ($^{15}\text{NH}_4$) $_2\text{SO}_4$ was added to the fermenters for use as a microbial marker. A sample of effluent was taken prior to the primed, continuous infusion for background ^{15}N analysis. One daily sample of the effluent was taken on d 8, 9, and 10 and composited by fermenter for analysis. All samples were immediately frozen at -20°C . Bacteria were separated from feed particles using a Waring blender and straining through two layers of cheesecloth. Differential centrifugation was used to first separate the bacteria from the feed (15 min at $500 \times g$) and then separate the bacteria from the liquid supernatant (15 min at $23,300 \times g$). Samples remained frozen until analysis. Effluent samples were analyzed for N using the Kjeldahl method and for ADF and NDF (Van Soest, 1991). Samples were acidified using 3 ml of 6 N HCl per 50 ml of sample to stop fermentation prior to analysis for VFA (Firkins et al., 1990) and $\text{NH}_3\text{-N}$ (Chaney and Marbach, 1962). Sub-samples of effluent to be used for ^{15}N analysis were raised to a pH of approximately 9 using 25% NaOH in order to volatilize ammonia from the sample. Bacterial and effluent samples were analyzed for ^{15}N by the Stable Isotope Laboratory (Utah State University, Logan). The analyses were performed by continuous-flow direct combustion and mass spectrometry using a Europa Scientific SL-2020 (PDZ Europa, Cheshire, England) system. Nitrogen-15 in ammonia samples was determined at the University of Illinois (Firkins et al., 1992).

DNA Extraction and PCR. Three samples per period were collected directly from the fermenters and frozen at -80°C . Total genomic DNA was extracted from the thawed composite samples using the bead beating method (Yu and Mohn, 1999) followed by purification of the DNA using a QIAamp column (Qiagen, Valencia, CA). The DNA samples were used in PCR amplification with primers S926f (5'-CTYAAAKGAATTGACGG-3') and L189r (5'-TACTGAGATGYTTMARTTC-3') as described by Yu and Mohn (1999). The resultant PCR products (amplicons) contain the complete RIS and parts of the flanking rDNA, (ca. 600 bp of 16S rDNA and 190 bp of 23S rDNA). The PCR conditions were as

follows: initial denaturation at 94°C for 3 min, annealing at 45°C for 1.5 min, and extension at 72°C for 2.5 min. Subsequent cycles consisted of a 1.5-min denaturation step at 94°C , a 1.5-min annealing step at 45°C , and a 2-min extension step at 72°C . After 30 cycles, there was a final 7-min extension step at 72°C .

Phylogenetic Analysis Based on RIS-LP. Ribosomal DNA-RIS fragments amplified by PCR were separated on a 4% polyacrylamide (37:1) gel, and the gel was stained with GelStar nucleic acid stain (BioWhittaker, Inc., Walkersville, MD). The RIS-LP banding patterns were documented using a FluorChem Imaging System (Alpha Innotech Corporation, San Leandro, CA). The gel image was then exported into the GelCompar II analysis software in the BioNumerics package (BioSystematica, Devon, UK) for cluster analysis and dendrogram construction. The UPGMA method in the software was used for dendrogram construction.

Statistical Analysis

Data were analyzed using Proc GLM of SAS (1999) according to the following model:

$$Y_{ijk} = \mu + T_i + F_j + P_k + e_{ijk}$$

where:

Y_{ijk} is the dependent, continuous variable,

μ is the overall population mean,

T_i is the fixed effect of the i^{th} treatment ($i = 1, 2, 3, 4$),

F_j is the fixed effect of the j^{th} fermenter ($j = 1, 2, 3, 4$),

P_k is the fixed effect of the k^{th} period ($k = 1, 2, 3, 4$), and

e_{ijk} is the residual error, assumed independent and $\sim N(0, \sigma_e^2)$.

Two preplanned contrasts were used to determine linear or quadratic response to 0, 0.055, and 0.110% of HMB, and a third compared the effects of similar concentrations of methionine supplied as dl-Met (0.097%) vs. HMB (0.110%). The Proc GLM procedure of SAS was used for regression analysis of data having significant quadratic effects to estimate peaks or nadirs.

RESULTS AND DISCUSSION

Digestibility of Nutrients

The alfalfa hay was lower in fiber than initially anticipated, causing the overall diet NDF and ADF concentration to be lower than expected. Low fiber concentration

Table 2. Nutrient digestibility, nitrogen fluxes, and ammonia utilization in continuous culture fermenters supplemented with two concentrations of HMB¹ or dl-Met.

	HMB (%)			dl-Met	SEM	Contrasts		
	0	0.055	0.110			Linear ²	Quadratic	HMB vs dl-Met ³
Digestibility (%)								
NDF	45.6	42.9	49.1	43.2	2.69	NS ⁴	NS	0.167
ADF	44.7	40.9	47.8	39.4	1.90	NS	0.061	0.021
Hemicellulose	46.7	45.3	50.8	47.8	4.21	NS	NS	NS
True OM	45.0	41.1	45.1	39.6	3.49	NS	NS	NS
Nitrogen flows								
NH ₃ -N (g/d)	0.24	0.29	0.24	0.21	0.05	NS	NS	NS
NAN (g/d)	2.39	2.54	2.38	2.66	0.15	NS	NS	NS
NAN (% of N intake ⁵)	90.6	96.6	90.2	100.9	5.55	NS	NS	NS
Bacterial N flow (g/d)	1.31	1.39	1.36	1.44	0.070	NS	NS	NS
NANBN (g/d) ⁶	1.08	1.16	1.02	1.22	0.090	NS	NS	0.192
NANBN (% of N intake)	41.0	43.8	38.8	46.2	3.56	NS	NS	0.192
Ammonia-N (mg/dl)	6.04	7.03	6.10	5.13	1.26	NS	NS	NS
Proportion of bacterial N from NH ₃ -N	0.70	0.60	0.62	0.68	0.021	0.032	0.054	0.101
Microbial efficiency ⁷	31.5	37.2	33.6	40.5	4.61	NS	NS	NS

¹HMB = 2-hydroxy-4-(methylthio) butanoic acid.²Linear and quadratic contrasts used 0, 0.055, and 0.110% HMB diets.³0.110% HMB diet vs dl-Met diet.⁴NS = nonsignificant; $P > 0.20$.⁵N intake was intake from feed sources and did not include N from the buffer solution.⁶NANBN = non-ammonia non-bacterial nitrogen.⁷grams of microbial N produced/kilogram OM truly digested.

in the diet should not be an issue with the automatic buffering system used in this experiment. Digestibilities of hemicellulose, NDF, and true OM were not significantly affected by HMB addition (Table 2). Digestibility of ADF was affected quadratically by concentration of HMB. The minimum digestibility, determined by the first derivative of the estimated quadratic function, was at 0.047% of HMB supplementation. Others have reported no changes in digestibility with addition of HMB (Vazquez-Anon et al., 2001; Patterson and Kung, 1988) in vitro, whereas some have noted increased digestibility of crude fiber (Holter et al., 1972) in lactating cows and increased cellulose digestibility in vitro (Gil et al., 1973a).

Digestibility of ADF was decreased for the dl-Met treatment compared with the diet containing the equivalent amount of Met supplied as HMB. Salisbury et al. (1971) found that methanethiol production was four times greater for dl-Met than HMB, indicating that dl-Met may be broken down more quickly and become less effective than HMB with time. Bach and Stern (1999) showed that dl-Met is one of the most degradable AA, with free Met in the rumen contributing less than 2% to total duodenal flow of Met in high yielding cows (Volden et al., 2001). Both dl-Met and HMB were supplied continuously to the fermenters in our trial. This may have synchronized the degradation and uptake

rates, decreasing the benefit of a slower degraded source compared with discontinuous feeding.

Nitrogen Fluxes and Ammonia Utilization

According to the NRC (2001), the control diet would supply 3319 g/d of RDP if fed to a 650-kg lactating cow producing 45 kg of milk per day with a DMI of 25.9 kg/d. The suggested requirement for RDP is 2516 g/d. Thus, our diet had an approximate excess of RDP of 32%, and therefore should not be limiting for microbial growth. Nitrogen fluxes and ammonia utilization are reported in Table 2. Addition of Met as either HMB or dl-Met had no effect on effluent flows of NH₃-N, NAN, bacterial N, nonammonia-nonbacterial nitrogen (NANBN), or NANBN as a percentage of N intake. If Met acts as a growth stimulant to bacteria in a situation in which Met is rate-limiting, an increase in bacterial N flow would be expected. Total RUP flow would either increase or remain the same. Kajikawa et al. (2002) found that Met did not increase microbial growth rate or efficiency when added alone to mixed ruminal bacteria cultured in vitro. However, it was one of seven AA that were stimulatory when supplied together. All AA were provided in isonitrogenous proportions, and potential interactions were not considered. Gil et al. (1973a) found NH₃-N concentrations in vitro were lower with

Table 3. Volatile fatty acid total production and concentration in continuous culture fermenters supplemented with two concentrations of HMB¹ or dl-Met.

	HMB (%)			dl-Met	SEM	Contrasts		HMB vs dl-Met ³
	0	0.055	0.110			Linear ²	Quadratic	
Volatile fatty acids (mol/100 mol)								
Acetate	66.7	66.7	66.9	70.1	0.79	NS ⁴	NS	0.031
Propionate	19.4	19.8	15.9	17.0	0.76	0.017	0.059	NS
Butyrate	13.2	12.5	15.3	12.9	1.10	NS	NS	NS
Valerate	2.26	2.41	2.26	2.23	0.026	NS	0.003	NS
Isovalerate	1.1	1.4	1.4	1.4	0.15	NS	NS	NS
Isobutyrate	0.36	0.38	0.43	0.39	0.016	0.023	NS	NS
Acetate:Propionate	3.57	3.40	4.22	4.26	0.14	0.017	0.028	NS
Total outflow (mmol/d)	343	374	358	329	25.9	NS	NS	NS

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

²Linear and quadratic contrasts used 0, 0.055 and 0.110% HMB diets.

³0.110% HMB diet vs dl-Met diet.

⁴NS = nonsignificant; $P > 0.20$.

Met supplemented as dl-Met or HMB over a control, whereas Bach and Stern (1999) found increased $\text{NH}_3\text{-N}$ and decreased NAN in continuous culture with supplemental Met. Vazquez-Anon et al. (2001) found no effects of HMB on $\text{NH}_3\text{-N}$ or NAN flows in continuous culture. Our results showed no difference in bacterial N flow from HMB supplementation, which is contrary to the findings of Vazquez-Anon (2001) and Gil et al. (1973a), who found increases in microbial CP synthesis with HMB, but results are in agreement with those of Bach and Stern (1999), who found no changes with dl-Met. Patton et al. (1970a) reported an increased flow of lipids that were serving as structural components related to microbial growth. They believed most of the additional lipid was associated with rumen protozoa. Because protozoa rapidly wash out of continuous culture fermenters of the type we used (Mansfield et al., 1995), effects on protozoa could not be determined. This may be why the expected increase in microbial protein has not been found with continuous culture systems (Bach and Stern, 1999) but has been found in *in vitro* systems without outflow such as those used by Patton et al. (1970a) and Gil et al. (1973a). Nitrogen outflows, expressed as a percentage of N intake, and microbial efficiency were not significantly different among the four treatments.

Ammonia-N concentrations were not different among the four treatments. However, there was a significant linear decrease ($P = 0.032$) in the amount of bacterial N obtained from $\text{NH}_3\text{-N}$ with amounts of HMB supplementation. Because of the quadratic effect of HMB concentration ($P = 0.054$), regression analysis was done and indicated that 0.0735% HMB supplementation of the diet would minimize the proportion of bacterial N obtained from $\text{NH}_3\text{-N}$. There was also a trend ($P = 0.10$) for the bacteria receiving dl-Met to obtain a greater

proportion of their N from ammonia than those receiving a similar amount of Met from HMB.

Volatile Fatty Acids

Volatile fatty acid concentration and total production are presented in Table 3. Acetate concentration was not affected by HMB supplementation. Acetate concentration was higher in the dl-Met treatment than with HMB. Propionate decreased linearly in concentration from HMB supplementation but a quadratic trend ($P = 0.13$) was noted for production (data not shown), which was estimated to peak at 0.0470% HMB. These changes were apparent in the acetate:propionate ratio, which showed a significant quadratic effect of HMB concentration. The lowest estimated acetate:propionate ratio would occur at 0.037% HMB. This calculated ratio of 3.35 is similar to that found at 0 and 0.055% when standard errors are considered. This indicates that HMB had basically no effect until more than 0.055% was fed, when a large increase in ratio occurs. Butyrate and isovalerate concentrations were not different. There were trends ($P < 0.15$) for a linear increase in production for isobutyrate and isovalerate (data not shown) in our trial with increasing concentration of HMB, and isobutyrate concentration increased linearly. Valerate concentration was affected quadratically by HMB, with estimated peak production at 0.0554%.

No consistent trends in VFA production have been observed from adding HMB. Acetate, propionate and butyrate concentrations have shown no changes in several studies (Lundquist et al., 1985; Windschitl and Stern, 1988; Vazquez-Anon et al., 2001). Researchers have reported increases (Lundquist et al., 1983) and decreases (Gil et al., 1973b) in acetate concentration

and decreases in propionate concentration (Gil et al., 1973b; Lundquist et al., 1983). Butyrate concentration usually decreased (Gil et al., 1973b) or was not changed (Lundquist et al., 1983). Valerate (Windschitl and Stern, 1988), isobutyrate, and isovalerate (Lundquist et al., 1983; Lundquist et al., 1985) concentrations were unchanged. Volatile fatty acid changes may be dependent on the populations of microbes in the cows. This could vary between experiments due to diet and other factors. Maintenance of pH in our system may also have influenced VFA profile when compared with in vivo data.

Relationship of VFA Metabolism to N Synthesis

Changes in VFA production from HMB supplementation are indicative of an effect on rumen microorganisms. Possible causes for these effects are: 1) microbial populations are changing, 2) different biochemical pathways are becoming more favored, or 3) a combination of both. Three of the predominant cellulolytic rumen bacteria, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *R. albus* require one or more of the branched-chain fatty acids (BCVFA) isobutyric, 2-methylbutyric, or isovaleric acids for the synthesis of valine, isoleucine, and leucine via reductive carboxylation and transamination of the fatty acid (Wallace, 1997). The decreased usage of these VFA for AA synthesis, indicated by increased production of isobutyrate and isovalerate with a decrease in utilization of $\text{NH}_3\text{-N}$ for bacterial N synthesis, could indicate a decrease in the population of microbes normally dependent on BCVFA for production of AA. A limitation of BCVFA growth factors seems unlikely, however, because fiber digestibility was not impaired in our study, and, in fact, ADF digestibility was numerically increased by HMB at 0.110%. It seems more likely that microbes were preferentially using other pathways to synthesize AA when HMB was infused in the fermenter. Met is normally synthesized using a pathway starting with oxaloacetate, an intermediate in the biosynthesis of many AA (Brock et al., 1994). Providing a readily available but slowly degradable source of Met may allow the cell to spare oxaloacetate for production of energy or de novo synthesis of other AA. Increased availability of oxaloacetate would also increase production of succinate, which is an intermediate step in the production of propionate (White, 2000). We observed a quadratic trend in propionate production with an estimated peak at 0.047% HMB in our study. These changes applied to an in vivo system could help to explain some of the effects on milk yield and composition commonly seen with feeding of HMB. Differences in VFA production may increase milk fat production and sparing of oxalo-

acetate could improve AA balance by providing more substrate for production of microbial CP.

RIS-LP Analysis

RIS-LP followed by cluster analysis based on pairwise comparison of the banding patterns obtained from gel electrophoresis of rDNA-RIS amplicons provides a reliable and fast method for comparison of microbial community profiles in environmental samples. Distinct RIS-LP banding patterns were obtained from gel electrophoresis of the PCR amplicons (Figure 1). The banding patterns showed that there were several distinct bands in each sample, including two to three major bands. These major bands represent the species that are the most abundant, possibly *Butyrivibrio fibrisolvens*, *Megasphaera elsdenii*, *Prevotella ruminicola*, and/or *Selenomonas ruminantium*. Cluster analysis showed that the treatments tended to group together, but no distinct pattern was detected because samples taken in the same period tended to cluster together irrespective of the treatment group from which the samples were collected, suggesting that period had a strong influence on bacterial community structure. Because each period was initiated by a new inoculation and the fermentations were controlled for pH and continuous feeding of highly processed feeds, the period effect might have hidden potential treatment effects. Alternatively, the treatments used in this experiment might not have caused a major shift in the community profile. Minor alterations in the community profile might be masked by the presence of large bands generated from species of bacteria that are usually present in large numbers in the inoculum. The use of genera- and species specific primers would allow more specific monitoring of the minor population changes.

CONCLUSIONS

Overall, the supplementation of HMB and dl-Met showed some effects on rumen bacterial growth and production of VFA in continuous culture. The lack of major effects may indicate that overfeeding of RDP from the diet provided enough excess AA so that Met was not limiting, the changes in bacterial populations were small and were concealed by the larger unaffected populations of common species of rumen bacteria, or that there may be a pronounced effect on protozoa in the rumen that would not be seen with continuous culture fermenters.

ACKNOWLEDGMENTS

The authors would like to thank Aventis Animal Nutrition for its funding of this research project.

Figure 1. Comparison of the bacterial community structure in fermenter samples; dendrogram shows cluster analysis performed based on percent similarities of the communities. P = Period 1 to 4; HMB = 2-hydroxy-4-(methylthio) butanoic acid. The length of scale depicts the percent similarity between different lanes.

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