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The Effects of HMTBa (2-hydroxy-4-methylthio-butanoic acid) Supplementation on Ruminal Microbial Crude Protein Synthesis and Community Structure in Dairy Cattle

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THE EFFECTS OF HMTBA (2-HYDROXY-4-METHYLTHIO-BUTANOIC ACID) SUPPLEMENTATION ON RUMINAL MICROBIAL CRUDE PROTEIN SYNTHESIS AND COMMUNITY STRUCTURE IN DAIRY CATTLE

by

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Metabolizable protein (MP) is protein that reaches the small intestine and is available for absorption and utilization by the cow. Dairy rations may be limited in the supply of MP essential to meeting the demands of milk synthesis, however as much as half of the MP flowing to the small intestine may be attributed to microbial origins and is referred to as microbial CP (MCP). Experiment 1 utilized a technique in which DNA was used as a microbial marker to estimate the concentration of bacterial CP (BCP) in the solid and liquid portions of rumen digesta. Rumen digesta was sampled and separated into solid and liquid fractions and microbes were isolated from whole ruminal digesta. Targeting bacterial DNA in samples using real-time PCR, in addition to N analysis, allowed for estimates of the concentration of BCP in the solid and liquid fractions to be attained. The concentration of BCP tended to be higher in the solid portion, highlighting the need to consider both particle and liquid associated bacteria when conducting experiments involving the microbial community. Experiment 2 focused on the ruminal effects of a commercial feed additive when fed with diets low or high in MP. The feed additive, 2-hydroxy-4-methylthio-butanoic acid (HMTBa) molecule (Alimet, Novus Internation, St. Charles, MO), a methionine analog, is believed to result in several positive effects on rumen fermentation, including increased MCP yield. Rumen pH was

decreased in response to the additive, while rumen VFA and ammonia were increased. The MCP yield was unaffected across treatments. Nutrient digestibility was increased in cows fed the diet low in MP. Rumen bacterial DNA was sequenced and analyzed bioinformatically; the proportion of *Fibrobacteres* were increased in cows receiving the additive, and a number of associations of the relative abundance of microorganisms with ruminal observations and treatments were observed.

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"How many are your works, LORD! In wisdom you made them all; the earth is full of your creatures."

Psalm 104:24

"For every animal of the forest is mine, and the cattle on a thousand hills."

Psalm 50:10

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INTRODUCTION

The formulation of dairy diets requires special focus on how protein supplied in various feed ingredients will be utilized in the cow (Cabrita et al., 2011). Because of the role of the microbial community in the rumen in the breakdown and restructuring of protein, actual protein and amino acid availability post-ruminally can be difficult to predict.

Dairy nutritionists refer to protein available for absorption and utilization by the cow as metabolizable protein (MP) (NRC, 2001). The concentration of MP can be manipulated firstly, because the proportion of protein degraded in the rumen varies across feedstuffs. A number of feedstuffs contain protein that is highly undegradable, such as many animal proteins, while others have been specifically designed to be protected from rumen microbial activity. Secondly, microbial crude protein (MCP) contributes to the overall MP available to cow (Korhonen et al., 2002). The MCP contributing to MP consists of protein contained in the microorganisms themselves--this protein becomes available for digestion and absorption when the microorganisms die and leave the rumen.

The concentration of microbial protein can be influenced by supporting favorable ruminal conditions, which involves the supply of nutrients required by the mixed microbial population. The two major nutrients required by rumen microbes are energy and N, which are generally supplied by carbohydrates and proteins, however the most ideal sources and proportions of each have not been clearly determined (Hoover and Stokes, 1991). A variety feed additives have been shown to promote beneficial microbial activity, including 2-hydroxy-4-methylthio-butanoic acid (HMTBa), an analog of the amino acid methionine (Rosser et al., 1971; Bull and Vandersall, 1973; Gil et al., 1973;

Lundquist et al., 1983). The HMTBa (Alimet, Novus International, St. Charles, MO) molecule is thought to have low ruminal escape (Vázquez-Añón et al., 2001) and promote microbial activity, potentially resulting in increased flow of MCP out of the rumen.

Because MCP is not a nutrient that is directly fed to the cow, but is rather derived by microbes in the rumen, it can be difficult to measure and ultimately predict. Several methods have been developed to track MCP leaving the rumen, all of which include the use of a marker. A marker is a substance closely related to or associated with the target nutrient, in that it flows at similar rates through the digestive tract, but is itself undigestible so that it can be measured in sample (Huhtanen et al., 1994).

Recent developments in DNA sequencing and bioinformatic technology can be used to gain further insights into the existence and function of microbes in the rumen. This new availability of information opens the door to a wealth of opportunity in terms of ruminant nutrition, including improved productivity and health (Krause et al., 2014). For example, bioinformatics technology may have the potential to lead to the design of probiotics, which by competitive exclusion of undesirable microbes, could support a healthy “core” microbial community (Tap et al., 2009).

CHAPTER I

REVIEW OF LITERATURE

Protein Digestion in Ruminants

Protein digestion in ruminant animals consists of a complex network of interacting factors that can be difficult to describe and predict. Van Soest et al. (1981) described dietary protein in three different categories; nonprotein N (NPN) ("A" fraction), true protein ("B" fraction), and unavailable N ("C" fraction). Nonprotein N accounts for essentially all of the soluble protein in silages and forages. Once consumed by the animal and entering the rumen, it is rapidly degraded by rumen microbes and converted into ammonia (Sniffen et al., 1992). Subsequently, ammonia is further utilized as the primary source of nitrogen for microbial growth and proliferation (Baldwin and Alison, 1983). In turn, the microbes incorporate this nitrogen into their own amino acids and proteins; eventually they die and their proteins become available to the cow post-ruminally, where it is digested and absorbed. Quantitatively, the microbial population of the rumen provides more than 50 % of the amino acids available for absorption via the small intestine of confined dairy cows (Dijkstra et al., 1998). In addition to microbial protein, proteins capable of escaping microbial degradation in the rumen flow to the small intestine and are available to the cow. Because of this initial division of available protein utilized by microbe and animal, ruminant nutritionists have devised a system of describing dietary proteins relative to their fate in the rumen.

Fractionation of Protein Entering the Rumen. Due to distinct differences in the extent and nature of rumen degradation of proteins, nutritionists characterize them by

using a system of fractionation. Those proteins undergoing ruminal degradation by microorganisms are described as rumen degradable proteins (RDP). Alternatively, proteins escaping ruminal degradation are referred to as rumen undegradable protein (RUP) (NRC, 2001). The characterization of protein into RDP or RUP is largely dependent upon chemical and physical characteristics of the proteins and their amino acids relative to their solubility in various solvents; rumen fluid, water, solutions of sodium chloride, phosphate buffer, detergents, and dilute acids or alkali have been used previously in literature to estimate the solubility of N in feed fed to ruminants (Blethen et al., 1990). For example, some heat treated proteins exhibit altered secondary structural characteristics, namely an increase in α-helix to β-sheet ratios, which has been correlated with total intestinally absorbed protein supply (Doiron et al., 2009). Furthermore, proteins and amino acids have been synthetically protected from rumen degradation by both physical and chemical means. For example, proteins and AA have been physically encapsulated with material which is in itself undegradable in the rumen, or chemically, by supplying an analog of a particular amino acid (Overton et al., 1996).

Another factor affecting the fractionation of protein entering is heavily dependent on rumen kinetics, that is, rate of passage from the rumen. For example, a dairy cow consuming a low forage to concentrate ratio diet will have a rapid rate of passage, resulting in more protein escaping ruminal degradation, thus increasing RUP (NRC, 2001). Conversely, a cow consuming a higher forage to concentrate ratio diet will have a lower rate of passage and consequently, protein will spend more time in the rumen, reducing RUP. Farmer et al. (2014) observed a rumen OM turnover rate of 6.98 % and 8.02 % per hour in cows consuming a 52 % and 39 % forage diet, respectively.

Fractionation of Protein Leaving the Rumen. Protein leaving the rumen eventually flows to the small intestine, where it contributes to MP. As its name implies, MP is assumed to be available for digestion and absorption by the cow. Fractions reaching the small intestine consist of RUP, MCP, and a small contribution of endogenous proteins, around 1 to 7 % of duodenal protein flow (Rulquin et al., 1998).

In the formulation of dairy diets, an adequate supply MP is essential in meeting production goals, as the efficiency of use of MP for lactation has been estimated to be 67 % (NRC, 2001). Many strategies exist for meeting this metabolic demand for MP, which is dramatically elevated during early lactation; recommendations for mature, dry Holstein cows fall between 1000 to 1200 g/d (Block, 2010), while the requirements for lactating cows more than doubles to around 2600 g/d (Lee et al., 2012). Logically, sources of RUP in the diet are increased, for example, by adding animal proteins and dried distillers grains and solubles (Hubbard et al., 2009). Additionally, strategies are implemented in order to increase MCP synthesis, including adequate supplies of RDP and fermentable energy, enhancing ruminal fermentation (NRC, 2001). As sources of RUP may be costly, maximizing microbial efficiency with lower quality, cost-effective inputs is often a prudent strategy implemented by nutritionists, as the AA supplied by microorganisms is similar to that which is required by the cow (Korhonen et al., 2002).

Microbes in the Rumen

The symbiotic relationship between the rumen microbial community and the animal is unique and essential to maximizing digestive efficiency. In terms of ruminant nutrition, the three most prominent categories of rumen microbes are bacteria, protozoa,

and fungi (Martin, 1994); yeasts and viruses are also present in the rumen, however the mechanisms of their effects on nutrition and the microbial community is less understood. The presence of these microbes in the rumen, along with favorable ruminal conditions for microbial life, mutually benefits both microbe and ruminant. Specifically, isoacidic (pH 6-7) and isothermal (39°C) conditions of the rumen, along with a continual supply of water and fermentative substrates, results in an ideal environment for microbial propagation, while microbial fermentation of otherwise indigestible complex carbohydrates release usable nutrients to the animal. Bacteria and protozoa offer substantial contributions to MP as MCP, as high as 89 % of nonammonia N (Shabi et al., 2000), and are further described below.

Bacteria. In terms of their contribution to digestion in ruminant animals, bacteria are perhaps the most influential of the ruminal microorganisms mainly due to their sheer numbers. Legions of microscopic bacterial species measuring 1 to 5 μm inhabit the rumen; it has been estimated that direct counts can be as high as 10^{10} cells per g of ruminal contents (wet basis) (Russell, 2002).

Bacteria play a key role in a number of digestive and fermentative processes in the rumen, and these supply nutrients to the animal which is essential for maintenance and production. Perhaps most notably, bacteria are capable of breaking down cellulose by secretion of cellulase enzymes. Carbohydrate fermentation by ruminal bacteria results in the production of volatile fatty acids (VFA), most notably acetic acid, proponoic acid, and butyric acid. Once absorbed through the rumen wall, VFA are further utilized by the ruminant for energy, and supply approximately 70 % of the animal's energy needs

(Bergman, 1990). In addition to supplying energy, bacteria contribute to a large proportion of the MCP available for digestion and absorption via the small intestine (NRC, 2001). Depending upon on the composition of the diet, over 80 % of MP may be traced to microbial origins, largely composed of bacterial species.

Through direct observation, and in recent years, whole-genome sequencing, pyrosequencing, proteomics, and transcriptomics (Krause et al., 2014) have allowed for myriads of bacterial genera to be identified in the rumen. In fact, a recent meta-analysis of 16s gene sequences in the Ribosomal Database Project (Michigan State University, East Lansing, MI) revealed that 5,271 bacterial operational taxonomic units (OTUs), clusters of similar 16s rRNA sequences, have been identified, representing 19 phyla, of which *Firmicutes* (2,958 OTUs), *Bacteroidetes* (1,610 OTUs), and *Proteobacteria* (226 OTUs) were the most prevalent (Kim et al., 2011). Other major bacterial organisms that have been identified in the rumen include: *Fibrobacter succinogenes, Ruminococcus albus, Ruminococcus flavenciens, Butyrivibrio fibrisolvens, Ruminobacter amylophilus, Selenomonas ruminantium, Prevotella sp., Succinomonas amylolytica, Succinivibrio dextrinosolvens, Eubacterium ruminantium, Magasphaera elsdinii, Lachnospira multiparus, Anaerovibrio lipolytica, Peptostreptococcus anerobious, Clostridium aminophilum, Clostridium sticklandii, Wolinella succinogenes* and *Methanobrevibacter ruminantiun* (Russell, 2002).

Protozoa. Being found in much lesser numbers in the rumen relative to bacteria, the presence of protozoa was estimated to be 10^7 cells per ml of ruminal digesta (Russell, 2002). Despite their relatively low number, they can, at times, account for half of the

ruminal biomass as a result of their large size relative to bacteria, measuring 20 to 200 μm.

Apart from size, the major differentiation between bacteria and protozoa is their eukaryotic nature. In addition, protozoa are grouped under two primary classifications, flagellated or ciliated. Flagellated protozoa are further categorized under five genera namely, *Chilomastix, Monocercomonoides, Monocercomonas, Tetratrichomonas,* and *Pentatrichomonas*. Ciliated protozoa are classified into two broad groups, Holotrichs and Entodiniomorphs, and the division is dependent on ciliary arrangement and presence or absence of skeletal plates (Williams and Coleman, 1992).

The coexistence of protozoa and bacteria in the rumen is an interesting one, resulting in several nutritional advantages to the animal (Firkins, 2012). First, protozoa benefit bacterial fermentation by stabilizing ruminal pH, stemming from consumption of starch granules, which they degrade more slowly than ruminal bacteria. Second, Entodiniomorphids function to stabilize pH by metabolizing lactate and producing butyrate (Brossard et al., 2004). The stabilization of rumen pH benefits the cow nutritionally in that, unregulated, pH levels may slip into acidotic conditions, hindering the function of the rumen. In addition to stabilizing ruminal pH, protozoa may benefit rumen function by promoting fiber degradation via fibrolytic enzymes, in addition to the incorporation of polyunsaturated fatty acids into their membranes (Firkins, 2012). However, some interactions between protozoa and bacteria may yield negative nutritional consequences. First, and possibly most importantly, protozoa may reduce bacterial numbers in the rumen by predation. After consumption of bacteria, protozoa may release up to 50 % of bacterial protein back into rumen fluid, resulting in a loss of what was

potentially protein that could be available post-ruminally to the cow as MCP (Firkins, 2012). Furthermore, protozoa may promote methane emissions; ruminal methanogens sometimes attach to protozoa species, which could suggest hydrogen transfer between species (Johnson and Johnson, 1995). Increased methane emissions may represent a loss of energy that could have otherwise aided production.

Fractionation and Chemical Composition of Rumen Microorganisms. Storm and Ørskov (1983) determined the chemical composition of individual and combined fractions of ruminal microorganisms by proximate analysis as listed in Table 1.1. Isolation of rumen microorganisms from ruminal digesta was achieved by a series of centrifugation steps. Storm and Ørskov (1983) considered rumen fluid that had been centrifuged at 1,200 × *g* for 4 minutes to be free of most protozoa. Next, centrifuging at 19,000 × *g* for 8 minutes, they considered the precipitate to contain the bulk of the microorganisms remaining, namely bacteria. Finally, after centrifugation at 19,500 × *g* for 15 minute, all remaining microorganisms were assumed to be present in the precipitate. This study provided an appropriate baseline for estimates of differing chemical compositions of ruminal microorganisms; however this study was limited in that microorganisms were isolated solely from ruminal fluid.

Several researchers have demonstrated differing compositions in bacteria associated with liquid and solid fractions of ruminal digesta (Craig et al., 1987; Legay-Carmier and Bauchart, 1989; Merry and McAllan, 1983), suggesting that the majority of ruminal bacteria are associated with the solid portion of ruminal digesta, in that they are physically attached to the particles that they are digesting. Consequently, methods have

been developed in attempt to dislodge ruminal bacteria associated with particles before isolation in order to obtain microbial isolates more representative of their existence in the rumen. Cecava et al. (1990) harvested ruminal bacteria by first straining rumen contents through four layers of cheesecloth, from which they would isolate bacteria considered to be associated with fluid. Next, they rinsed the remaining particulate matter with a volume of saline equal to that of the filtrate and blended the mixture in a commercial blender in order to extricate bacteria associated with the particulate matter. The blended mixture was subsequently strained through four layers of cheesecloth and the filtrate was used to isolate bacteria considered to be associated with particles. Analysis of the chemical composition of these bacterial fractions isolated while steers were fed high and low forage diets revealed differences in nitrogen composition as illustrated in Table 1.2. These findings mark the importance of the consideration of liquid and particle associated fractions of ruminal microorganisms in experiments measuring nutritive components of microbial origin.

Effects of HMTBa Supplementation on Ruminal Microorganisms

The 2-hydroxy-4-methylthio-butanoic acid (HMTBa) molecule, an analog (structurally similar compound) of methionine (represented in Figure 1.1), has long been of interest in dairy nutrition for its apparent enhancing effects on microbial protein yield, fiber digestibility, VFA production, and consequently, the potential for improved lactational performance (Rosser et al., 1971; Bull and Vandersall, 1973; Gil et al., 1973; Lundquist et al., 1983; Vázquez-Añón et al., 2001). Specifically, the commercial product Alimet (Novus International, St. Charles, MO) is the focus of the following project and was used

as the source of HMTBa. It has been observed previously that methionine hyrdoxy analogs similar to HMTBa have between 0 and 40 % rumen escape, and are therefore largely utilized in the rumen, apparently stimulating microbial activity resulting in increased microbial protein yield, fiber digestibility, and concentration of VFA (Vázquez-Añón et al., 2001). However, the mechanisms of these effects resulting from ruminal utilization are not completely understood. One reason for this may be the fact that the bulk of previous work has employed in vitro techniques, which do not account for the ruminal absorption of HMTBa, or the ability of the ruminant to recycle nitrogen. Irrespective of these potential limitations, the enhancing effects of HMTBa supplementation on several ruminal digestive properties have been explored in the literature.

Microbial Protein Yield. Studies utilizing in vitro techniques have led researchers to believe that HMTBa also has a positive effect on the amount of microbial protein synthesized in the rumen. Gil et al. (1973) conducted an in vitro fermentation experiment in which mixed populations of ruminal bacteria were exposed to a methionine hydroxy analog (MHA) treatment, with glucose and cellulose used as fermentative substrates, and urea as the source of nitrogen. They concluded that MHA accelerated bacterial nitrogen incorporation and a congruent increase in glucose and cellulose digestion rate was observed. As a consequence, bacterial dry matter and nitrogen yield were more than twice that of the control after 6 hours of fermentation (Table 1.3). More recently, an experiment in which continuous culture fermenters were fed a 50 % grain mixture (containing high moisture shelled corn, corn distiller's grains, cooked soybeans,

wheat middlings, blood meal, fish meal, feather meal, and vitamin and mineral premix) and 50 % forage diet which included four concentrations of HMTBa (0, 0.20, 0.77, and 1.43 % DM basis) added twice daily, bacterial protein synthesis and efficiency were increased when 0.20 and 0.77 % HMTBa were added (Vázquez-Añón et al., 2001). Interestingly, supplementing HMTBa at 1.43 % resulted in a negative effect on bacterial protein synthesis and efficiency, suggesting over supplementation of methionine analog may result in adverse effects relative to microbial productivity. Nevertheless, the results of these studies suggest utilization of HMTBa by ruminal microorganisms may increase rumen microbial protein yield.

Protozoa Number. Potentially related to microbial efficiency and synthesis, it has been observed that supplemental HMTBa may result in an increase in ruminal protozoa numbers in the rumen. As previously discussed, there are several advantages and disadvantages related to the presence ruminal protozoa; the advent of increased protozoa may have positive or negative effects on the efficiency of microbial fermentation, heavily dependent on diet type and subsequent ruminal environments, for example, the stabilization of rumen pH (Brossard et al., 2004).

In order to test if the methionine analog would increase protozoal numbers within the rumen, researchers fed three groups of four whether lambs either a grain based diet, grain plus hay, or grain plus MHA at a rate of 11 g/kg of the total ration in pelletized form (Patton et al., 1970). After three weeks of adaptation, rumen samples were collected via stomach tube twice a week for three successive weeks. The number of protozoa in rumen samples was attained by staining and direct counts. The researchers

observed an increase in protozoa concentration in grain diet containing MHA in comparison to the grain only diet; protozoa concentrations increased from 1.19 protozoa/mL $\times 10^5$ in the grain only diet to 22.8 protozoa/mL $\times 10^5$ in the grain plus MHA diet. Similarly, when the trial was repeated, there were 0.86 protozoa/mL $\times 10^5$ observed in the grain only diet and 50.0 protozoa/mL $\times 10^5$ in the grain plus MHA diet. In both trials, protozoal counts were slightly higher in the grain plus hay diets than in the grain plus MHA diets. The results are listed in Table 1.4. These results were expected, as grain based diets tend to reduce protozoal numbers (Owens et al., 1998), sometimes resulting in complete defaunation, while the inclusion of higher proportion of forage promotes protozoal competence. In this experiment, supplemental MHA seemed to adequately restore protozoal concentrations in a grain based diet.

Fiber Digestibility. In addition to increasing microbial protein yield in vitro, it has been observed that HMTBa may stimulate ruminal microorganisms to increase fiber digestibility. Bull and Vandersall (1973) observed an increase in the in vitro digestion of cellulose from 48.5 % to above 82.0 % with the addition of methionine hydroxy analog at 0.08 % of substrate dry matter. Exploring these observations, a more recent study was conducted in which HMTBa was fed to eight ruminally cannulated cows at 0.10 % of the diet dry matter. With the inclusion of HMTBa verses the control, the researchers observed an increase in apparent ruminal digestibility of organic matter from 43.8 % to 51.6 %, as well as an increase in the ruminal digestibility of NDF from 37.2 % to 40.7 % (Noftsger et al., 2005). These data support the previous in vitro work, suggesting ruminal

digestion is altered by the inclusion of HMTBa, perhaps as a result of stimulatory effects on the rumen microbiome (the ecological community of microbes inhabiting the rumen).

Volatile Fatty Acids. A product of microbial fermentation and an essential source of energy to the cow, VFA play a central role in ruminal digestion and utilization of substrates. Several studies have indicated that supplemental HMTBa may alter ratios of ruminal concentration of VFA.

In order to test the effects of HMTBa on concentration of VFA, Rosser et al. (1971) included 40 g of methionine analog per day in diets fed to 24 Holstein cows in early to midlactation. Rumen digesta samples were collected via stomach pump at the end of each period and analyzed for VFA concentrations. An increase in the concentration of butyrate was observed in comparison to the control, accounting for 15.3 % of total VFAs in the experimental treatment and 11.8 % in the control diets.

In accordance with these observations, two experiments were conducted involving 59 and 63 lactating Holstein cows, respectively (Lundquist et al., 1983). The cows were fed either 40 % or 60 % concentrate diets with or without the inclusion of methionine analog at a rate of 0.25 % of the diet dry matter. On weeks 14 and 16 of each trial, samples of rumen fluid were taken via stomach tube from all cows and analyzed for VFAs by gas chromatography. While ruminal VFA concentrations remained similar between control and methionine analog cows on the 40 % concentrate diet, VFA concentrations seemed to be altered in cows receiving the 60 % concentrate diet; methionine analog increased acetate and decreased propionate. Similar to Rosser and others' observations, rumen butyrate was lower for cows on the 60 % concentrate control

diet than those on the 40 % diet including methionine analog. These data suggest that the inclusion of methionine analog may increase the ratio of acetate to propionate in the rumen as well as butyrate concentrations.

Supplementation of HMTBa in rations fed to dairy cattle is believed to improve cow productivity resulting from enhanced microbial activity, measured by the rumen microbial digestive properties described above. However, past experiments have yielded mixed results (Table 1.5), and a clear mechanism of these effects has not been described.

Estimation of Rumen Microbial Protein Production and Utilization

For microbial protein generated in the rumen to be utilized by the cow, it must flow out of the rumen and to the small intestine for digestion and absorption. Inherent of the kinetic nature of this process, estimation of MCP in the rumen alone is not necessarily representative of protein available for utilization by productive mechanisms, as MCP must first reach the small intestine. As a result, several techniques have been developed which attempt to quantify the flow of microbial protein out of the rumen by the combined use of digesta flow markers along with markers of nitrogen of microbial origin.

The basic process of the estimation of microbial protein available post-ruminally is as follows (Zinn and Owens, 1986; Aharoni and Tagari, 1991; Hristov et al., 2005; Castillo-Lopez et al., 2014;): 1) digesta flow to the small intestine is estimated via the appearance of an indigestible marker (such as Cr_2O_3, TiO_2, indigestible ADF) in duodenal digesta samples; 2) total nitrogen is estimated in duodenal digesta samples and is multiplied by the estimate of total daily duodenal digesta flow in order to attain an approximation of total daily nitrogen flow; and 3) markers indicating nitrogen assumed to

have originated from microbes (such as purines, diaminopimelic acid (DAPA), or DNA) are measured in duodenal digesta samples and is multiplied by the daily digesta flow rate to attain an estimate of daily microbial nitrogen flow, which can be expressed on a percentage of total daily nitrogen flow to the small intestine.

Digesta Flow Markers. In order to estimate post-ruminal availability of microbial nitrogen, the amount of digesta flowing daily to the small intestine must first be measured. Commonly, digesta flow is estimated by measuring the concentration of indigestible markers in samples, given that the daily dosage of the marker is known.

Several external markers have been used extensively in the literature. Utilized as a digesta flow marker for many years (Waller et al., 1980; Firkins et al., 1986; Christiansen and Webb, 1990), chromic oxide (Cr_2O_3) is an inorganic compound employed as an external marker by virtue of its apparent indigestibility. Chromic oxide has been used less frequently in recent years due to concerns of its carcinogenicity and has been replaced by the similar use of titanium dioxide (TiO_2). Apart from fewer concerns regarding handling relative to chromic oxide, titanium dioxide may also be advantageous for use as a maker as it can be legally added directly to diets, while chromic oxide cannot (Titgemeyer et al., 2001).

In addition to external makers, internal markers have been utilized as digesta flow markers, including acid detergent insoluble nitrogen (ADIN), acid insoluble ash, lignin, and indigestible ADF (Cochran et al., 1986; Sunvold and Cochran, 1991). Similar to the external markers discussed, these nutritive entities are selected for use as markers due to their apparent indigestibility in the animal.

Unfortunately, a perfect marker of digesta flow does not exist, as there are several limitations associated with digestion and absorption. Consequently, a number of assumptions must be made regarding digesta flow markers. First, it must be assumed that there is no absorption of the marker from the digestive tract. Second, it is assumed that the marker is not affected by the digestive tract or microbial populations. Third, it must be assumed that the marker flows parallel with, and is physically similar to and associated with the material it is intended to mark (Owens and Hanson, 1991).

Microbial Markers. After digesta flow has been estimated as described above, the total amount of microbial protein flowing to the small intestine daily can be estimated by use of microbial markers. As the name implies, microbial markers are designed to determine the proportion of nitrogen in a sample derived from microbial origins. Several methods have been developed including use of (DAPA), nitrogen-15, purines, and DNA (Castillo-Lopez et al., 2010). Dehority (1995) suggested that the ideal microbial marker should, not be present in the feed, not be absorbed, be biologically stable, have a relatively simple assay procedure, occur in a similar percentage between the various types of microbes, be a constant percentage of the microbial cell in all stages of growth, and all forms should flow at a similar rate. Unfortunately, similar to digesta flow markers, a perfect microbial marker does not exist and these assumptions must be borne in mind when designing experiments.

Purines. Developed by Zinn and Owens (1986), the use of purines as microbial markers for net ruminal protein synthesis has been extensively utilized and is perhaps the

most commonly used microbial marker. The procedure takes advantage of the fact that bacteria are inherently high in nucleic acids, including the purine bases adenine and guanine, while feeds are typically low in adenine and guanine. Subsequently, the digesta being analyzed is hydrolyzed with perchloric acid, followed by the precipitation of the purines with silver nitrate ($AgNO_3$). The isolated purine bases are then measured via spectrophotometry or high-performance liquid chromatography (HPLC). Net microbial protein synthesis is estimated by first establishing a ratio of purine to nitrogen in isolated ruminal microbial pellets, which then allows for an estimate of microbial nitrogen to be calculated based on the amount of purine quantified in samples of duodenal digesta collected over the period of a day.

Several assumptions must be made with the use of purines as microbial markers, giving rise to limitations with this method. First, it is assumed that purines originating from the feed are completely degraded in the rumen, as dietary purines escaping ruminal degradation would lead to overestimation of microbial purines. Second, it must be assumed that the purine to nitrogen ratio is held constant among microbial communities and in digesta samples from which they are isolated; in reality, this ratio varies with bacterial growth rate and digesta components, as purines tend to be higher in the liquid fraction. Third, purines in the duodenum are assumed to be purely microbial—Zinn et al. (1986) suggested that sloughed epithelial cells and feed particles might also contribute to purines present in the digesta. Belanche et al. (2011) observed greater purine concentrations in the duodenum (54.5 mmol/g) than in the abomasum (26.7 mmol/g) of lambs, which they attributed to sloughed epithelial cells.

DNA for Microbial Markers. A recently developed approach, real-time polymerase chain reaction (PCR) may be used for the detection and quantification of microbial populations (Castillo-Lopez et al., 2010; Yu et al., 2005) by use of a fluorescence probe in addition to two primers. As real-time PCR can be used to target a group of microorganisms (Nadkarni et al., 2002), the technology can be used as a marker for microbial protein, and unlike purines, can differentiate between proteins generated from bacteria or protozoa by targeting genes unique to either population.

The PCR reaction involves the "unwinding" of each strand of the parent DNA, which is used as a template to produce a complementary daughter strand. The synthesis of DNA by DNA polymerase is primed by a short DNA sequence which is complementary to the template sequence that is being targeted. The process of unwinding and replicating the DNA strand is dependent on temperature cycling, occurring in three basic steps: denaturation, annealing, and extension. First, at high temperatures (typically around 94-95°C), denaturing of the strands of the DNA template occurs. Second, the temperature is lowered specific to the primers being used in order for them to anneal to the template strand (55-72°C dependent on the types of primers). Third, the temperature is raised to around 72°C, at which DNA polymerase activity occurs. Temperature cycles are repeated a handful of times (typically around 25 to 40) and the DNA is exponentially replicated as a result (McPherson and Møller, 2006). As the name implies, the difference between real-time PCR and PCR alone is that real-time PCR allows for the monitoring and visualization of DNA amplification in real-time.

Several technologies exist which emit a fluorescent signal during the real-time PCR reaction, allowing for the amplification of DNA to be quantified as the reaction

progresses. The fluorescent signal associated with each technology increase their signaling proportional to the exponential increase in DNA products. Three basic approaches are used in order for signaling to occur: One, free dye is incorporated into the newly formed, double-stranded DNA; two, dye-primer based signaling; and three, fluorescently labeled oligonucleotide probes between two primers (Dorak, 2006). One commercially available fluorescent signaling technology, TaqMan (Applied Biosystems, Foster City, CA), involves a fluorescently labeled probe as well as forward and reverse primers represented in Figure 1.2. While the TaqMan probe is free in solution, the 6-carboxy fluorescein molecule (FAM), the reporter dye at the 5' end of the probe, is quenched by the 6-carboxy-tetra-methyl-rhodamine (TAMRA) molecule, which is located at the 3' end of the probe. In the annealing phase of the PCR reaction, the probe binds to the complementary template strand. While primer extension is occurring via Taq DNA polymerase, the probe is released from the 5' end by a nuclease contained in the Taq DNA polymerase. When the probe is released, it is no longer quenched and the signal can be detected by the real-time PCR instrumentation.

In order to estimate microbial protein in digesta samples using DNA as a marker, forward and reverse primers, as well as a fluorescence probe, are designed specific to a gene common to the microbial population of interest. Ruminal microorganisms are isolated and their DNA is extracted. The DNA isolated from ruminal microbes is then exposed to the real-time PCR procedure; the cycle threshold values attained are used to calculate the relative abundance of target DNA in the sample according to the equation derived by Castillo-Lopez et al. (2010). The isolated microbial samples are then analyzed for protein content in order for a ratio of relative abundance of DNA to protein

to be established. Finally, DNA is isolated from samples of duodenal digesta and is exposed to the real-time PCR procedure using the same primers and probe. The relative abundance of target DNA in digesta samples is then calculated, and the previously established ratio of abundance of DNA to nitrogen in microbial isolates is used in order to estimate nitrogen in the samples which originated from the target rumen microorganisms.

Compared with purines, DNA markers yield consistently lower estimates of intestinal microbial protein, potentially due to the contribution of purines from feed and sloughed epithelial cells (Zinn and Owens, 1986; Belanche et al., 2011). However, differences in intestinal microbial protein observed as a result of varying dietary experimental treatments are reflected similarly when both purines and DNA are used as makers, validating the use of DNA as a microbial marker (Castillo-Lopez et al., 2014).

DNA Sequencing

The advent of the use of high throughput sequencing technology on the ruminal microbial community offers new insight into the structure of the rumen microbiome relative to varying nutritional factors. Sequencing technology offers a plethora of exciting new opportunities when utilized as an approach to determine optimal rumen environments to support and sustain different functions of the microbiome. Krause et al. (2014) suggested that the manipulation of ruminal fermentation and microbial populations via ionophores, antimicrobials, colicins and bacteriocins, and diet change can now be examined in detail with this technology in order to better understand the impacts they may have on the rumen microbiome, as well as how they can be replicated to

enhance productive efficiency. Several technologies currently exist which allow for high throughput DNA sequencing.

Pyrosequencing. Based on real-time monitoring of DNA synthesis, the technique known as pyrosequencing utilizes four-enzyme DNA sequencing technology by monitoring DNA synthesis via bioluminescence. Nucleotides are added sequentially to a primed template while the sequence of nucleotides being incorporated into the growing DNA chain are traced and recorded (Ronaghi, 1998).

The 4 enzymes used in the reaction are the Klenow fragment of DNA polymerase I, ATP sulfurylase, Luciferase, and Apyrase (Ahmadian et al., 2006). The steps of pyrosequencing are outlined by Ahmadian et al., (2006): Firstly, DNA polymerization occurs if the added nucleotide forms a base pair with the sequencing template, leading to the incorporation of the growing strand of DNA; Secondly, inorganic pyrophosphate (PPi) is released by the Klenow DNA polymerase and becomes a substrate for ATP Sulfurylase, producing ATP; Thirdly, ATP is converted to light by Luciferase which is detected by the sequencing instrumentation. Apyrase removes nucleotides and ATP which were not incorporated between additions of different bases; this reaction insures that light is only produced when the correct nucleotide is added.

Semiconductor-based Sequencing. A recently developed sequencing technology, semiconductor-based sequencing involves the detecting of protons that are released while nucleotides are incorporated during synthesis (Rothberg et al., 2011).

One such platform utilizing this technology is known as the Ion Torrent Personal Genome Machine (Life Technologies, Carlsbad, CA). Within the Ion Torrent Personal Genome Machine, semiconductor-based sequencing begins on the surface of 3-micron diameter beads called Ion Sphere Particles, where fragments of DNA with specific adapter sequences are linked and amplified by emulsion PCR (Quail et al., 2012). Next, beads containing the amplified DNA are loaded into wells situated above complementary metal-oxide semiconductor pH-sensitive field effect transistors (Merriman and Rothberg, 2012) and sequencing is primed based on the location of the adapter sequence. At this point, the DNA fragments are single stranded, and in the presences of polymerase, sequencing begins as each of the four bases (A, C, G, T) are introduced sequentially. If a base is incorporated into the strand, H ions are released as a result of polymerase activity. As a result, the pH of the solution is altered and can be detected by the pH sensors. Conversely, if the next base in the sequence is not incorporated, little or no change in pH is detected.

Semiconductor-based sequencing platforms such as the Ion Torrent Personal Genome Machine, sometimes referred to as “next-generation sequencers”, offer the potential for affordably reducing workloads and the rapid acquisition of genomic data (Ulrich et al., 2012). The use of next-generation sequencers for the evaluation of the microbial community in the rumen is an emerging field which will contribute to advancements in our understanding of microbial ecology in the context of the rumen.

SUMMARY

One of the most important considerations when formulating diets to enhance lactation performance in dairy cows is the supply of MP, as the efficiency of its use for lactation is around 67 % (NRC, 2001). Contributions to MP include RUP, MCP, and endogenous proteins, of which MCP typically represents the largest fraction. Of protein originating from microbial populations, bacteria contribute the majority post-ruminally and play perhaps the most important role in the digestion of fermentative substrates provided in the diet. The HMTBa molecule has been utilized as a supplement in dairy diets in order to improve microbial efficiency, and consequently, improve lactation performance. In several experiments, microbial protein yield, as well as protozoal concentration, has increased in the presence of MHA. Increased digestibility of cellulose, NDF, and organic matter have also been observed in vitro as well as in vivo. Additionally, MHA supplementation appears to increase acetate to propionate ratios, as well as butyrate concentration, in ruminal fluid. Few studies have been conducted in vivo with regards to MCP synthesis resulting from HMTBa supplementation; an in vivo approach is warranted to account for nitrogen recycling and potential ruminal absorption of the molecule. The use of purines and DNA as microbial markers, as well as DNA sequencing and bioinformatics analysis, will yield further insight into the effects of HMTBa on the efficiency and community structure in the rumen.

General Objectives

The general objectives of this research were to measure several in vivo effects resulting from HMTBa supplementation in diets that were formulated to be either high or

low in metabolizable protein. The factors of interest were; 1) intake and digestibility; 2) lactation performance; 3) ruminal pH, VFAs, and ammonia; 4) MCP flow to the duodenum; and 5) microbial community structure.

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TABLES AND FIGURES

Table 1.1 Mean chemical compositions with their pooled standard errors of individual and combined fractions of rumen microorganisms (Storm et al., 1983)

Centrifugal fraction	1[a]	2[b]	3[c]	2+3	SEM
Proximal analysis (g/kg DM)					
Crude fiber	17.1	9.40	4.20	5.80	1.93
Moisture	76.2	62.0	49.5	55.9	2.33
Ash	143	116	98.3	104	4.02
Lipid	---	---	---	92.1	6.65
Carbohydrate	---	---	---	93.2	7.05
Nitrogen	84.4	99.7	103	102	1.59
Composition of N (g/kg N)					
RNA-N	96.4	112	114	113	7.45
DNA-N	33.4	40.6	42.9	41.3	9.20
Amino acid-N	799	810	806	808	5.06

[a]Supernatant centrifuged at 1200 x g for 4 minutes considered to be free of most protozoa and most of the remaining dietary matter.
[b]Supernatant re-centrifuged at 19000 x g for 8 minutes considered to contain the bulk of the microorganisms.
[c]Supernatant re-centrifuged at 19500 x g for 15 minutes considered to harvest virtually all remaining microorganisms.

Table 1.2 Composition of ruminal bacteria isolated from steers fed two energy levels (Cecava et al., 1990)

	Energy level,[1] Mcal ME/kg DM		
Item	2.24 (HF)	2.29 (LF)	SEM
Organic matter, mg/g DM			
Mixed bacteria[2]	822	842	
FAB[3]	815	845	8.80
PAB[4]	825	845	
Nitrogen, mg/g OM			
Mixed bacteria	97.9[a]	94.7[a]	
FAB	101[a]	97.8[a]	1.40
PAB	93.5[a]	93.6[a]	
Nitrogen:purines			
Mixed bacteria	0.77[a]	0.77[a]	
FAB	0.64[b]	0.64[b]	0.02
PAB	0.72[c]	0.75[a]	

[a-c]Means in the same column, and within the same item, that do not have a common superscript differ ($P < 0.05$).
[1]HF = High forage; ME = Metabolizable energy.
[2]Mixed bacteria = Bacterial isolate prepared from fresh, homogenized ruminal contents; number of observations per isolate fraction was 64.
[3]FAB = fluid-associated bacteria.
[4]PAB = particle-associated bacteria.

Table 1.3 Effect of methionine hydroxy analog (MHA) (8 mg/ml) on bacterial protein nitrogen (N), bacterial dry matter (DM), and production percent glucose disappearance (% GLU. Dis.) measured at 0, 3, 6, and 13.5 hours of fermentation (Gil et al., 1973)

Item	Fermentation time, hours	Control[1]	MHA[1]
DM, mg[2]	0	62.4	56.0
N, mg[2]	0	2.32	1.92
% GLU. Dis.	0	0.00	0.00
DM, mg	3	54.4	88.8**
N, mg	3	3.44	3.44
% GLU. Dis.	3	13.0	11.0
DM, mg	6	157	398**
N, mg	6	6.08	14.6**
% GLU. Dis.	6	44.0	100**
DM, mg	13.5	272.8	271
N, mg	13.5	9.52	8.40
% GLU. Dis.	13.5	100	100

[1]Averages of four determinations.

[2]Milligrams per 160 ml of medium.

**$P < 0.01$, value different from control.

Table 1.4 Average concentration of protozoa in rumen fluid of lambs fed grain with or without methionine hydroxy analog and orchard grass hay (Patton et al., 1970)

Ration	Protozoa/ml x 10^{5a}
Trial 1	
A, grain[1]	1.19[a]
B, grain + MHA	22.8[a]
C, grain + hay	37.5
Trial 2	
A, grain[2]	0.86
B, grain + MHA	50.0
C, grain + hay	68.9

[1]74.0 % ground shelled corn, 13.4 % dehydrated alfalfa meal, 5.0 % ground oats, 5.0 % molasses, 1.0 % urea, 0.8 % dicalcium phosphate, 0.8 % trace-mineral salt.

[2]64.3 % ground shelled corn, 10.0 % dehydrated alfalfa meal, 10.0 % ground oats, 7.9 % soybean oil meal, 3.2 % beat pulp, 3.0 % molasses, 0.8 % dicalcium phosphate, 0.8 % trace-mineral salt.

[a]The difference between each ration and each other ration in a trial is significant $P < 0.01$, except B vs. C in Trial 1 where $P < 0.05$.

Table 1.5 The effects of methionine analog supplementation on rumen digestive conditions; the '+' represents a positivie effect, '-' a negative effect, and '■' no effect

	Rumen Microbes		Digestibility		VFA			Rumen Measurements		
Study	MCP Yield	Protozoa Count	Fiber	Starch	Acetate	Propionate	Butyrate	Ammonia	pH	Pool
Patton et al., 1970[1]		+								
Rosser et al., 1971[2]					+	-	+			
Gil et al., 1973[3]	+		+	+				-		
Bull and Vandersall, 1973[2]			+							
Lundquist et al., 1983[2]					+	-	■			
Vázquez-Añón et al., 2001[3]	+		■		■	■	■	-	■	
Noftsger et al., 2005[2]	■	■	+	■	■	■	■	■	■	■

[1]Experiment was conducted in vivo (ovine).
[2]Experiment was conducted in vivo (bovine).
[3]Experiment was conducted in vitro.

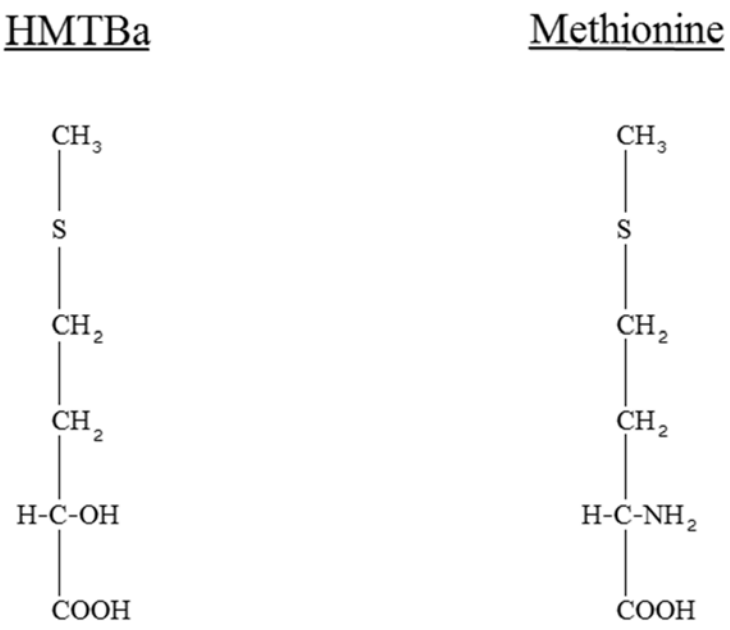

Figure 1.1 The structure of 2-hydroxy-4-methylthio-butanoic acid (HMTBa) (left), or methionine analog, versus the structure of methionine (right)

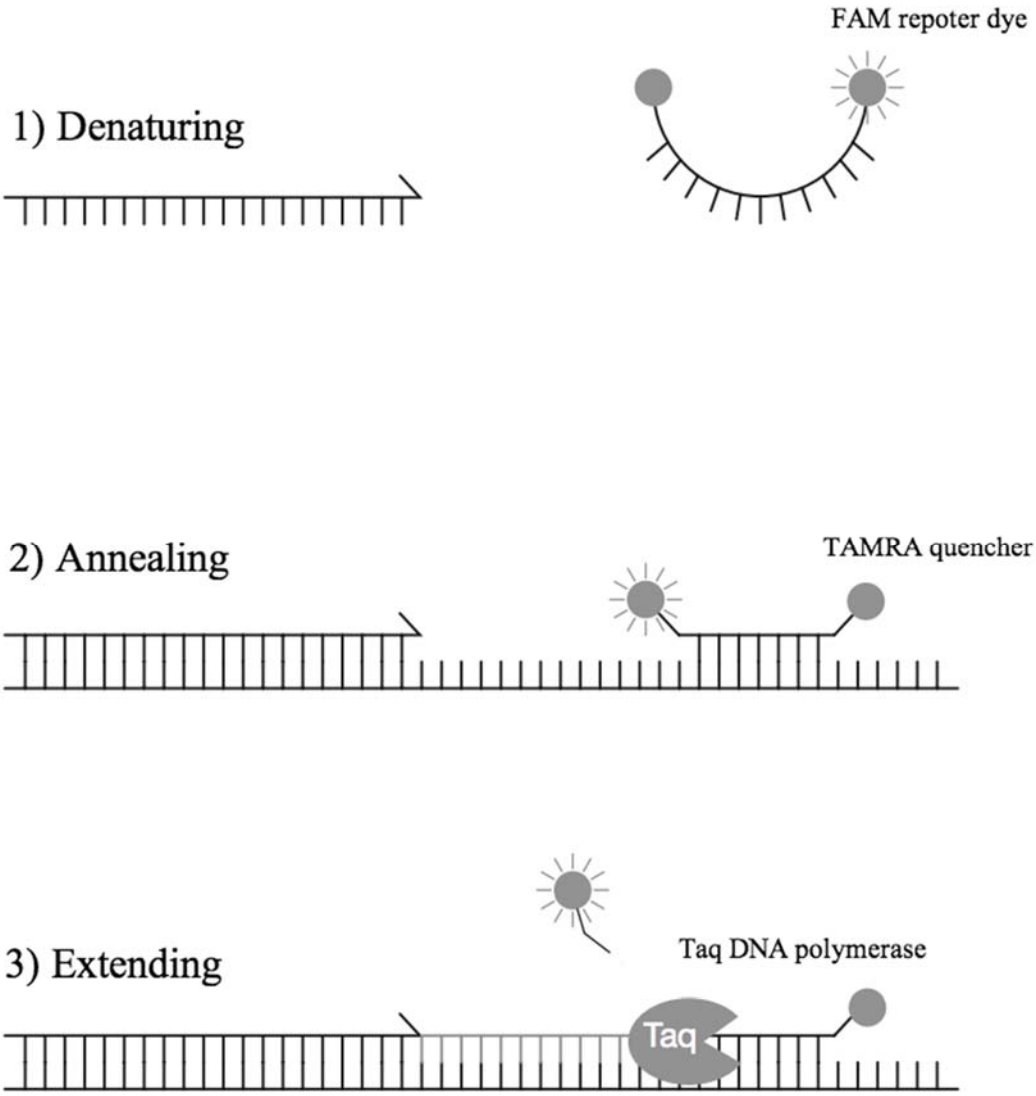

Figure 1.2 Fluorescent signal generation via the TaqMan probe, adapted from Dorak, 2006

CHAPTER II

Short Communication: Estimation of bacterial protein in rumen digesta using DNA markers

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INTERPRETIVE SUMMARY

The experiment evaluated the difference in bacterial crude protein concentration between the solid and liquid portions of rumen digesta using DNA as a bacterial marker. The concentration of bacterial crude protein tended to be higher in the solid portion of rumen digesta than in the liquid portion. The results emphasize the need to sample both the solid and liquid portions of rumen digesta in experiments evaluating the bacterial community in the rumen.

ABSTRACT

The objective of this study was to use DNA as bacterial markers to estimate and compare the concentration of bacterial crude protein (BCP) in solid and liquid portions of rumen digesta. Using a completely randomized design, 2 multiparous, lactating Holstein cows (average days in milk 14 ± 4 d, average BW 618 ± 40 kg, average DMI 23 ± 4 kg/d, average milk yield 34 ± 10 kg/d), fitted with ruminal cannulae were fed the same diet once daily at 0930 h. Every 4 h over a 24 h period, a sample of approximately 1.5 kg of rumen contents was collected from each cow and was strained through 4 layers of cheesecloth. Particle associated bacteria (PAB) was separated from the solid portion of rumen contents by adding phosphate buffered saline (PBS) and blending the mixture in a commercial blender, followed by straining through four layers of cheesecloth. Fluid collected after blending, as well as fluid retained from the initial straining, underwent differential centrifugation, yielding bacterial pellets consisting of fluid associated bacteria (FAB) and PAB. Next, DNA was then extracted from bacterial pellets and from the non-centrifuged samples of rumen fluid and particles. The DNA from the bacterial pellets, rumen fluid, and rumen particle samples were subjected to real-time PCR using the TaqMan assay. Primers and a probe were designed from DNA encoding part of the 16s rRNA. The relative abundance of bacterial DNA tended to be higher ($P = 0.09$) in the solid portion (209.5 ± 26.6 mg BCP/g DM) than in the liquid portion (106.4 ± 43.6 mg BCP/g DM). Results suggest that BCP is detected in both the solid and liquid portion of rumen digesta and that it is found in higher concentrations in the solid portion.

Key words: bacteria, DNA marker, particle associated bacteria, fluid associated bacteria

INTRODUCTION

Rumen digesta is often sampled for the purpose of bacterial analysis. The presence of ruminal bacteria is believed to be different between the solid and liquid portions, however the extent of the difference has not been clearly outlined. Isolated bacteria that are found to be more commonly attached or "associated" with feed particles are known as particle associated bacteria (PAB), while bacteria isolated from ruminal fluid are known as liquid associated bacteria (LAB) (Martin, 1994). The extent of differentiation of PAB and LAB between rumen digesta fractions becomes important when considering sampling method for bacterial analysis. For example, if only the liquid portion of rumen digesta was sampled, bacterial concentration of whole rumen digesta may be misrepresented. We hypothesized that, using DNA as bacterial markers, we would observe greater concentrations of BCP in the solid portion of rumen contents than in the liquid portion.

MATERIALS AND METHODS

Animals and Treatments

The experimental cows were managed according to the guidelines stipulated by the University of Nebraska Animal Care and use Committee. Two lactating, multiparous Holstein cows fitted with rumen cannulas were used (n = 2). The cows were 14 ± 4 DIM and averaged 34 ± 10 kg/d milk yield and 618 ± 40 kg BW throughout the experiment.

Cows were fed the same diet (Table 2.1). The total mixed ration (TMR) was mixed daily and was fed once daily at 0930 h with feed offered for *ad libitum* consumption (5 % refusals). Water was available for *ad libitum* consumption.

Feed Sampling

Approximately 2.5 kg (wet basis) of individual feed ingredients as well as samples of the TMR were collected immediately after feeding on the day of collections. Feed samples were frozen at -20˚C 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, 2000), N (Leco FP-528 N Combustion Analyzer; Leco Corp., St. Joseph, MI), soluble protein (Krishnamoorthy et al., 1982), RDP (Krishnamoorthy et al., 1983), NDF (Van Soest et al. 1991), ADF (method 973.18; AOAC, 2000), ADIN and NDIN (Leco FP-528 N Combustion Analyzer; Leco Corp., St. Joseph, MI), lignin (Goering and Van Soest, 1970), starch (Hall, 2009), sugar (DuBois et al., 1956), ether extract (method 2003.05; AOAC, 2006), ash (method 942.05; AOAC, 2000), and minerals (method 985.01; AOAC 2000).

Ruminal Digesta and Microbe Sampling

Ruminal contents were sampled every 4 h over the course of one day. Separate samples of the solid and liquid portions of rumen digesta were taken by straining through four layers of cheesecloth. Sample collection occurred at 0400, 0800, 1200, 1600, 2000, and 0000. Ruminal bacteria were isolated according to the procedure described by Histrov et al. (2005). 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 phosphate-buffered saline equal to the volume of the filtrate, and blended in a commercial blender in attempt 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). From this sample, bacteria were harvested immediately via differential centrifugation (Hristov and Broderick, 1996) with an initial low-speed centrifugation at 400 × *g* for 5 min at 4˚C and a subsequent high-speed centrifugation at 20,000 × *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.

Estimation of BCP using DNA Markers

DNA Extraction. Bacterial CP was estimated based on the ratio bacterial DNA marker:N. Bacterial DNA was extracted from rumen solid, liquid, and bacterial samples by the repeat bead beating plus column method according to the extraction method for PCR-quality DNA described by Yu and Morrison (2004). Briefly, collected samples of rumen solids, liquids, and bacteria were combined with lysis buffer and beads, and then they were shaken for physical disruption of cells and exposure of cellular contents. Then, DNA and RNA were precipitated. Next, DNA was purified by applying a series of centrifugation steps and by eliminating the RNA and proteins. The concentration of DNA in each sample was measured by spectrophotometry (NanoDrop ND-1000 Spectrophotometer, NanoDrop Technologies, Inc. Wilmington, DE) and stored at -20 ºC in aliquots of 25 μL for later analysis for BCP using real-time PCR.

Real-time PCR. The bacterial DNA marker used in this study has been reported elsewhere (Yu et al., 2005) and is part of the gene encoding the 16S rRNA, which has been shown to be highly preserved in bacteria (Ogier et al., 2002; Zimmermann et al.,

2010). The National Center of Biotechnology Information (NCBI) accession number of the targeted bacterial DNA marker is FJ715623. The marker is composed of a forward primer, a TaqMan probe, and a reverse primer (Castillo-Lopez et al., 2010). Forward primer: 5'-act cct acg gga ggc agc ag-3'. TaqMan probe: 5'-FAM/tgc cag cag ccg cgg taa tac/TAMRA-3'. Reverse primer: 5'-gac tac cag ggt atc taa tcc-3'.

Real-time PCR reactions were as follows, 4 µL of DNA sample were combined with 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 0.25 µL of 10 µM TaqMan probe, 7.5 µL of TaqMan Master Mix (Applied Biosystems, Foster City, CA, USA) and 1.25 µL of nanopure water. Two samples with no DNA were included and used as non-template controls. Each sample was run in duplicate in separate wells of the 384-well real-time PCR plate. DNA samples were subjected to real-time PCR using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Temperature cycling was as described by Moya et al. (2009) with some variations, specific conditions were as follows: stage 1**:** 50 ºC for 2 minutes; stage 2: 95 ºC for 10 minutes; stage 3: 50 cycles alternating denaturation at 95 ºC for 15 seconds, then annealing and polymerization at 60 ºC for 1 minute.

Calculation of BCP. Results from real-time PCR were used to estimate BCP according to calculations described by Castillo-Lopez et al. (2010) and expressed in mg of CP/g of DM. Real-time PCR results and concentration reactions (amount of DNA placed in each well, and amount of total DNA recovered from each sample) were used to calculate the abundance of DNA marker per g of DM. In addition, the abundance of DNA marker per g of CP from a pure sample of bacteria was estimated to obtain the ratio of

DNA marker to CP. To do so, the pure bacterial samples were analyzed for CP. Bacterial CP content from either cow averaged 48.9 ± 0.05 % and 49.7 ± 0.26 %, respectively. From those values, the amount of BCP was calculated and reported in mg of CP per unit of DM.

Statistical Analysis

Data collected on the concentration BCP in the solid and liquid portions of rumen digesta were analyzed using the GLIMMIX procedure of SAS (Version 9.2; SAS Institute, Inc., Cary, NC) as a completely randomized design. Fixed model effect included digesta sample type as treatment with cow as the random effect, representing 2 replicates. Means were generated using the LSMEANS statement.

RESULTS AND DISCUSSION

Bacterial crude protein concentrations tended to be higher ($P = 0.09$) in the solid portion of rumen digesta than in the liquid portion as illustrated by Figure 2.1. The relative abundance of BCP in the solid and liquid portions averaged 209.5 ± 26.6 mg BCP/g DM and 106.4 ± 43.6 mg BCP/g DM, respectively. This trend we observed favors our initial hypothesis that BCP concentrations would be greater in the solid portion of rumen digesta than in the liquid portion. These results are not surprising, in that it has been observed in previous studies that most rumen microorganisms are associated with feed particles in the rumen (Forsberg and Lam, 1977; Olubobokun and Craig, 1990); up to 70-80 % of microbial organic matter in whole rumen contents may be associated with the particulate phase (Craig et al., 1987). However, to our knowledge, we are the first to estimate the differentiation of BCP concentration between solid and liquid portions of rumen digesta using DNA as a microbial marker. These results emphasize the need to sample both the solid and liquid fractions when estimating bacterial crude protein in rumen digesta. Future research that evaluates the bacterial community structure within these sample types may contribute to further understanding of the nature of the observed differences.

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TABLES AND FIGURES

Table 2.1 Ingredient and chemical composition of TMR fed during the experiment[1]

Ingredient, % DM	
Corn silage[2]	31.4
Ground corn	22.9
Alfalfa hay[3]	18.4
Soy bean meal, 47.5 % CP	14.8
Ground Soybean Hulls	7.90
Soy pass[4]	2.00
Calcium carbonate	0.90
Sodium bicarbonate	0.70
Dicalcium phosphate, 18.5 % P	0.30
Sodium chloride	0.20
Magnesium oxide	0.20
Trace Min/Vit premix[5]	0.20
Chemical, % DM[6]	
CP	17.9
NDF	29.2
Starch	20.3
Ether Extract	3.40

[1]Values determined by Cumberland Valley Analytical Services, Hagerstown, MD.
[2]NDF = 39.5 ± 0.60 %, CP = 7.4 ± 0.10 %.
[3]NDF = 39.4 ± 0.90 %, CP = 19.3 ± 0.65 %.
[4]LignoTech, Overland Park, KS.
[5]Formulated to supply approximately 120, 000 IU/d vitamin A, 24, 000 IU/d of vitamin D, and 800 IU/d Vitamin E, 1.0 % Ca, 0.50 % P, 0.36 % Mg, and 1.3 % K in total ration.
[6]According to the CPM Dairy Ration Analyzer (v3.0.8.1; Boston et al., 2000).

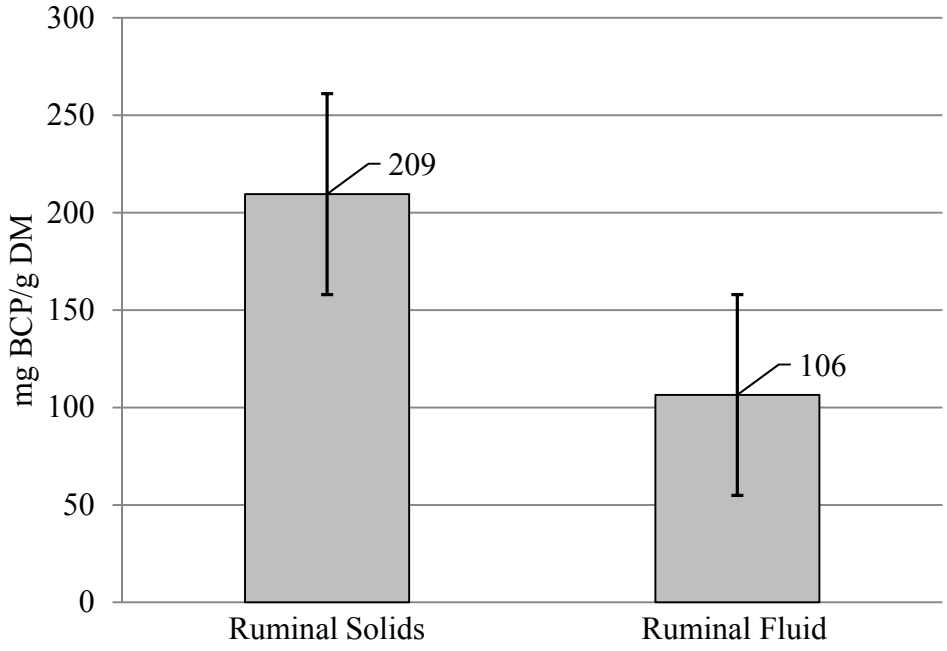

Figure 2.1 Bacterial crude protein (BCP) concentration in rumen contents

CHAPTER III

Flow of microbial crude protein out of the rumen when dairy cattle are supplemented with HMTBa

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INTERPRETIVE SUMMARY

This experiment evaluated the effects of 2-hydroxy-4-methylthio-butanoic acid (Alimet, Novus International, St. Charles, MO) on milk production and composition, rumen microbial activity and protein flow out of the rumen, and rumen microbial community composition when fed with diets deficient or in excess of metabolizable protein. Milk production and composition was similar among treatments and the flow of microbial protein was not affected by the supplement, but rumen VFA and ammonia concentrations were increased with the addition of 2-hydroxy-4-methylthio-butanoic acid. The rumen microbial community was also affected at the phylum level by the supplement, and a number of associations were drawn between microorganisms and treatments and observations.

ABSTRACT

Four multiparous, lactating Holstein cows (average DIM 169.5 ± 20.5 d), fitted with ruminal and duodenal cannulae, 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 (HMB; Alimet, Novus International, St. Charles, MO) when fed with diets deficient or in excess of metabolizable protein (MP) on milk production and composition, rumen microbial activity and protein flow out of the rumen, and rumen microbial community composition. Experimental periods were 28 d in length. Cows were housed in individual tiestalls and fed diets designated as "Low MP" or "High MP", which were top dressed with 250 g of HMB, once daily at 0930 h. No interactions were observed between HMB and level of dietary MP, with the exception of ruminal acetate to propionate ratio ($P = 0.04$). Milk yield was not affected by treatment and averaged 23.8 ± 2.06 kg. There was a tendency ($P = 0.06$) for increased milk protein percent in cows receiving Low MP diets, averaging 3.30 ± 0.09 % and 3.21 ± 0.09 % for Low MP and High MP, respectively. The DM, OM, NDF, and N digestibilities were greater ($P \leq 0.03$) in cows consuming the Low MP diet. Rumen pH was lower ($P = 0.05$) in cows consuming High MP diets as well as in those consuming HMB ($P < 0.01$). Rumen kinetics were not affected among treatments. Rumen ammonia concentrations tended to be greater ($P = 0.06$) in cows consuming HMB. Rumen VFA concentrations were greater ($P = 0.02$) in cows consuming HMB. Duodenal DM flow, N flow, and bacterial N flow did not differ between treatments ($P \geq 0.15$). The microbial community structure of cows receiving HMB was affected at the phylum level, as the proportion of *Fibrobacteres* was increased ($P = 0.04$). A number of association ($P \leq 0.05$) of specific microorganisms and

metadata were observed, including animal, HMB supplementation, level of dietary MP, DMI, digestibility, rumen ammonia, microbial N flow, and milk production and components. Results suggests that HMB affects rumen microbial activity, irrespective of dietary MP level. Consequently, further investigation is warranted into the mechanism of these effects in the rumen.

Key words: HMB, microbial protein, rumen, bioinformatics

INTRODUCTION

Metabolizable protein (MP) is protein which is available for absorption and utilization by the cow via the small intestine. Cows consuming rations which are low in MP may harm productivity, as milk and protein yields can be decreased (Cabrita et al., 2011; Lee et al., 2012). One approach to meet the needs of MP is to formulate diets to support a high plane of microbial activity and growth in the rumen, which results in increased microbial crude protein (MCP) available to the cow post-ruminally (NRC, 2001), as the AA profile supplied by microorganisms is believed to be similar to that required by the cow (Korhonen et al., 2002). This practice is frequently achieved by supplying adequate energy and proteins (Hoover and Stokes, 1991). Additionally, the concentration of MP in the diet can be altered by feeding sources of RUP, for example, animal proteins and dried distillers grains and solubles (Hubbard et al., 2009). As feeds high in RUP are may be costly, promoting microbial protein productivity with cheaper feeds is often a prudent strategy.

The commercial HMTBa product Alimet (Novus International, St. Charles, MO), when supplemented in dairy diets, has been shown to have enhancing effects on rumen microbial activity, including MCP yield, fiber digestibility, and VFA production. (Rosser et al., 1971; Bull and Vandersall, 1973; Gil et al., 1973; Lundquist et al., 1983; Vázquez-Añón et al., 2001). 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 MP supplied by the ration. Furthermore, much of the past research focusing on the effects of HMTBa on rumen microbial productivity have 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 in an in vivo experiment, rumen microbial activity would be increased by HMTBa supplementation with the High MP diet, and that the responses would be lower or nonexistent when fed with the Low MP diet. In addition, we hypothesized that the rumen microbial community structure would be altered by diet type and HMTBa supplementation.

MATERIALS AND METHODS

Animals 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 cannulae were used in this experiment, which was a replicated 4 × 4 Latin square design (Kononoff and Hanford, 2006). Cows received each treatment once on 1 of four 28-d experimental periods. Cows were 169.5 ± 20.5 DIM at the start of the experiment and averaged 690.9 ± 98.5 kg of BW (as determined by weight tape) throughout. 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 tiestalls equipped with rubber mats.

Treatments were formulated to be either low or high in MP in the diet (stated as "Low MP" or "High MP" within the context of these diets) according to the dairy NRC (2001) model (Appendix II). A portion of ground corn was replaced by soybean meal in the High MP diet in order to achieve a concentration of MP accepted as more than

sufficient 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, while lowering MP down to a concentration considered to be deficient. Low and High MP diets were top dressed with 25 g of 2-hydroxy-4-methylthio-butanoic acid (HMB) (Alimet, Novus Interrnational, St. Charles, MO), a methionine analog, with 225 g of ground corn as a carrier. Additionally, Low and High MP diets were top dressed with 250 g of ground corn to serve as negative controls. The HMB 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 with feed offered for ad libitum consumption (5 % refusals). Water was available for ad libitum consumption. Cows were milked twice daily at 0700 and 1800 h. Chromic oxide (Cr_2O_3) was used as a marker for the estimation of digesta flow (Harvatine et al., 2002; Sylvester et al., 2005). Seven and a half grams of Cr_2O_3 was weighed and placed in gelatin capsules (Torpac Inc., Fairfield, NJ), then dosed into the rumen via the ruminal cannula twice daily at approximately 0730 and 1830 h on d 17 through 26 of each experimental period to provide a marker to estimate digesta flow.

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 four layers of cheesecloth. The pH of the filtrate was then measured with a hand held pH probe (model M90, Corning Inc., Corning, NY) and was then 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 in subsequent collection d, so that every 60-minute interval in a 24-h period was represented (6 samples per d and a total of 24 samples per cow per period). Samples were collected on d 23 at 0100, 0500, 0900, 1300, 1700, and 2100; d 24 at 0200, 0600, 1000, 1400, 1800, and 2200; d 25 at 0300, 0700, 1100, 1500, 1900, and 2300; d 26 at 0400, 0800, 1200, 1600, 2000, and 0000. 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 27 and 28. Feed samples were frozen at -20˚C for later analysis.

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

Milk Sampling

Milk yields were recorded daily. Milk samples were collected twice daily for 7 consecutive days on d 20-26. Milk samples were placed in 50 mL conical tube and

immediately frozen at -20˚C for later analysis. Additional samples were collected in 50 mL tubes and shipped to DHIA (Heart of America DHIA, Manhattan, KS) where they were analyzed for fat, true protein, lactose, and SNF (AOAC, 200) using a B2000 Infrared Analyzer (Bentley Instruments. Chaska, MN). Milk urea nitrogen was determined by the same laboratory using a modified Berthelot reaction concentration using a ChemSpec 150 Analyzer (Bentley Instruments. Chaska, MN). Yields of milk components were estimated according to milk weight and time of collection. During the last 7 d of each period, milk yield was averaged.

Rumen Evacuation and Kinetics

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

Rumen kinetics were calculated according to the equations described by Van Soest (1994). First, rate of disappearance calculated by dividing DMI (kg DM per day) by the rumen pool (kg DM). The resulting value was then divided by 24 and multiplied by 100, so that rate of disappearance was expressed as % per hour. Second, rate of passage (k_p) was calculated by dividing fecal output (kg DM per day) by the rumen pool (kg DM). The resulting value was then divided by 24 and multiplied by 100, so that k_p was expressed as % per hour. Third, rate of digestion (k_d) was calculated by subtracting k_p from the rate of disappearance, and was expressed as % per hour.

Isolation of Ruminal Bacteria and Protozoa

Ruminal bacteria were isolated according to the procedure described by Histrov 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). From this sample, bacteria were harvested immediately via differential centrifugation (Hristov and Broderick, 1996) with an initial low-speed centrifugation at 400 × *g* for 5 min at 4˚C and a subsequent high-speed centrifugation at 20,000 × *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 composed 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.

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 A. 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, 2000), N (Leco FP-528 N Combustion Analyzer; Leco Corp., St. Joseph, MI), soluble protein (Krishnamoorthy et al., 1982), RDP (Krishnamoorthy et al., 1983), NDF (Van Soest et al. 1991), ADF (method 973.18; AOAC, 2000), ADIN and NDIN (Leco FP-528 N Combustion Analyzer; Leco Corp., St. Joseph, MI), lignin (Goering and Van Soest, 1970), starch (Hall, 2009), sugar (DuBois et al., 1956), ether extract (method 2003.05; AOAC, 2006), ash (method 942.05; AOAC, 2000), and minerals (method 985.01; AOAC, 2000). Samples of TMR were used to determine particle size according to Kononoff et al. (2003) using the Penn State Particle Separator.

Feed samples, orts, and fecal samples were also analyzed at the University of Nebraska-Lincoln for N (Leco FP-528, Leco Corp., St. Joseph, MI), NDF (Van Soest et al., 1991), starch (Megazyme, AOAC method 996.11 and AACC method 76.13), and ash (method 942.05; AOAC, 2000). Heat stable α-amylase (number A3306; Sigma Chemical Co., St. Louis, MO) was included in the NDF procedure (0.5 mL per 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 (Chaney and Edward, 1962). A stock solution was prepared using known amounts of ammonium chloride. The rumen fluid was centrifuging at 12,000 × *g* for 20 min at 4°C to obtain a

clear supernatant. Next, 40 µL of the supernatant or standard was combined with 40 µL of water in glass test tubes. 2.5 mL of phenol reagent, containing sodium nitroprusside and dry phenol, was added along with 2.0 mL of alkaline hypochlorite. Samples and standards were then incubated in a 37°C water bath for 10 min. Last, 300 µL of samples and standards were added to a microtiter plate, and the absorbance was read at a wavelength of 500 nm on a SpctraMax 250 (Molecular Devices, Sunnyval, CA) spectrophotometer. Ammonia concentration was estimated using linear regression, where x = absorbance and y = concentration.

The concentration of VFA were estimated according to Erwin et al. (1961). Rumen fluid samples were first centrifuged at 5,000 × *g* for 10 min. An aliquot of 2.0 mL of supernatant was combined with 0.5 mL of 25 % meta-phosphoric acid and 25 mM 2-ethybutyrate solution. A stock standard was prepared containing known amounts of VFA, and 2.0 mL of this solution was also combined with 0.5 mL of 25 % meta-phosphoric acid and 25 mM 2-ethybutyrate solution. Samples and standards were refrigerated for 30 min and then centrifuged at 10,000 × *g* for 15 min. The supernatant was filtered through a 25-mm Whatman (GE Healthcare Life Sciences, Pittsburgh, PA) syringe filter using a 3 mL BD (Becton, Dickinson and Company, Franklin Lakes, NJ) tuberculin syringe into a 2 mL screw top vial. The vials were then analyzed for VFA using gas chromatography by a Thermo Scientific Trace 1300 (Thermo Fisher Scientific, Waltham, MA) gas chromatographer.

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 Company, Philadelphia, PA). Then, ground samples were analyzed for DM (100°C oven for 24 h). Subsamples of isolated ruminal microbial 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) were used as a microbial marker to measure duodenal flow of total microbial N. The analysis of purines was conducted according to the procedure described by Aharoni and Tagari (1991). Approximately 50 mg of lyophilized microbial and duodenal samples were placed in Pyrex (Corning Inc., Corning, NY) screw-cap tubes. Then, samples were combined with 2.5 ml of 70 % $HCLO_4$. Samples were then vortexed and incubated in a 90-95˚C water bath for 15 min before an additional vortexing and 45 min incubation. Next, 17.5 mL of 28.5 mM H_6NPO_4 was added and the samples were reinserted into the water bath for 15 min. Samples were then filtered through Whatman #1 filter paper (Whatman Inc., Florham Park, NJ) into 60 × 125 mm disposable glass culture tubes. An aliquot of 0.25 mL of the filtrate was transferred into 165 × 125 mm disposable glass culture tubes and combined with 0.25 mL 0.4 M $AgNO_3$ and 4.5 mL 0.2 M H_6NPO_4 and were allowed to stand overnight in the refrigerator. Samples were then centrifuged for 10 minutes at 1,000 × *g* and the supernatant was removed. The pellet was washed with 4.5 mL of washing solution and 250 µL of $AgNO_3$. Samples were then incubated in the water bath for 30 minutes and centrifuged again for 10 minutes at 1,000 × *g*. After cooling, 200 µL of the supernatant was pipette into a microtiter plate and total purines were measured using a 717 HPLC system (Waters Corp. Inc., Milford, MA). 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.

Estimation of MCP using DNA as a Microbial Marker. Bacterial CP was also estimated based on the ratio bacterial DNA marker:N. To do so, bacterial DNA was extracted by the repeat bead beating plus column method according to the extraction method for PCR-quality DNA from digesta samples described by Yu and Morrison (2004) using a PowerMag Soil DNA Isolation Kit (Mo Bio, Carlsbad, CA). Collected samples duodenal digesta and rumen bacteria were combined with lysis buffer and beads, and then they were shaken for physical disruption of cells and exposure of cellular contents. Then, DNA and RNA were precipitated and DNA was purified by applying a series of centrifugation steps and by eliminating the RNA and proteins. Finally, the MagMAX Express-96 Deep Well Magnetic Particle Processor (Applied Biosystems, Foster City, CA) was used for magnetic bead-based extraction of DNA. The concentration of DNA in each sample was measured by spectrophotometry (NanoDrop ND-1000 Spectrophotometer, NanoDrop Technologies, Inc. Wilmington, DE) and stored at -20 ºC in aliquots of 90 µL for later analysis for BCP using real-time PCR.

The bacterial DNA marker used in this study has been reported elsewhere (Yu et al., 2005) and is part of the gene encoding the 16S rRNA, which has been shown to be highly preserved in bacteria (Ogier et al., 2002; Zimmermann et al., 2010). The National Center of Biotechnology Information (NCBI) accession number of the targeted bacterial DNA marker is FJ715623. The marker is composed of a forward primer, a TaqMan probe and a reverse primer. Forward primer: 5'-act cct acg gga ggc agc ag-3'. TaqMan

probe: 5'-FAM/tgc cag cag ccg cgg taa tac/TAMRA-3'. Reverse primer: 5'-gac tac cag ggt atc taa tcc-3'. For protozoa, marker (composed of forward primer, reverse primer and probe) was designed from DNA encoding part of the 18S rRNA gene. The NCBI accession number of the targeted DNA is EU796177. Forward primer: 5'-gct ttc gat ggt agt gta tt-3'. TaqMan Probe: 5' -FAM/cgg aag gca gca ggc gc/TAMRA- 3'. Reverse primer: 5'-act tgc cct cta atc gta ct-3'.

Real-time PCR reactions were as follows, 6.25 µL of DNA sample were combined with 0.5 µL of 10 µM forward primer, 0.5 µL of 10 µM reverse primer, 0.25 µL of 10 µM TaqMan probe, 7.5 µL of TaqMan Master Mix (Applied Biosystems, Foster City, CA, USA). Two samples with no DNA were included and used as non-template controls. Each sample was run in duplicate in separate wells of the 384-well real-time PCR plate. DNA samples were subjected to real-time PCR using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Temperature cycling was as described by Moya et al. (2009) with some variations, specific conditions were as follows: stage 1**:** 50 ºC for 2 minutes; stage 2: 95 ºC for 10 minutes; stage 3: 50 cycles alternating denaturation at 95 ºC for 15 seconds, then annealing and polymerization at 60 ºC for 1 minute.

Results from real-time PCR were used to estimate BCP and PCP according to calculations described by Castillo-Lopez et al. (2010) and expressed in mg of CP/g of DM. Real-time PCR results and concentration reactions (amount of DNA placed in each well, and amount of total DNA recovered from each sample) were used to calculate the abundance of DNA marker per g of DM from each dietary treatment. In addition, the abundance of DNA marker per g of CP from a pure sample of each microbial type

(bacteria, protozoa) was estimated to obtain the ratio of DNA marker to CP. To do so, pure bacterial and protozoal samples were isolated from a portion of the ruminal fluid by differential centrifugation as described by Shabi et al. (2000). From those values, the amount of BCP, PCP will be calculated and reported in mg of CP/g of DM.

Rumen Microbial Community Analysis. DNA was extracted as described above and analysis of rumen microbiota composition was performed by deep sequencing of 16S rDNA tags on rumen digesta collected at four different time-points (0, 4, 12 16 h after feeding) which were composited by day. Semiconductor-based sequencing was performed on the Ion Torrent Personal Genome Machine (Life Technologies, Carlsbad, CA) according to manufacturer protocols. Sequencing was obtained at a depth of approximately 30,000 raw reads per sample (top 99.99 % of the microbiota) using the 341F-518R (V3) segment of the 16S rRNA gene. Conditions for PCR were as follows: stage 1: 95°C for 2 minutes; stage 2: 25 cycles of 95°C for 30 seconds, 52°C for 30 seconds, and 72°C for 30 seconds; stage 3: 72°C for 2 minutes. Negative control and a positive control reactions were also performed to control for any PCR contamination. Routinely, the output of greater than 3 million filter-pass reads is achieved from amplicon runs using 200 bp chemistry and custom bar-coded fusion primers. Amplicons from 96 samples were multiplexed after barcoding and sequenced on a single chip. The methods used for emPCR, 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 the quality filter and analysis pipeline developed in Dr. Fernando's Lab as described by

Sasso, et al. (2014). The processed data was then used for microbial community analysis. To identify taxonomic diversity, the resulting reads were classified using the CLASSIFIER (Wang et al., 2007) algorithm (trained using Greengenes database 12_10) and BLAST similarity matches to the National Center for Biotechnology Information (NCBI) non-redundant database. It was expected that a portion of the sequences would have no genus level similarity to published sequences present in the databases, therefore we also employed an Operational Taxonomic Unit (OTU) based analysis, by clustering sequences into OTUs based on sequence similarity (eg. >97 %). The pre-processed reads were clustered into OTUs using USEARCH (Edgar, 2010; www.drive5.com/usearch/) at 97 % similarity (Castillo-Lopez et al., 2014) to generate Operational Taxonomic Units (OTUs) after screening for chimeric sequences using UCHIME (Edgar et al., 2011). To identify the taxonomic distribution of the OTUs generated, the CLASSIFIER algorithm described above was used. Following taxonomic identification of the OTUs, clustering of communities/OTUs based on phenotype was performed using weighted unifrac analysis. Ordination plots were generated using principal component analysis and non-metric multi-dimensional scaling. In addition, the OTU distribution among treatments was used to obtain quantitative insight into the microbial ecology as affected by dietary treatment and was analyzed using the GLIMMIX procedure of SAS (Version 9.2; SAS Institute, Inc., Cary, NC).

A statistical tool developed by The Huttenhower Lab (Department of Biostatistics, Harvard School of Public Health, Boston, MA) entitled “MaAsLin” (Multivariate Association with Linear Models), was used to find associations between metadata and microbial community abundance utilizing OTU distribution in samples.

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. For any association with a *q*-value ≤ 0.05 (the minimum false discovery rate), the MaAsLin tool generated knotched box plots for factor data, such as animal or diet type, and scatter grams with a line of best fit for continuous data, such as MY or rumen ammonia concentration. At the top of the plots, the coefficient effect size (r) is listed, followed by the standard deviation, *P*-value, and *q*-value. In addition to the knotched box plots or scatter plots, a complementary partial residual plot was generated. In the partial residual plot, the residuals are plotted against the independent variable, which is useful in order to detect outliers or assess the presence or absence of inhomogeneity of variance (Larsen and Mccleary, 1972). The DNA sequences represented by OTUs that were identified to be associated with the various metadata were then submitted to the NCBI Standard Nucleotide BLAST tool in order to identify specific bacterial microorganisms. After submission of sequence data to the BLAST tool, the user is presented with a listing of potential matches to microorganisms in the database, along with query cover %, E-value, and maximum identity %. The query cover % represents the percentage of the sequence that overlaps with the potentially matching microorganism's sequence. The E-value represents the number of expected hits that occur by chance when searching the database; the smaller the E-value, the more significant the match. The identity % is the percent identity between the query and the hit in a nucleotide-to-nucleotide alignment (Agostino, 2013a).

Calculation of Digesta Flow of DM. The use of Cr_2O_3 as a marker for the estimation of digesta flow has been reported elsewhere (Christiansen and Webb, 1990; Titgemeyer, 1997; Kozloski et al., 1998). The concentration of Cr_2O_3 in samples of TMR, rumen, duodenal, and fecal samples were determined by an external laboratory (Analab, Fulton, IL) by inductively coupled plasma mass spectrometry (Vista MPX, Agilent Technologies, Inc., Santa Clara, CA). Fecal output was calculated by dividing the intake of Cr_2O_3 by the concentration of Cr_2O_3 in the feces. Duodenal flows were calculated as described by Erasmus et al. (1992). The flow of DM was calculated by dividing the amount of daily Cr_2O_3 dosed by Cr_2O_3 concentration in ground duodenal samples. Then, DM flow was expressed in g per day. Duodenal flow of CP was calculated by multiplying DM flow by the concentration of CP (N × 6.25) in duodenal samples (DM basis).

Furthermore, indigestible acid detergent fiber (iADF; Ramirez-Ramirez et al., 2011; Huhtanen et al., 1994) and indigestible NDF (iNDF; Ahvenjärvi et al., 2003) were determined for use as digesta flow markers. The iADF and iNDF procedures for TMR, rumen solids, duodenal, and fecal samples were carried out in order to estimate as additional measures of flow (Huhtanen et al., 1994). Approximately 1.25 g of 1-mm ground subsamples were weighed in triplicate into 5 × 10 cm Dacron nylon bags (Ankom Inc., Fairport, NY) with a pore size of 50 μm. 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 two secured 100 g weights. Nylon mesh bags were incubated for 12 days (Wu 2005) in the ventral sac of the rumen of steers fitted with ruminal cannulae 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 days, the Dacron bags were removed and machine washed using five, 3 minute cycles consisting of a 1 minute wash and a 2 minute spin, rinsed in distilled water, and dried in a 60ºC forced air oven for 12 hours. After drying, NDF and ADF were determined using an Ankom Fiber Analyzer (Ankom Technology, Fairport, NY). Fecal output was calculated by determining the intake of iADF or iNDF and dividing by the concentration of iADF or iNDF in the feces. Duodenal flow was calculated by dividing the amount of iADF or iNDF intake by the concentration of iADF or iNDF in duodenal samples and expressed in g per day.

Statistical Analysis

Data collected on the effects of HMB 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 HMB 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 × 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 HMB and interaction as planned a priori.

RESULTS

Diet and Ingredient Composition

Ingredient composition of the experimental diets is listed in Table 3.1. 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 in order to achieve a concentration of MP accepted 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, while 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 supplied 2132 g MP/day (94.4 % of requirement), while the high MP diet supplied 2267 g MP/day (100.4 % of requirement) (Appendix II).

Table 3.2 lists the chemical composition of the experimental diets. The CP content of the Low MP and High MP diets were similar with 16.6 ± 0.34 % and 16.6 ± 0.33 % (DM basis) respectively, however, soluble protein differed as expected with 6.00 ± 0.35 % and 4.53 ± 0.59 %. Particle size distribution is also listed in Table 3.2. The proportion of material retained on the > 19.0 mm, 19.0 – 8.0 mm, 8.00 -1.18 mm, and < 1.18 mm screens were not different and averaged 5.77 ± 1.70 %, 29.4 ± 0.07 %, 48.1 ± 0.57 %, and 16.8 ± 0.99 %, respectively.

The chemical composition of forages, concentrates and top dress is listed in Table 3.3. The CP content of corn silage was 7.80 ± 0.40 %, while alfalfa and brome hays averaged 21.5 ± 0.61 % and 15.5 ± 0.48 %. The NDF content of corn silage was 38.0 ± 2.43 % and alfalfa and brome hays were 43.8 ± 2.28 % and 49.5 ± 3.00 %. By design,

Low and High MP concentrates were similar in all aspects except soluble protein, which was 8.68 ± 0.90 % for Low MP concentrate and 5.55 ± 1.46 % for High MP concentrate, while CP content remained similar at 22.3 ± 0.64 % and 22.2 ± 1.33 % for Low and High MP concentrates, respectively.

Dry Matter Intake, Milk Production and Composition

The results of production performance are listed in Table 3.4. The DMI, milk yield, 3.5 % FCM, milk fat %, milk fat yield, protein yield, and MUN were not affected by either level of MP or HMB supplementation, nor did we observe an interaction between the two ($P \geq 0.35$), and averaged 23.9 ± 1.15 kg/d, 28.2 ± 3.18 kg/d, 28.7 ± 2.55 kg/d, 3.7 ± 0.33 %, 1.02 ± 0.09 kg/d, 0.91 ± 0.8 kg/d, and 11.6 ± 0.97 mg/DL across treatments. Milk protein percent tended to be affect by level of MP ($P = 0.06$) but not HMB and averaged 3.30 ± 0.09 % with the Low MP diet and 3.21 ± 0.0 9% for the High MP diet.

Nutrient Digestibility

Table 3.5 lists the results of apparent total tract apparent digestibility of nutrients estimated with the use of Cr_2O_3, iNDF, and iADF digesta flow markers. No effects of HMB supplementation or an interaction between HMB and level of MP on digestibility were observed ($P \geq 0.21$). Digestibility estimates obtained using iNDF as a digesta flow marker suggested that nutrient digestibilities were increased ($P \leq 0.03$) in cows consuming the Low MP diet. In cows consuming the Low MP diet, the DM, N, NDF, and OM digestibilities averaged 69.4 ± 0.40 %, 69.6 ± 0.25 %, 54.2 ± 1.10 %, and 66.9 ±

0.45 %, respectively, and 67.7 ± 0.45 %, 65.5 ± 0.55 %, 50.9 ± 0.80 %, and 64.5 ± 0.20 % in cows consuming the High MP diet, respectively,

No effects of level of MP on digestiblity were observed ($P \geq 0.11$) in estimates obtained using iADF as a digesta flow marker. Nutrient digestibility estimates were numerically greater in cows consuming Low MP diets. When using Cr_2O_3 as a digesta flow marker, the DM digestibility tended to increase ($P = 0.07$) from the Low to High MP diets, averaging 60.3 ± 1.45 % and 64.8 ± 0.55 %, respectively. Relatedly, OM digestibility tended to increase ($P = 0.08$) from Low to High MP diets. The OM digestibility averaged 57.0 ± 1.60 % and 61.7 ± 0.5 % in Low and High MP diets, respectively.

Rumen pH, Pool Size, Kinetics, Ammonia and VFA

The results of several observed rumen measurements are listed in Table 3.6. Rumen pH was affected by both level of MP and HMB supplementation. Rumen pH was lower ($P = 0.05$) in High MP diets. The average rumen pH of cattle consuming the Low MP diets was 5.89 ± 0.04, while in cattle consuming the High MP diet averaged 5.83 ± 0.05. Additionally, rumen pH was lower ($P < 0.01$) in diets supplemented with HMB; control diets averaged 5.90 ± 0.03, while HMB supplemented diets averaged 5.82 ± 0.04.

Rumen pool of DM was not affected by HMB supplementation ($P = 0.41$), however, the rumen pool size tended to be greater ($P = 0.08$) in High MP diets. The rumen pool of cows consuming Low MP diets averaged 10.4 ± 0.25 kg of DM, while cows consuming High MP diets was increased to 11.9 ± 0.45 kg of DM. Rumen kinetics of DM were not affected ($P \geq 0.15$) by level of MP, HMB, or an interaction between the

two. The DM k_p averaged 3.00 ± 0.13 %/h while the DM k_d averaged 6.46 ± 0.38 %/h across treatments. No effects of HMB, level of MP, or an interaction between the two were observed for NDF k_p and averaged 2.72 ± 0.33 %/h across treatments. The NDF k_d was greater ($P = 0.04$) in cows consuming the Low MP diet than in the High MP diet and averaged 3.31 ± 0.04 %/h and 2.77 ± 0.16 %/h, respectively.

There was a trend for the concentration of ammonia in the rumen to be greater ($P = 0.06$) in cows receiving HMB. On average, rumen ammonia concentration in cows receiving the control top dress was 16.5 ± 0.52 mg/dl, while cows consuming HMB averaged 17.2 ± 0.09 mg/dl. The total VFA concentration for cows receiving HMB was greater ($P = 0.02$) than those receiving the control. The total VFA concentration for cows consuming the control top dress was 119 ± 1.02 mM, while cows consuming HMB averaged 126 ± 0.22 mM. The concentration of acetate in the rumen was greater ($P < 0.01$) in cows consuming the control diet, averaging 63.0 ± 0.05 mM, while cows receiving HMB averaged 61.8 ± 0.41 mM. Rumen propionate concentration was greater ($P < 0.01$) in cows receiving HMB than those that did not. The average propionate concentration in cows receiving the control top dress was 23.2 ± 0.24 mM, while cows receiving HMB averaged 24.8 ± 0.31 mM. Consequently, the ratio of acetate to propionate (A:P) was greater ($P < 0.01$) in cows receiving the control than those receiving HMB. Furthermore, an interaction ($P = 0.04$) of HMB and diet type was observed for A:P, which seemed to be less affected by HMB in cows receiving the High MP diet. In cows receiving the control top dress, the A:P averaged 2.78 ± 0.03, while cows receiving HMB averaged 2.58 ± 0.06. Butyrate concentration was greater ($P = 0.05$) in cows consuming the control than those consuming HMB. In cows consuming

the control, butyrate concentration averaged 11.0 ± 0.08 mM, while cows consuming HMB averaged 10.7 ± 0.07 mM. Rumen isovalerate concentration was greater in cows consuming HMB than in those consuming the control ($P = 0.03$). The rumen isovalerate concentration in cows receiving the control averaged 0.99 ± 0.04 mM, while those consuming HMB averaged 1.13 ± 0.07. No rumen VFA concentrations were affected by level of MP except for valerate; cows consuming the High MP diet had greater ($P < 0.01$) valerate concentrations than those consuming the Low MP diet.

Duodenal Flow of Dry Matter, Nitrogen, and Bacterial Nitrogen

Table 3.7 lists estimates of duodenal flow of DM, nitrogen, and bacterial nitrogen, which were measured using purines and Cr_2O_3, iNDF, and iADF as digesta flow markers. No effects of HMB supplementation, level of MP, or an interaction between the two were observed ($P \geq 0.15$) on duodenal flow of DM, N, and bacterial N in estimates attained using any of the digesta flow markers. The concentration of purines in isolated microbial samples averaged 17.7 ± 4.36 mg/g DM.

Using Cr_2O_3 as a digesta flow marker, duodenal flow, duodenal N flow, bacterial N flow, and bacterial N flow as a % of total N flow averaged 17.7 ± 1.91 kg DM/d, 770 ± 48.3 g DM/d, 353 ± 55.4 g DM/d, and 44.7 ± 5.27 % across treatments, respectively. Using iNDF as a digesta flow marker, duodenal flow, duodenal N flow, bacterial N flow, and bacterial N flow as a % of total N flow averaged 15.8 ± 1.18 kg DM/d, 708 g ± 62.5 DM/d, 315 ± 44.3 g DM/d, and 45.6 ± 7.06 % across treatments, respectively. Using iADF as a digesta flow marker, duodenal flow, duodenal N flow, bacterial N flow, and

bacterial N flow as a % of total N flow averaged 20.5 ± 1.88 kg DM/d, 921 ± 91 g DM/d, 403 ± 65.2 g DM/d, and 45.6 ± 6.11 % across treatments, respectively.

Table 3.8 lists estimates of the abundance of target bacterial and protozoal DNA in duodenal fluid. No effects of HMB, level of MP, or an interaction between the two were observed ($P \geq 0.22$) on bacterial Ct or abundance of target DNA in duodenal fluid, and averaged 40.4 ± 0.29 cycles and 1.07E-02 ± 0.02 abundance/g DM across treatments. No effect of HMB, level of MP or and interaction between the two was observed for abundance of target protozoal DNA in duodenal fluid, and averaged 3.01 ± 3.93 abundance/g DM across treatments. An effect of HMB on protozoal Ct was observed ($P = 0.05$) and averaged 25.3 ± 0.05 cycles for the control, while those of HMB averaged 26.1 ± 0.25 cycles.

Community Composition of Bacteria in the Rumen

Proportions of bacterial phyla relative to total number of reads recovered from rumen digesta are listed in Table 3.9. The results from the bioinformatic analysis of total OTUs are listed along with those from the analysis of core OTUs, which was defined as 95 % of OTUs being present in each treatment. In the total analysis of OTUs, proportion of bacterial phyla did not differ ($P \geq 0.11$) between concentration of MP or HMB supplementation. There was a trend ($P = 0.11$) for cows consuming HMB to have increased proportions of *Fibrobacteres* present. The proportion of *Fibrobacteres* in cows consuming the control top dress was 0.35 ± 0.06 %, while cows receiving HMB averaged 0.45 ± 0.06 %. In the analysis of core OTUs, the proportion of *Fibrobacteres* present was greater ($P = 0.04$) in cows consuming HMB versus those consuming the control.

Cows consuming the control averaged 0.29 ± 0.04 % *Fibrobacteres*, while cows consuming HMB averaged 0.41 ± 0.04 %. There was a tendency for proportion of *Verrucomicrobia* to be decreased in cows consuming HMB. *Verrucomicrobia* were present in cows offered the control averaging 0.09 ± 0.02 %, while they averaged 0.07 ± 0.02 % in cows offered HMB.

Associations of OTUs and Metadata

Results generated by the MaAsLin analysis identified significant ($P \leq 0.05$) associations between OTUs and HMB, level of dietary MP, DMI, microbial N flow, % microbial N flow of total N flow, MY, % milk fat, % milk lactose, % milk protein, milk fat yield, ammonia, N digestibility, and NDF digestibility. The association scatter grams and complementary partial residual plots, along with the top 3 microorganism identified by NCBI Single Nucleotide BLAST (Bethesda, MD) analysis of OTUs, are represented in Figures 3.1—3.18. A total of 94 OTUs were identified to be associated with difference in experimental animal. Of these OTUs, the relative abundance of 86 were found to be different ($P \leq 0.05$) in one experimental cow than in the other three, which were similar in every case.

DISCUSSION

Metabolizable protein is protein that reaches the small intestine and is available for absorption and utilization by the cow. In the formulation of dairy diets, MP is an important consideration as it delivers the AA required for the synthesis of protein necessary for lactation (NRC, 2001). Diets deficient in MP reduce milk and milk protein yield (Cabrita et al., 2011), while those in excess decrease efficiency of N utilization and contribute to higher concentrations of N in feces and ultimately excretion of N into the environment (St-Pierre and Thraen, 1999). The HMB molecule is an analog of methionine and has been supplemented in dairy diets because of its apparent contributions to MP resulting from increased MCP yield (Vázquez-Añón et al., 2001). In addition to increased MCP yield, HMB has been observed to positively affect other facets of rumen microbial activity, including fiber digestibility and VFA production (Rosser et al., 1971; Bull and Vandersall, 1973; Gil et al., 1973; Lundquist et al., 1983; Vázquez-Añón et al., 2001). Many of these effects on rumen microbial activity have been observed utilizing in vitro techniques, which do not account for rumen absorption of HMB or N recycling. Furthermore, the effectiveness of HMB fed with diets deficient or in excess of MP required for lactation is not readily available in literature. We hypothesized that in an in vivo experiment, rumen microbial activity would be increased by HMB supplementation with the High MP diet, and that the responses would be lower or nonexistent when fed with the Low MP diet. Additionally, we hypothesized that the rumen microbial community structure would be altered by diet type and HMB supplementation. In order to test this, rations were formulated to be either deficient or in excess of MP, were top dressed with HMB or a control, and were fed to lactating,

ruminally and duodenally cannulated cows. The effects to be observed were intake and digestibility, lactation performance, ruminal pH, VFAs, and ammonia, and MCP flow to the duodenum using purines and DNA as microbial markers. In addition, microbial community structure was assessed using high throughput sequencing of rumen microbial DNA.

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 four 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 HMB supplementation, where this type of experimental design is more appropriate. Larger studies may be required in order to determine differences in milk production variables. Using sixty-one Holstein cows, St-Pierre and Sylvester (2005) determined that significant responses in milk protein yield as an effect of feeding different methionine sources (including HMB) were not observed until after 5 weeks of supplementation.

In every measurement of the experiment, no interactions were observed between HMB and level of MP in the diet (with the exception of the ruminal acetate to propionate ratio). This is noteworthy in terms of our hypothesis, in that the effects of HMB were seemingly unaltered under conditions in which dietary MP was adequate or deficient.

Supplementation of HMB 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 low in MP. The rate of NDF digestion was decreased in cows receiving the High MP diet, which may have been a consequence of decreased ruminal pH in cows consuming this diet, as a reduction in pH hinders rate of NDF digestion (Grant and Mertens, 1992). We observed an average rate of NDF digestion of 3.04 %/h, which was similar to observations by Ramirez (2013) which averaged 2.81 %/h. In order 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 fiber analyses of the TMR, as the low MP diet is higher in starch and lower in NDF and ADF. 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). In order to estimate digestibility, three different digesta flow markers were used to calculate fecal output; this included one external marker, Cr_2O_3, and two internal

markers, iNDF and iADF. Digestibility estimates attained by using Cr_2O_3 and iADF did not yield significant results. As suggested by Ipharraguerre et al. (2007) estimations of digesta flow attained from the Cr_2O_3 markers may be less sensitive to contributions of digesta phases in duodenal contents, as Cr_2O_3 does not associate with any phase as it flows through the digestive system (Merchen, 1988). Huhtanen et al. (1994) suggested that digesta flow estimates may be variable when using iNDF or iADF as markers, as there is a potential for the loss of particles of feed and fecal samples from the nylon bags during ruminal incubation. Due to our observed differences in nutrient digestibility estimates, which are potentially explained by dietary differences discussed above, we decided to rely heavily on iNDF as a marker for digesta flow.

Several unexpected effects of HMB supplementation were observed in rumen measurements. Firstly, acetate concentration was decreased while propionate concentration was increased. This is contrary to what Rosser et al. (1971) and Lundquist et al. (1973) observed in vivo. In two more recent studies (Vázquez-Añón et al., 2001; Noftsger et al., 2005), an effect of HMB on VFA production was not observed. The only interaction of HMB and diet type observed in this study was the acetate to propionate ratio, which decreased when HMB was supplemented with 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 HMB was supplemented with the Low MP diet. As no others have observed an increase in the concentration of propionate in response to HMB, we have no explanation for this interaction. Secondly, rumen pH was decreased with HMB supplementation, while no effect on rumen pH was observed in work by Vázquez-Añón et al. (2001) and Noftsger et al. (2005). Our observations of

increased rumen VFA concentration were the likely driver of the reduction in pH. Thirdly, 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 HMB supplementation may stimulate protein digestion, releasing more ammonia in the rumen. With mixed results, the effects of HMB on VFA concentration, ammonia, and pH in the rumen remains unclear and warrants further investigation.

An effect of HMB supplementation on MCP yield was not observed in this study, similar to work by Noftsger et al. (2005). This may have been partially due to the large amount of variability in estimates of duodenal MCP concentration we observed using purines as markers. Variation of observations in MCP flow using purines as markers is not uncommon due to the apparent variability in the concentration of purines in bacteria; Obispo and Dehority (1999) observed a variation of the concentration of purines as a percent of DM ranging from 0.69 to 5.57 % in 10 pure cultures. A meta-analysis of studies involving purines (Clark et al., 1992) found a range of estimates of purine concentration in mixed ruminal bacteria from 2.40 to 13.02 %. Furthermore, our methodology did not account for the contributions of endogenous protein to duodenal purine concentration, which may account for 1 to 7 % of duodenal protein flow (Rulquin et al., 1998). These limitations may be overcome experimentally by increasing sample size, replication, and therefore, statistical power. In addition, variation of duodenal digesta flow estimates and may ultimately contribute to variation in MCP flow observations. For example, using iADF as a digesta flow marker, Castillo-Lopez et al. (2014) observed an average of 15.2 kg duodenal DM flow, while our observations

averaged 20.5 kg duodenal DM flow using iADF; differences in duodenal flow estimates will largely impact calculated MCP flow.

Unfortunately, microbial protein flow using DNA markers was not calculated, as the initial mass of samples used for DNA extraction were not recorded. Rather, equal volumes of samples were used, and mass was assumed to be the same. Due to the analytical precision needed to attain reasonable estimates of MCP, especially in light of the exponential amplification of PCR, we found that small variations in initial sample mass largely impacted final estimates of MCP, therefore MCP flow estimates were not reported. Alternatively, results were expressed as Ct values derived from RT-PCR targeting either bacterial or protozoal DNA in equal volumes of duodenal fluid, and as abundance of target DNA per g of duodenal fluid DM. No effects of HMB, level of MP, or an interaction between the two on abundance of target DNA or Ct were observed for bacteria, supporting our observations of MCP flow made using purines as microbial markers, where no differences were observed. The Ct values which we observed when targeting bacterial DNA were higher than expected and need to be revisited. The supplementation of HMB appeared to increase Ct when targeting protozoal DNA in duodenal fluid, suggesting that, because more thermal cycles were required to reach the amplification threshold, less protozoal DNA was present in the duodenal fluid of cows receiving HMB than those receiving the control.

A plethora of data were generated as a result of bioinformatics analysis of rumen microbial DNA. At the phylum level, the rumen microbiome was largely unaffected by treatment (Table 3.8), however, HMB supplementation seemed to increase the proportion of *Fibrobacteres*. Ramirez et al. (2012) observed that proportion of *Fibrobacteres*

relative to the total bacterial population tended to be sensitive to dietary changes. Increased proportions of *Fibrobacteres* may contribute to the enhanced fiber digestibilities others have observed in response to HMB supplementation (Gil et al., 1973; Bull and Vandersall, 1973; Noftsger et al., 2005), although we did not observe increased fiber digestibility in this study. Community structure was not affected by Low or High MP diets; while minor adjustments in RDP and available carbohydrates were made in order to alter concentration of dietary MP between diets, these did not result in changes in the microbial community structure. A number of associations were drawn between OTUs and metadata resulting from the MaAsLin analysis, suggesting specific microorganisms were related to metadata. Only one OTU was found to be associated with HMB supplementation and was identified by the BLAST analysis as *Anoxynatronum sibricum*. When HMB was supplemented, the relative abundance of *Anoxynatronum sibricum* decreased. As *Anoxynatronum sibricum* is a true alkaliphile (Garnova, et al., 2003), our observations of decreased ruminal pH with HMB supplementation may explain the decrease in the relative abundance of this OTU. One of the strongest associations discovered by the analysis was with rumen ammonia concentration and OTU 738, which the BLAST analysis identified as *Eubacterium coprostanoligenes*, or two strains of *Clostridium clariflavum*, DSM 19732 and EBR45. *Eubacterium coprostanoligenes* may not be the proper microorganism represented by this OTU, as ruminal pH is too low to support its growth (Vos et al., 2009). A more likely candidate, *Clostridium clariflavum* DSM 19732 contains the gene encoding for aspartate-ammonia ligase (Izquierdo et al., 2012), which catalyzes the conversion of L-asparate to L-asparagine in the presence of ATP and ammonia (Hinchman et al., 1992). Asparagine

plays an important role in the synthesis of ammonia (Bishop et al., 2013). Another association drawn by the analysis was between OTU 451, which the BLAST analysis identified as *Butyrvibrio crossotus*, and g microbial N flow per day as well as microbial N flow as a % of the total N flow. This association, albeit weaker than others observed within this study, could be explored in future studies for use as a marker for microbial N flow. The MaAsLin analysis also revealed associations of a total of 94 OTUs related to specific experimental animals, 86 of which were found to be unique to a single animal, cow number 3069; the difference in the rumen microbial community structure of this cow is illustrated in a principal coordinate analysis in Figure 3.19. The difference in this cow's rumen microbial community structure may be explained by the fact that she was a relatively new animal to the research facility, being purchased from a commercial farm several weeks prior to the commencement of the experiment. Additionally, the cow underwent rumen and duodenal cannulation surgery several weeks prior to collection. Further research needs to be conducted in order to determine if environment, cannulation surgery, or drugs administered during and after surgery played a role in differing community structures. Sequencing technology is an emerging science and application of this information is not completely developed, however, it presents vast opportunities for discoveries related to host-bacterial interactions, opening the door for advances in animal health, productivity, and food safety (Krause et al., 2014).

In conclusion, continued inclusion of HMB in dairy diets is advisable due to its enhancing effects on milk production and milk fat yield (Zanton et al., 2014). However, a better understanding of its effects in the rumen, including potential influences on the

microbial community structure, would help nutritionists know how to best utilize it as a supplement in dairy rations.

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TABLES AND FIGURES

Table 3.1 Ingredient and chemical composition of TMR fed during the experiment formulated to supply concentrations of metabolizable protein either deficient or in excess of requirements for lactating dairy cows[1]

	Level of Metabolizable Protein	
Ingredient, % DM	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[2]	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[3]	0.17	0.17
Mineral premix[4]	0.10	0.10
Vitamin premix[5]	0.10	0.10
Chemical composition		
DM, %	59.5	59.4
CP, %	15.4	15.4
RUP, % CP	33.3	37.1
RDP, % CP	66.7	62.9
NDF, %	33.7	33.8
ADF, %	22.1	22.2
Starch, %	27.1	24.9
Ether extract, %	2.71	2.62
MP, kg[6]	2.13	2.27
NE_L, Mcal[6]	33.2	33.5

[1]According to the CPM Dairy Ration Analyzer (v3.0.8.1; Boston et al., 2000).
[2]Dried distillers grains with solubles.
[3]Megalac (Church & Dwight Co. Inc., Princeton, NJ).
[4]Formulated to contain 1.0 % Ca, 0.50 % P, 0.36 % Mg, and 1.3 % K.
[5]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.
[6]According to the dairy NRC (2001) model.

Table 3.2 Chemical composition and particle size of Low and High MP TMR fed during the experiment[1]

	Level of Metabolizable Protein			
	Low MP		High MP	
	Mean	SD	Mean	SD
Chemical, % 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
ADICP[2]	1.15	0.04	1.23	0.10
NDICP[3]	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
Lignin, % NDF	9.46	2.04	9.89	2.66
NFC[4]	38.2	1.18	37.0	2.42
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
Fe, ppm	370	24.8	383	20.3
Zn, ppm	95.8	5.56	103	5.94
Cu, ppm	27.3	1.71	28.8	0.96
Mn, ppm	87.5	5.80	93.0	2.58
Particle Size, %[5]				
> 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

[1]Values determined by Cumberland Valley Analytical Services, Hagerstown, MD.
[2]Acid detergent insoluble crude protein.
[3]Neutral detergent insoluble crude protein.
[4]NFC = Nonfiber carbohydrate calculated by difference 100 - (% NDF + % CP + % Fat + % Ash).
[5]Determined using the Penn State Particle Separator on wet basis (Heinrichs and Kononoff, 2002).

Table 3.3 Chemical composition of corn silage, alfalfa hay, brome hay, Low and High MP concentrates, and control and 2-hydroxy-4-methylthio-butanoic acid (HMB) top dress fed during the experiment[1] (n = 4)

	Corn Silage		Alfalfa Hay		Brome Hay		Low MP Concentrate		High MP Concentrate		Control Top Dress		HMB Top Dress	
Chemical, % DM	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[2]	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[3]	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
RDP	5.98	0.45	12.9	0.34	9.70	0.56	--	--	--	--	--	--	--	--
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[4]	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, ppm[5]	310	195	252	51.6	192	29.0	595	85.7	600	60.6	66.8	10.1	98.8	12.2
Zn, ppm	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, ppm	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, ppm	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

[1]Values determined by Cumberland Valley Analytical Services, Hagerstown, MD.
[2]Acid detergent insoluble crude protein.
[3]Neutral detergent insoluble crude protein.
[4]NFC = Nonfiber carbohydrate calculated by difference 100 - (% NDF + % CP + % Fat + % Ash).
[5]Parts per million.

Table 3.4 Effects of feeding 2-hydroxy-4-methylthio-butanoic acid (HMB) with Low and High MP diets on milk production and composition

	Treatment							
	Low MP		High MP			*P*-value[1]		
	Control	HMB	Control	HMB	SEM[2]	MP	HMB	MP × HMB
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[4], mg/DL	11.6	12.0	11.0	11.8	0.97	0.41	0.21	0.66

[1]*P*-values for contrasts of level of metabolizable protein and HMB supplementation.
[2]The highest standard error of treatment means is shown.
[3]Energy corrected milk = 0.327 × milk yield [kg] + 12.95 × fat [kg] + 7.20 × protein [kg] adjusted for 3.5 % fat and 3.2 % total protein (DHI Glossary, 2014).
[4]Milk Urea Nitrogen.

Table 3.5 Effects of feeding 2-hydroxy-4-methylthio-butanoic acid (HMB) with high or low MP diets on nutrient digestibility estimated using chromium oxide, iNDF or iADF as digesta flow markers

	Treatment							
	Low MP		High MP			*P*-value[2]		
	Control	HMB	Control	HMB	SEM[1]	MP	HMB	MP × HMB
Cr_2O_3 marker								
DM, %	58.8	61.7	64.2	65.3	2.45	0.07	0.37	0.66
N, %	58.8	62.0	61.7	64.0	2.39	0.25	0.21	0.83
NDF, %	38.8	42.2	45.7	47.9	3.80	0.11	0.44	0.87
OM, %	55.4	58.6	61.1	62.2	2.66	0.08	0.37	0.66
iNDF marker								
DM, %	69.8	69.0	67.2	68.1	1.80	0.03	0.94	0.21
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
iADF marker								
DM, %	62.6	61.5	57.4	62.1	3.18	0.45	0.56	0.34
N, %	63.0	61.8	54.4	60.9	2.93	0.11	0.33	0.18
NDF, %	44.2	41.4	34.9	42.8	5.17	0.44	0.62	0.32
OM, %	59.5	58.3	53.7	58.7	3.44	0.41	0.56	0.34

[1]The highest standard error of treatment means is shown.
[2]*P*-values for contrasts of level of metabolizable protein and HMB supplementation.

Table 3.6 Effects of feeding 2-hydroxy-4-methylthio-butanoic acid (HMB) with Low or High MP diets on rumen pH, pool, kinetics, and concentration of ammonia and volatile fatty acids

	Treatment							
	Low MP		High MP			*P*-value[1]		
	Control	HMB	Control	HMB	SEM[2]	MP	HMB	MP × HMB
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
Rates[3], %/h								
DM k_p	2.89	3.17	2.87	3.07	0.36	0.72	0.19	0.82
DM k_d	6.60	6.95	5.91	6.39	0.57	0.15	0.31	0.86
NDF k_p	2.72	2.93	2.58	2.64	0.33	0.34	0.53	0.71
NDF k_d	3.34	3.27	2.61	2.92	0.31	0.04	0.57	0.39
Ammonia, mg/dl	17.0	17.3	16.0	17.2	0.88	0.14	0.06	0.30
Total VFA, (mM)	120	126	118	126	6.36	0.77	0.02	0.65
VFA mol/100 mol								
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
Valarate	1.45	1.45	1.59	1.67	0.09	<0.01	0.33	0.33
A:P[4]	2.82	2.52	2.75	2.64	0.20	0.60	<0.01	0.04

[1]*P*-values for contrasts of level of metabolizable protein and HMB supplementation.
[2]The highest standard error of treatment means is shown.
[3]Rate of passage (k_p); rate of digestion (k_d).
[4]Ratio of acetate to propionate.

Table 3.7 Effects of feeding 2-hydroxy-4-methylthio-butanoic acid (HMB) with Low and High MP diets on duodenal digesta and N flows estimated using purines and either chromium oxide, iNDF or iADF as digesta flow markers

	Treatment							
	Low MP		High MP			*P*-value[1]		
	Control	HMB	Control	HMB	SEM[2]	MP	HMB	MP × HMB
Cr_2O_3 marker								
Duodenal Flow, kg DM/d	17.8	17.5	17.7	17.7	1.91	0.97	0.90	0.92
Duodenal N Flow, g DM/d	766	772	787	755	48.3	0.94	0.66	0.51
Microbial N Flow, g DM/d	367	303	385	355	55.4	0.35	0.23	0.65
Microbial N of total N Flow, %	48.5	39.7	47.3	43.2	5.27	0.78	0.15	0.57
iNDF marker								
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 DM/d	687	703	670	773	62.5	0.60	0.27	0.41
Microbial N Flow, g DM/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
iADF marker								
Duodenal Flow, kg DM/d	20.8	21.4	18.4	21.5	1.88	0.56	0.36	0.53
Duodenal N Flow, g DM/d	911	964	825	982	91.0	0.72	0.29	0.59
Microbial N Flow, g DM/d	469	345	390	406	65.2	0.89	0.43	0.32
Microbial N of total N Flow, %	49.8	39.7	49.2	43.8	6.11	0.72	0.15	0.64

[1]*P*-values for contrasts of level of metabolizable protein and HMB supplementation.
[2]The highest standard error of treatment means is shown.

Table 3.8. Effects of feeding 2-hydroxy-4-methylthio-butanoic acid (HMB) with Low or High MP diets on bacterial and protozoal DNA abundance in duodenal fluid as estimated by real-time PCR

	Treatment							
	Low MP		High MP			P-Value[2]		
	Control	HMB	Control	HMB	SEM[1]	MP	HMB	MP × HMB
Bacteria								
Ct[3]	39.9	40.6	40.5	40.4	0.29	0.46	0.30	0.22
Abundance/g DM[4]	2.28E-03	7.66E-03	3.26E-02	1.18E-04	0.02	0.48	0.41	0.26
Protozoa								
Ct[5]	25.3	25.8	25.2	26.3	0.54	0.53	0.05	0.46
Abundance/g DM	2.00	0.20	0.61	9.24	3.93	0.37	0.42	0.23

[1]Highest standard error of treatment means is shown.

[2]P-values for contrasts of level of MP and HMB supplementation.

[3]Cycle thresholds resulting from RT-PCR targeting DNA encoding part of the bacterial 16S rRNA gene.

[4]Abundance of targeted DNA per gram of duodenal fluid DM; Abundance = (1/Efficency^Ct) (Castillo-Lopez et al., 2010).

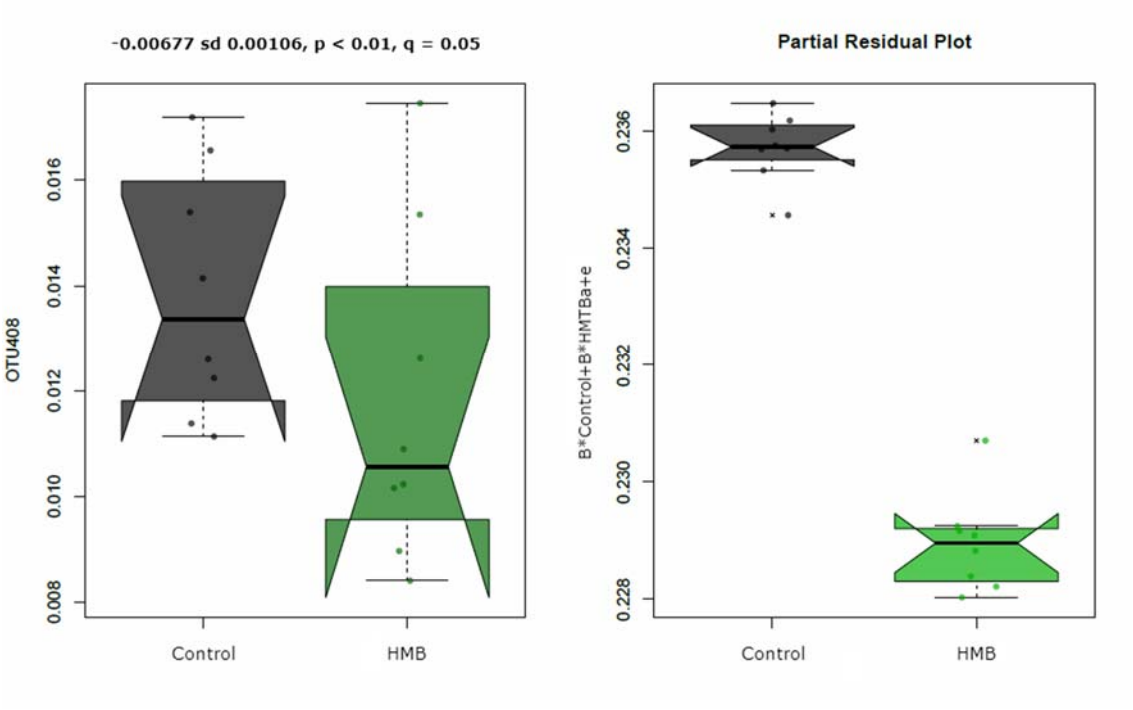
[5]Cycle threshold resulting from RT-PCR targeting DNA encoding part of the protozoal 18S rRNA gene.

Table 3.9 Effects of feeding 2-hydroxy-4-methylthio-butanoic acid (HMB) with Low and High MP diets on proportion on proportion of phyla of rumen bacterial populations relative to total number of reads recovered from ruminal digesta attained from bioinformatics analysis of total and core OTUs

	Treatment							
	Low MP		High MP			*P*-Value[1]		
	Control	HMB	Control	HMB	SEM[2]	MP	HMB	MP × HMB
Total Analysis								
Firmicutes	59.4	56.8	56	57.1	2.92	0.51	0.74	0.43
Bacteroidetes	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
Proteobacteria	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
Tenericutes	1.10	1.03	1.25	1.18	0.14	0.34	0.62	1.00
Actinobacteria	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
Spirochaetes	0.50	0.58	0.60	0.68	0.13	0.20	0.33	1.00
Fibrobacteres	0.31	0.46	0.39	0.44	0.06	1.00	0.11	0.21
Verrucomicrobia	0.10	0.05	0.15	0.10	0.03	0.17	0.17	0.62
Core Analysis								
Firmicutes	61.4	59.3	58.2	58.9	2.76	0.43	0.77	0.54
Bacteroidetes	27.0	28.3	29.7	29.0	2.23	0.34	0.86	0.58
Other	3.70	3.15	2.68	3.18	0.39	0.25	0.95	0.23
Proteobacteria	1.53	2.70	2.73	2.03	0.65	0.67	0.70	0.17
TM7	1.80	2.00	1.98	2.10	0.37	0.34	0.26	0.78
Tenericutes	1.00	0.90	1.10	1.08	0.13	0.32	0.64	0.78
Actinobacteria	0.60	0.05	0.45	0.08	0.36	0.87	0.24	0.81
SR1	0.75	0.73	0.78	1.03	0.24	0.40	0.56	0.47
Spirochaetes	0.38	0.38	0.38	0.43	0.07	0.62	0.62	0.62
Fibrobacteres	0.25	0.43	0.33	0.38	0.04	0.79	0.04	0.21
Verrucomicrobia	0.08	0.08	0.10	0.05	0.02	1.00	0.13	0.13

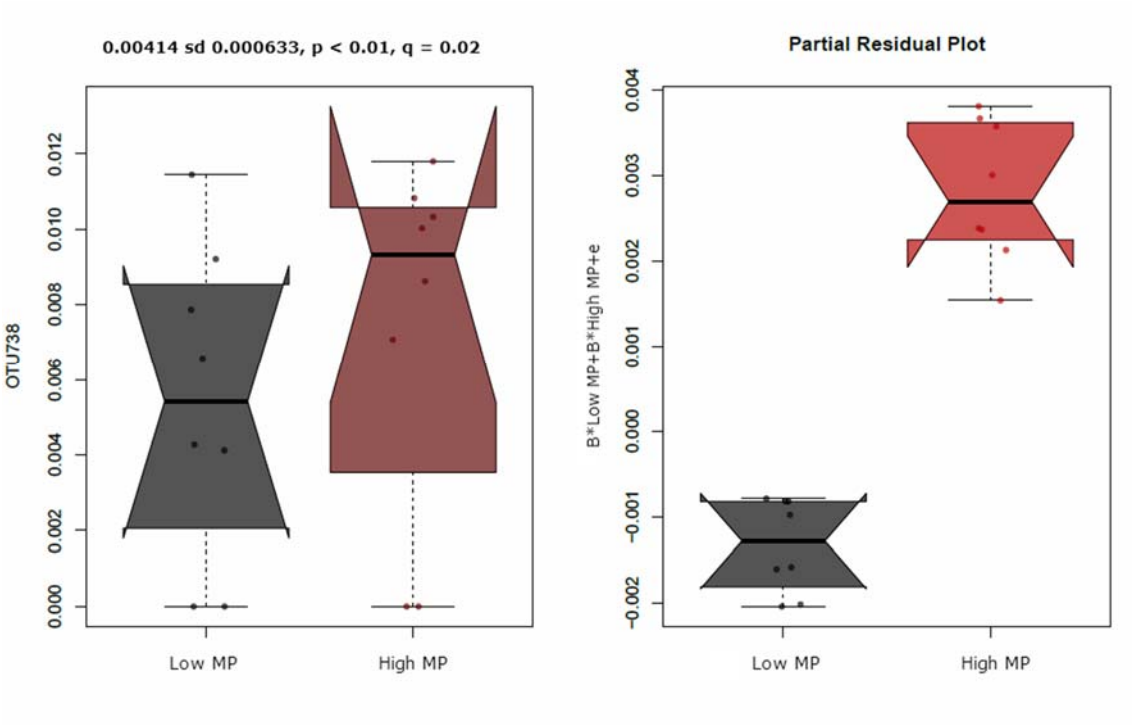
[1]*P*-values for contrasts of level of metabolizable protein and HMB supplementation.

[2]The highest standard error of treatment means is shown.



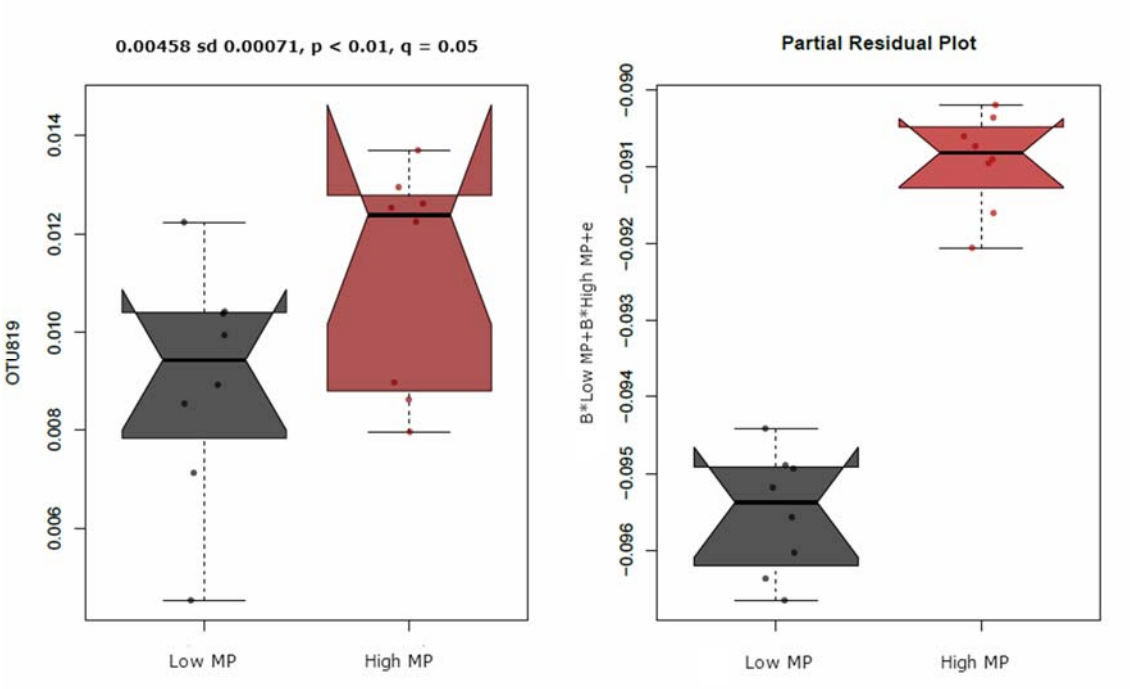
OTU 408	Strain	Query cover, %	E value	Identity, %
Anoxynatronum sibricum	Z-7981	100	2E-60	94
Clostridium sticklandii	DSM 519	100	6E-60	94
Acetoanaerobium noterae	NOT-3	100	6E-60	94

Figure 3.1 The association of the relative abundance of OTU 408 and the control or 2-hydroxy-4-methylthio-butanoic acid (HMB) top dress, along with the complementary partial residual plot. The r (sd) coefficient effect size, and *P* and *q* value tests for significance, are shown above the figure. Below the figure, the top three microorganisms matching the OTU as identified by NCBI Standard Nucleotide BLAST (Bethesda, MD) are listed.



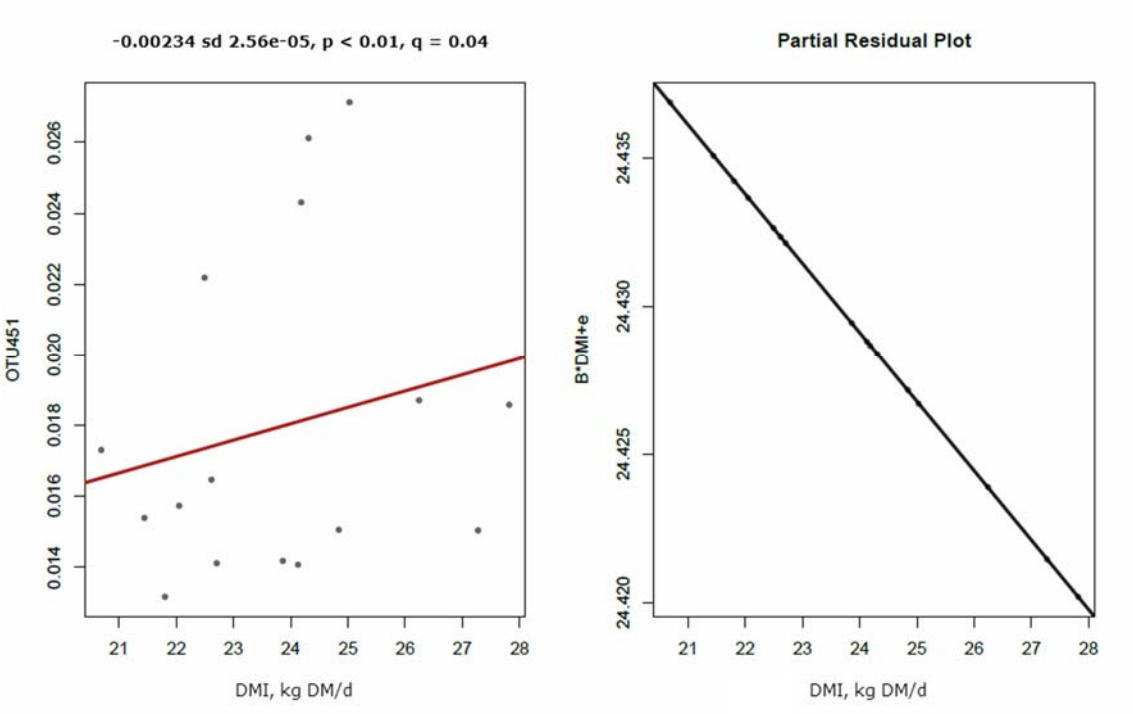
OTU 783	Strain	Query cover, %	E value	Identity, %
Prevotella bivia	JCM 6331	100	2E-60	91
Prevotella saccharolytica	JCM 17484	100	1E-58	90
Prevotella oralis	JCM 12251	100	1E-58	90

Figure 3.2 The association of the relative abundance of OTU 783 and Low or High MP diets, along with the complementary partial residual plot. The r (sd) coefficient effect size, and *P* and *q* value tests for significance, are shown above the figure. Below the figure, the top three microorganisms matching the OTU as identified by NCBI Standard Nucleotide BLAST (Bethesda, MD) are listed.



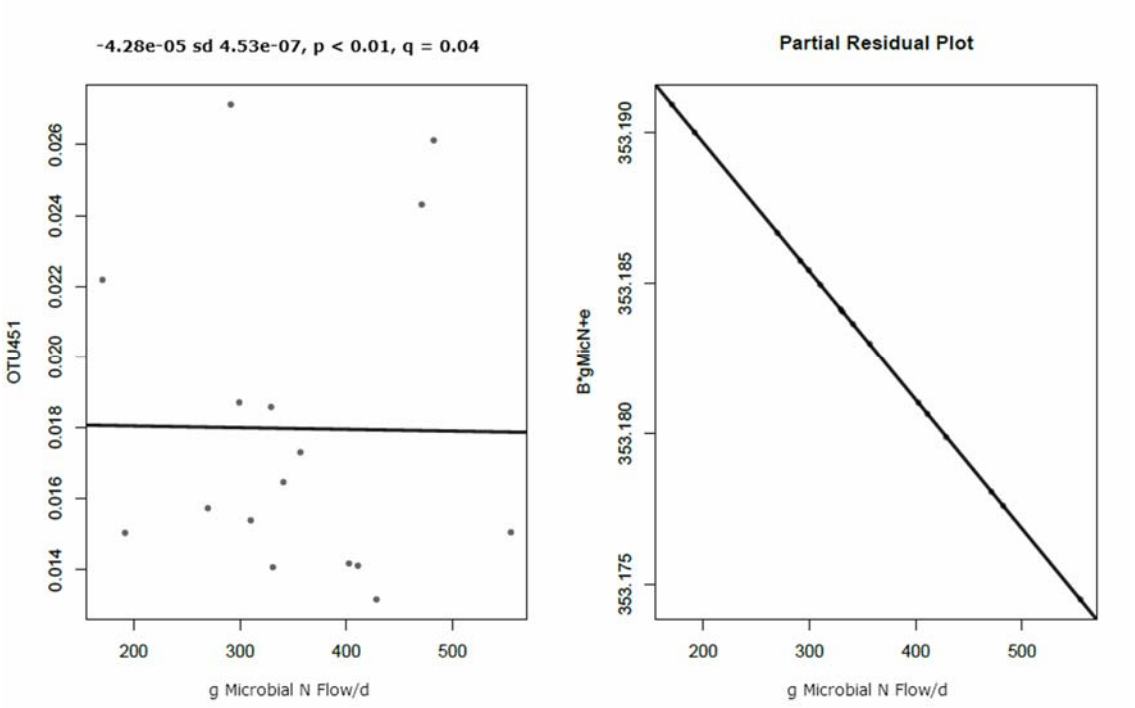
OTU 819	Strain	Query cover, %	E value	Identity, %
Galbibacter mesophilus	NBRC 101624	100	2E-45	86
Galbibacter mesophilus	Mok-17	100	2E-45	86
Gangjinia marincola	GJ16	100	5E-45	84

Figure 3.3 The association of the relative abundance of OTU 819 and Low or High MP diets, along with the complementary partial residual plot. The r (sd) coefficient effect size, and *P* and *q* value tests for significance, are shown above the figure. Below the figure, the top three microorganisms matching the OTU as identified by NCBI Standard Nucleotide BLAST (Bethesda, MD) are listed.



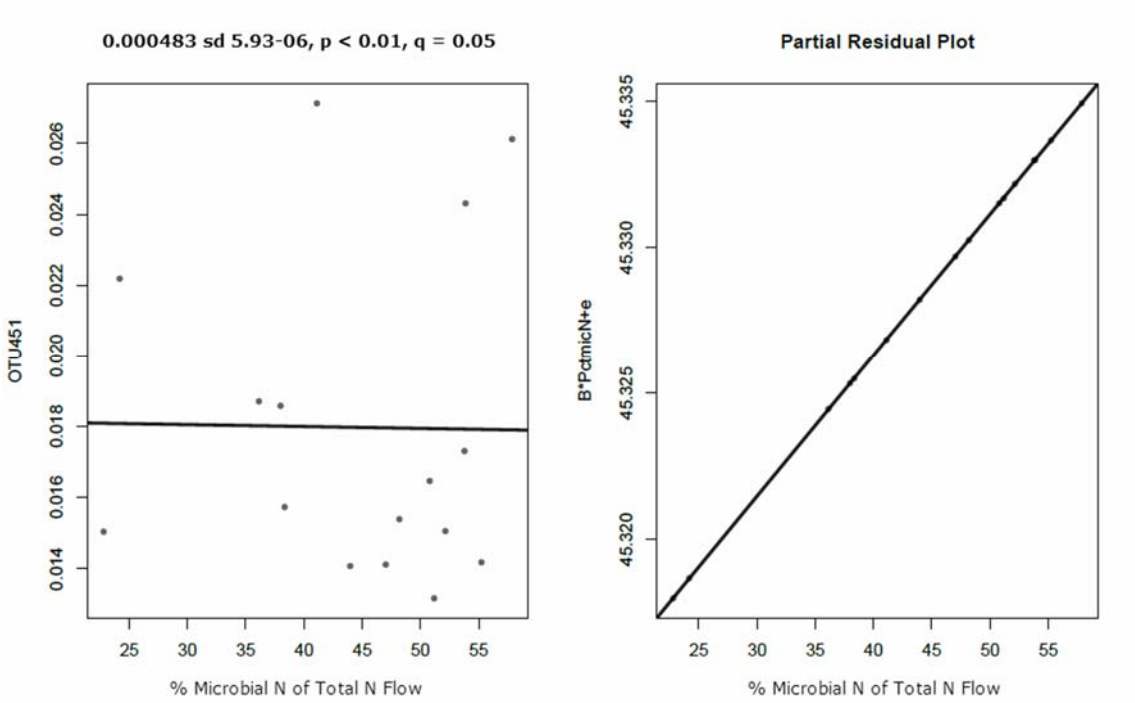
OTU 451	Strain	Query cover, %	E value	Identity, %
Butyrivibrio crossotus	DSM 2876	100	5E-71	98
Colstridium hathewayi	1313	100	2E-69	97
Eubacterium rectale	ATCC 33656	100	1E-67	97

Figure 3.4 The association of the relative abundance of OTU 451 and DMI (kg DM/d), along with the complementary partial residual plot. The r (sd) coefficient effect size, and *P* and *q* value tests for significance, are shown above the figure. Below the figure, the top three microorganisms matching the OTU as identified by NCBI Standard Nucleotide BLAST (Bethesda, MD) are listed.



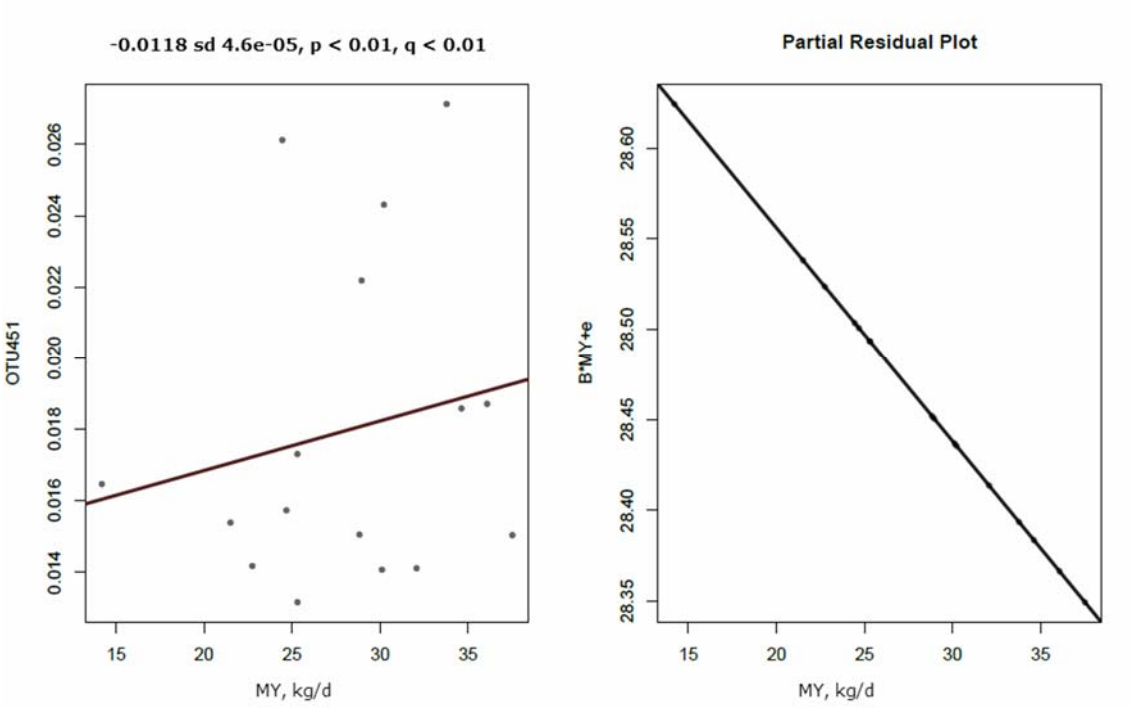
OTU 451	Strain	Query cover, %	E value	Identity, %
Butyrivibrio crossotus	DSM 2876	100	5E-71	98
Colstridium hathewayi	1313	100	2E-69	97
Eubacterium rectale	ATCC 33656	100	1E-67	97

Figure 3.5 The association of the relative abundance of OTU 451 and g microbial N flow per day, along with the complementary partial residual plot. The r (sd) coefficient effect size, and *P* and *q* value tests for significance, are shown above the figure. Below the figure, the top three microorganisms matching the OTU as identified by NCBI Standard Nucleotide BLAST (Bethesda, MD) are listed.



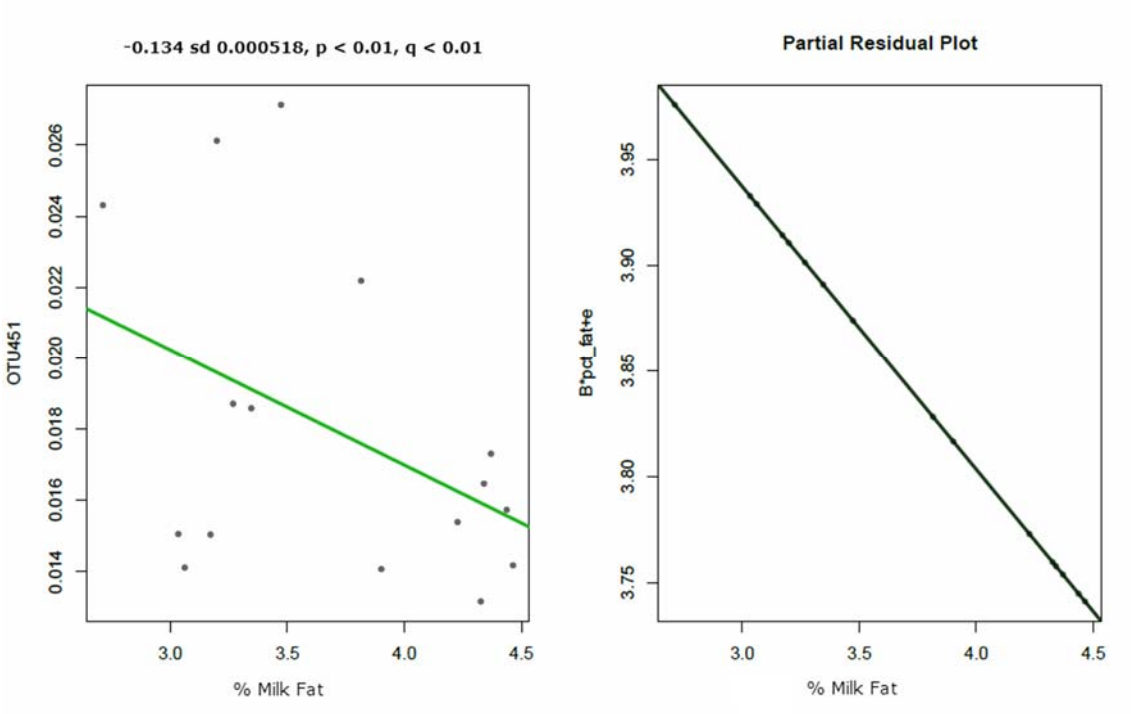
OTU 451	Strain	Query cover, %	E value	Identity, %
Butyrivibrio crossotus	DSM 2876	100	5E-71	98
Colstridium hathewayi	1313	100	2E-69	97
Eubacterium rectale	ATCC 33656	100	1E-67	97

Figure 3.6 The association of the relative abundance of OTU 451 and percent microbial N of total N flow per day, along with the complementary partial residual plot. The r (sd) coefficient effect size, and *P* and *q* value tests for significance, are shown above the figure. Below the figure, the top three microorganisms matching the OTU as identified by NCBI Standard Nucleotide BLAST (Bethesda, MD) are listed.



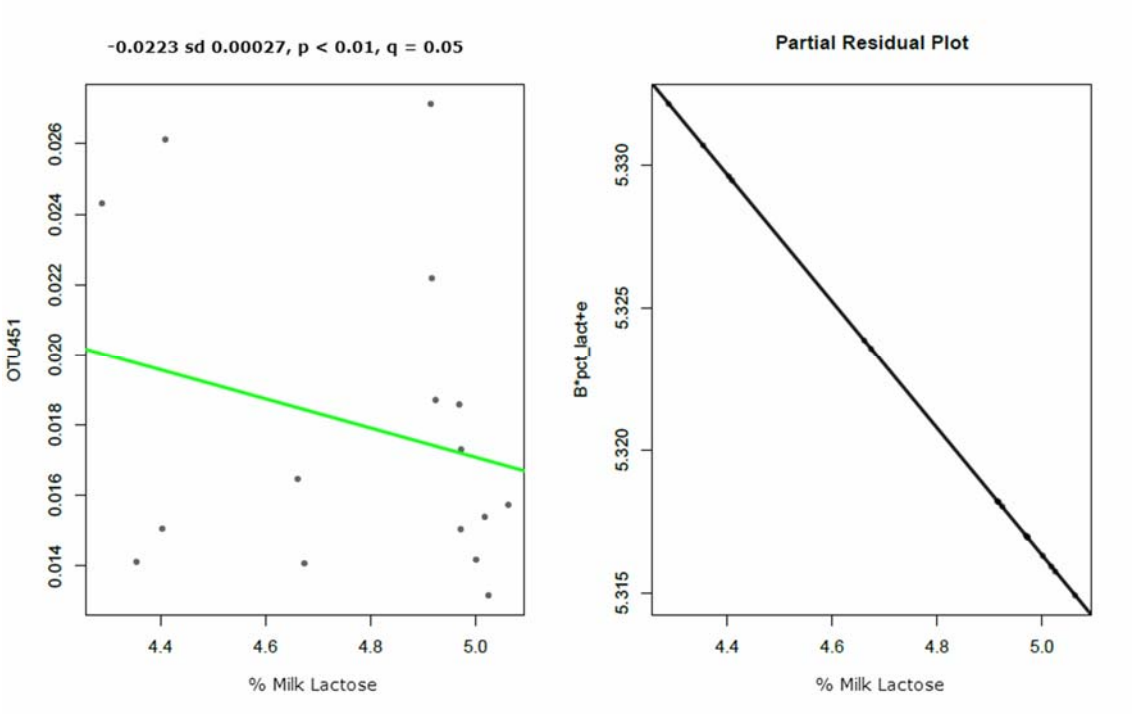
OTU 451	Strain	Query cover, %	E value	Identity, %
Butyrivibrio crossotus	DSM 2876	100	5E-71	98
Colstridium hathewayi	1313	100	2E-69	97
Eubacterium rectale	ATCC 33656	100	1E-67	97

Figure 3.7 The association of the relative abundance of OTU 451 and milk yield (kg/d), along with the complementary partial residual plot. The r (sd) coefficient effect size, and *P* and *q* value tests for significance, are shown above the figure. Below the figure, the top three microorganisms matching the OTU as identified by NCBI Standard Nucleotide BLAST (Bethesda, MD) are listed.



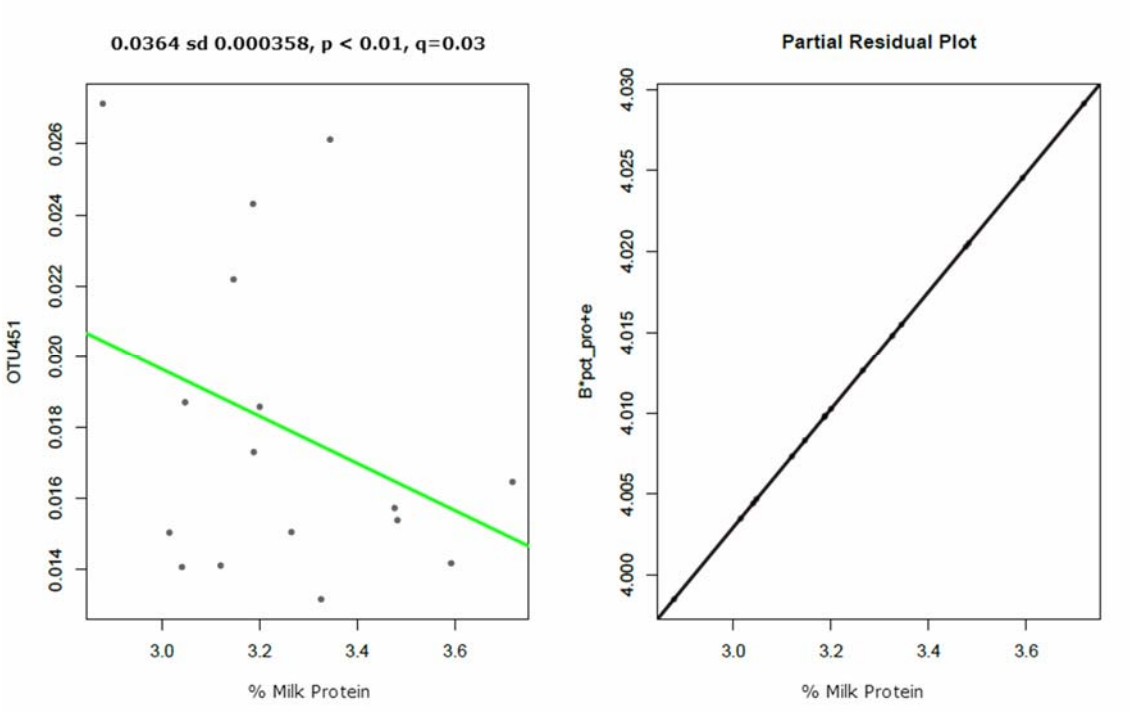
OTU 451	Strain	Query cover, %	E value	Identity, %
Butyrivibrio crossotus	DSM 2876	100	5E-71	98
Colstridium hathewayi	1313	100	2E-69	97
Eubacterium rectale	ATCC 33656	100	1E-67	97

Figure 3.8 The association of the relative abundance of OTU 451 and percent milk fat, along with the complementary partial residual plot. The r (sd) coefficient effect size, and *P* and *q* value tests for significance, are shown above the figure. Below the figure, the top three microorganisms matching the OTU as identified by NCBI Standard Nucleotide BLAST (Bethesda, MD) are listed.



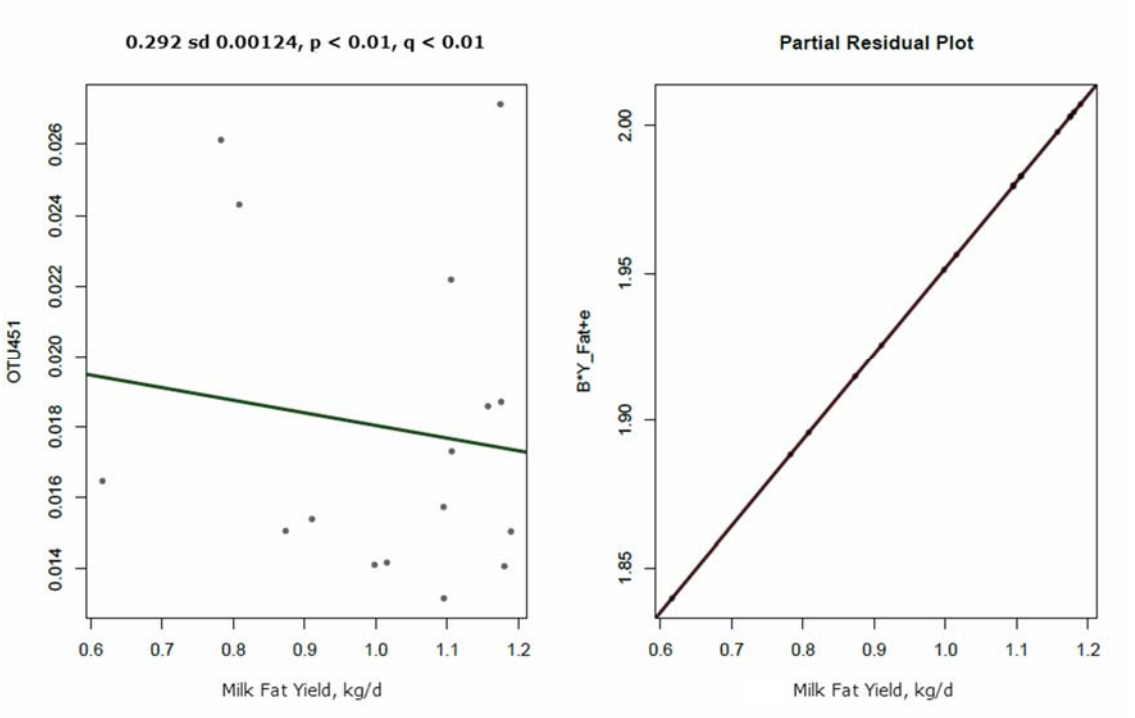
OTU 451	Strain	Query cover, %	E value	Identity, %
Butyrivibrio crossotus	DSM 2876	100	5E-71	98
Colstridium hathewayi	1313	100	2E-69	97
Eubacterium rectale	ATCC 33656	100	1E-67	97

Figure 3.9 The association of the relative abundance of OTU 451 and percent milk lactose, along with the complementary partial residual plot. The r (sd) coefficient effect size, and *P* and *q* value tests for significance, are shown above the figure. Below the figure, the top three microorganisms matching the OTU as identified by NCBI Standard Nucleotide BLAST (Bethesda, MD) are listed.



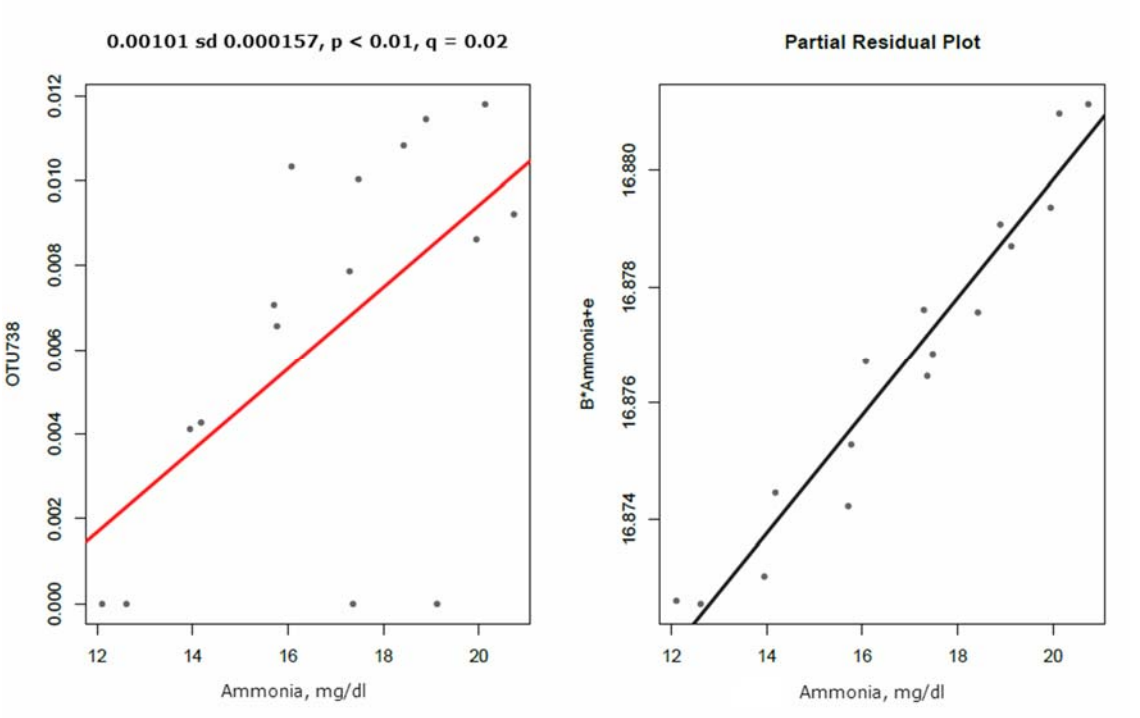
OTU 451	Strain	Query cover, %	E value	Identity, %
Butyrivibrio crossotus	DSM 2876	100	5E-71	98
Colstridium hathewayi	1313	100	2E-69	97
Eubacterium rectale	ATCC 33656	100	1E-67	97

Figure 3.10 The association of the relative abundance of OTU 451 and percent milk protein, along with the complementary partial residual plot. The r (sd) coefficient effect size, and *P* and *q* value tests for significance, are shown above the figure. Below the figure, the top three microorganisms matching the OTU as identified by NCBI Standard Nucleotide BLAST (Bethesda, MD) are listed.



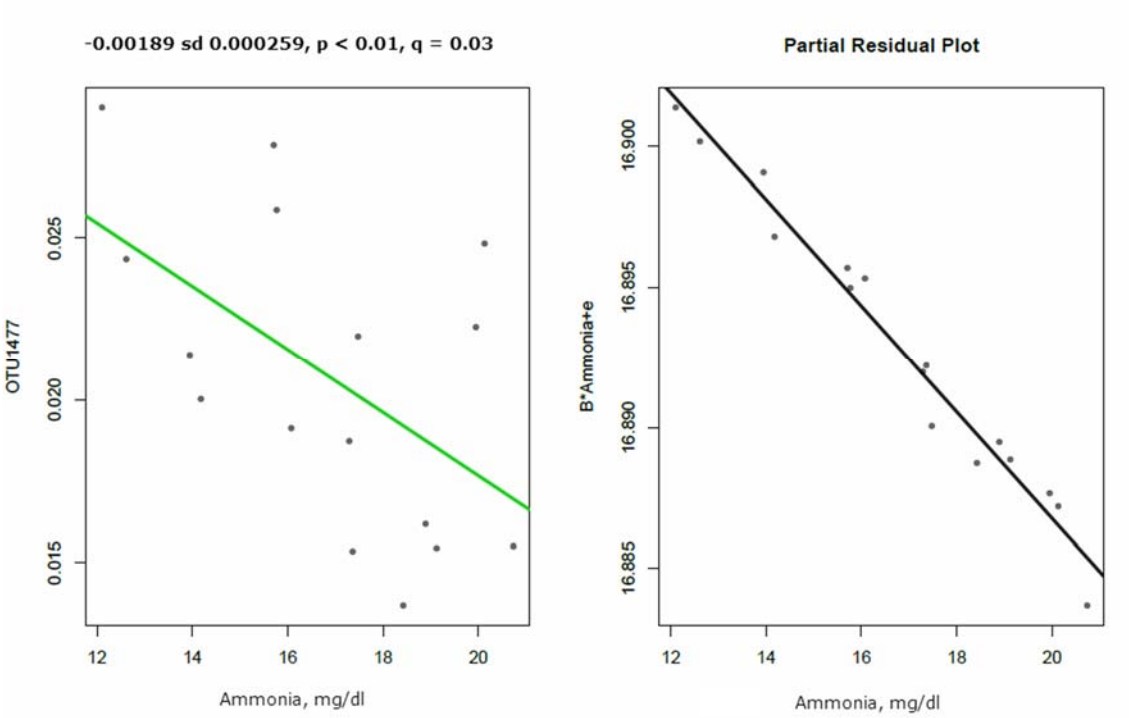
OTU 451	Strain	Query cover, %	E value	Identity, %
Butyrivibrio crossotus	DSM 2876	100	5E-71	98
Colstridium hathewayi	1313	100	2E-69	97
Eubacterium rectale	ATCC 33656	100	1E-67	97

Figure 3.11 The association of the relative abundance of OTU 451 and milk fat yield (kg/d), along with the complementary partial residual plot. The r (sd) coefficient effect size, and *P* and *q* value tests for significance, are shown above the figure. Below the figure, the top three microorganisms matching the OTU as identified by NCBI Standard Nucleotide BLAST (Bethesda, MD) are listed.



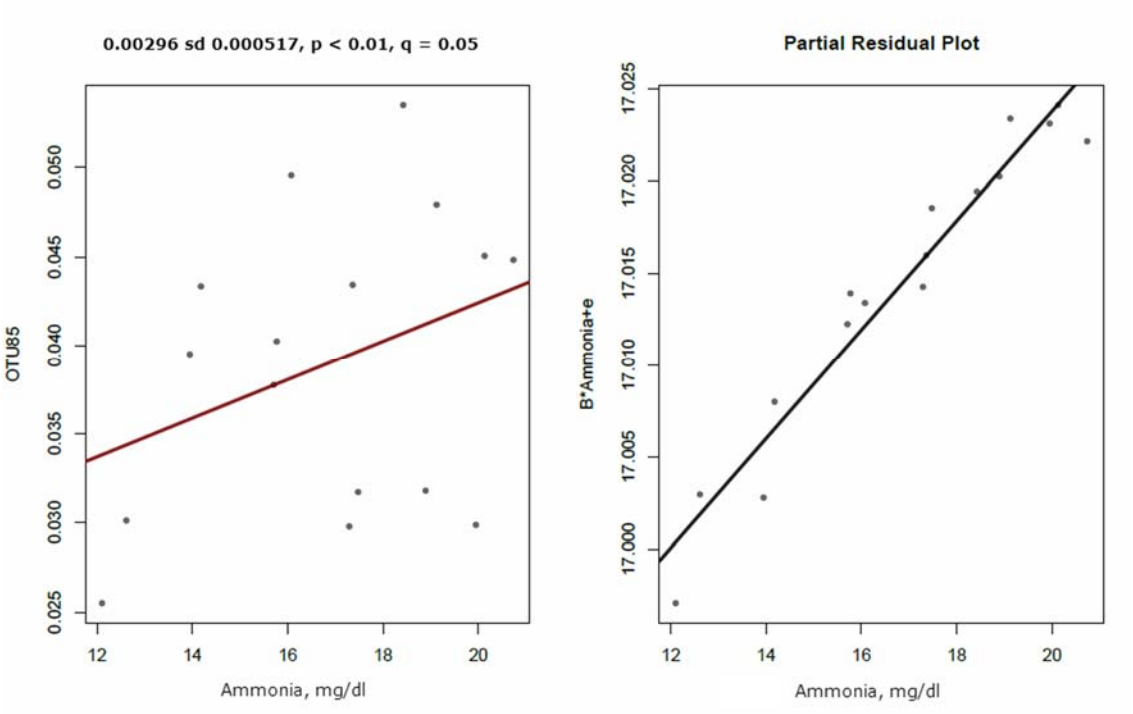
OTU 738	Strain	Query cover, %	E value	Identity, %
Eubacterium coprostanoligenes	HL	100	1E-51	90
Clostridium clariflavum	DSM 19732	100	2E-49	90
Clostridium clariflavum	EBR45	100	2E-49	90

Figure 3.12 The association of the relative abundance of OTU 738 and rumen ammonia (mg/dl), along with the complementary partial residual plot. The r (sd) coefficient effect size, and *P* and *q* value tests for significance, are shown above the figure. Below the figure, the top three microorganisms matching the OTU as identified by NCBI Standard Nucleotide BLAST (Bethesda, MD) are listed.



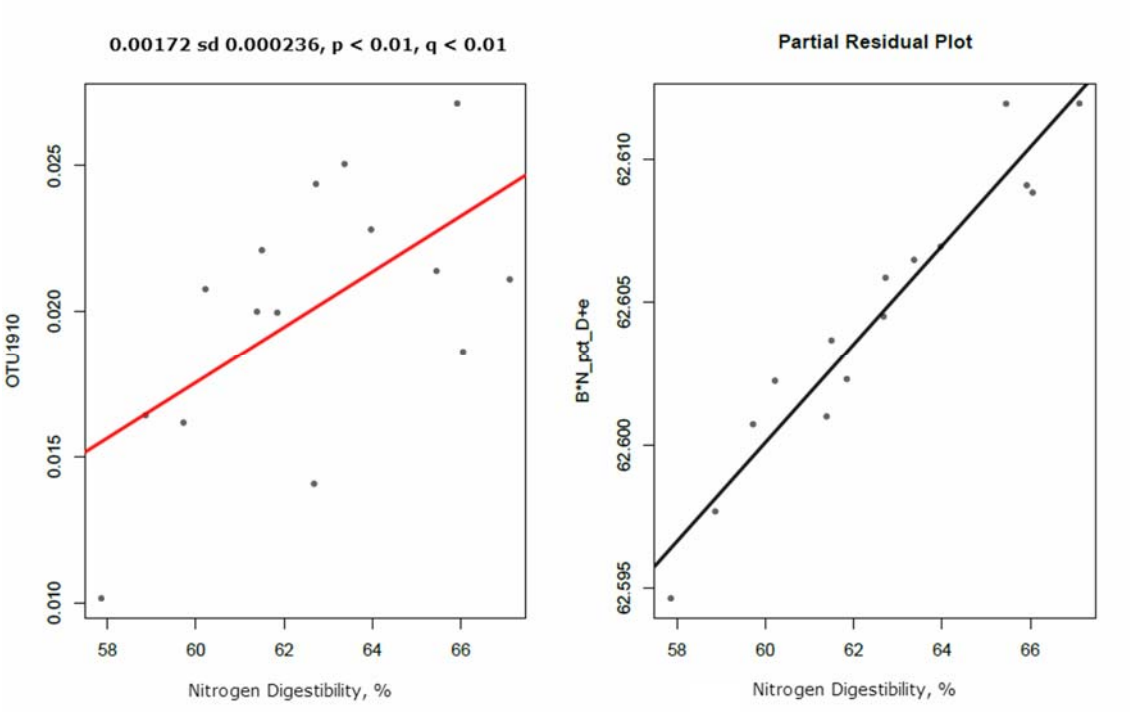
OTU 1477	Strain	Query cover, %	E value	Identity, %
Eubacterium hallii	ATCC 27751	99	4E-72	99
Coprococcus eutactus	ATCC 27759	99	8E-69	97
Clostridium herbivorans	54408	99	4E-67	97

Figure 3.13 The association of the relative abundance of OTU 1477 and rumen ammonia (mg/dl), along with the complementary partial residual plot. The r (sd) coefficient effect size, and *P* and *q* value tests for significance, are shown above the figure. Below the figure, the top three microorganisms matching the OTU as identified by NCBI Standard Nucleotide BLAST (Bethesda, MD) are listed.



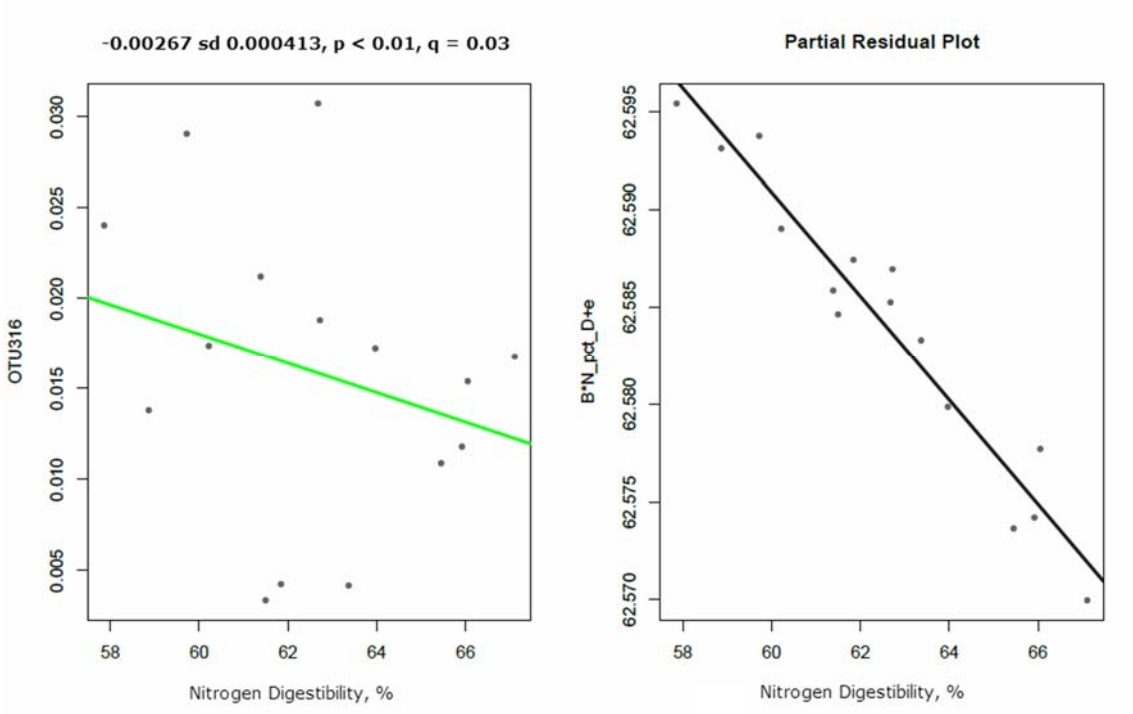
*OTU 85	Strain	Query cover, %	E value	Identity, %
Prevotella bivia	JCM 6331	100	2E-76	96
Prevotella ruminicola	Bryant 23	100	9E-74	95
Prevotella saccharolytica	JCM 17484	100	9E-74	95

Figure 3.14 The association of the relative abundance of OTU 85 and rumen ammonia (mg/dl), along with the complementary partial residual plot. The r (sd) coefficient effect size, and *P* and *q* value tests for significance, are shown above the figure. Below the figure, the top three microorganisms matching the OTU as identified by NCBI Standard Nucleotide BLAST (Bethesda, MD) are listed.



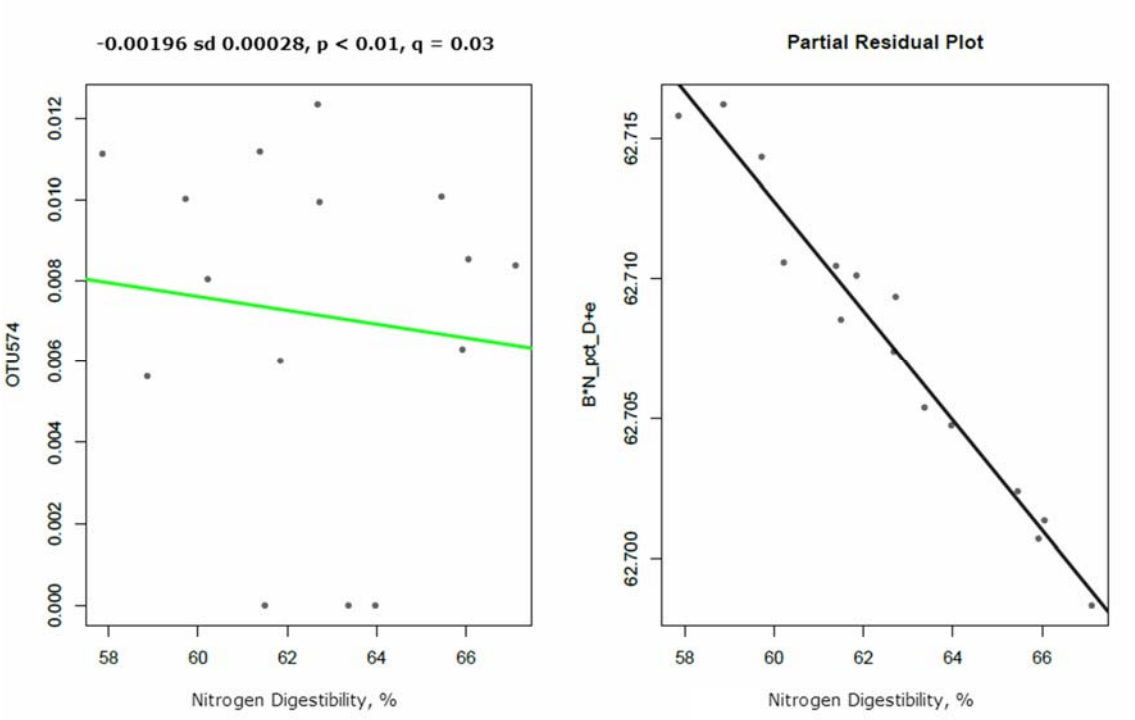
OTU 1910	Strain	Query cover, %	E value	Identity, %
Prevotella amnii	JCM 14753	100	9E-69	94
Prevotella oulorum	WPH 179	100	9E-69	94
Prevotella olorum	JCM 14966	100	4E-67	93

Figure 3.15 The association of the relative abundance of OTU 1910 and percent N digestibility, along with the complementary partial residual plot. The r (sd) coefficient effect size, and *P* and *q* value tests for significance, are shown above the figure. Below the figure, the top three microorganisms matching the OTU as identified by NCBI Standard Nucleotide BLAST (Bethesda, MD) are listed.



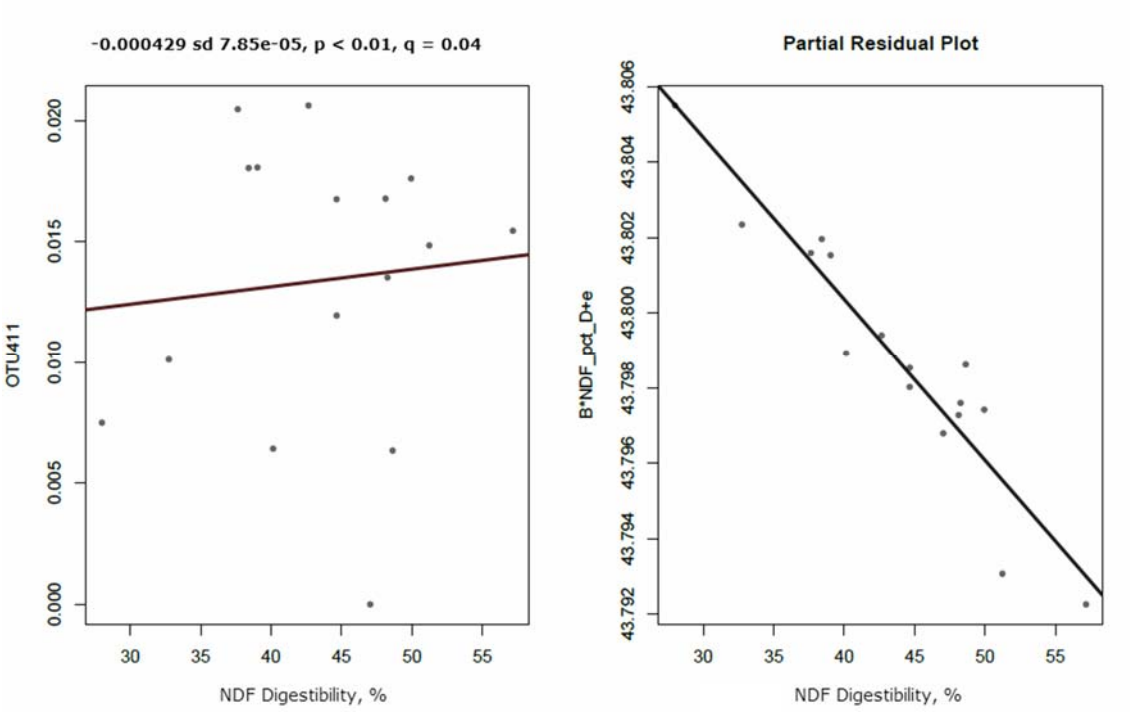
OTU 316	Strain	Query cover, %	E value	Identity, %
Mucilaginibacter kameinonensis	NBRC 102645	100	4E-47	86
Galbibacter mesophilus	NBRC 101624	100	4E-47	86
Mucilaginibacter kameinonensis	SCK	100	4E-47	86

Figure 3.16 The association of the relative abundance of OTU 316 and percent N digestibility, along with the complementary partial residual plot. The r (sd) coefficient effect size, and *P* and *q* value tests for significance, are shown above the figure. Below the figure, the top three microorganisms matching the OTU as identified by NCBI Standard Nucleotide BLAST (Bethesda, MD) are listed.



OTU 574	Strain	Query cover, %	E value	Identity, %
Intestinimonas butyriciproducens	SRB-521-5-I	100	2E-59	93
Pseudoflavonifractor capillosus	ATCC 29799	100	2E-59	93
Ruminiclostridium thermocellum	ATCC 27405	100	1E-57	92

Figure 3.17 The association of the relative abundance of OTU 574 and percent N digestibility, along with the complementary partial residual plot. The r (sd) coefficient effect size, and *P* and *q* value tests for significance, are shown above the figure. Below the figure, the top three microorganisms matching the OTU as identified by NCBI Standard Nucleotide BLAST (Bethesda, MD) are listed.



OTU 411	Strain	Query cover, %	E value	Identity, %
Bacteroides coprosuis	PC139	100	1E-58	90
Prevotella amnii	JCM 14753	100	1E-58	90
Paraprevotella clara	JCM 14859	100	1E-58	90

Figure 3.18 The association of the relative abundance of OTU 411 and percent NDF digestibility, along with the complementary partial residual plot. The r (sd) coefficient effect size, and *P* and *q* value tests for significance, are shown above the figure. Below the figure, the top three microorganisms matching the OTU as identified by NCBI Standard Nucleotide BLAST (Bethesda, MD) are listed.

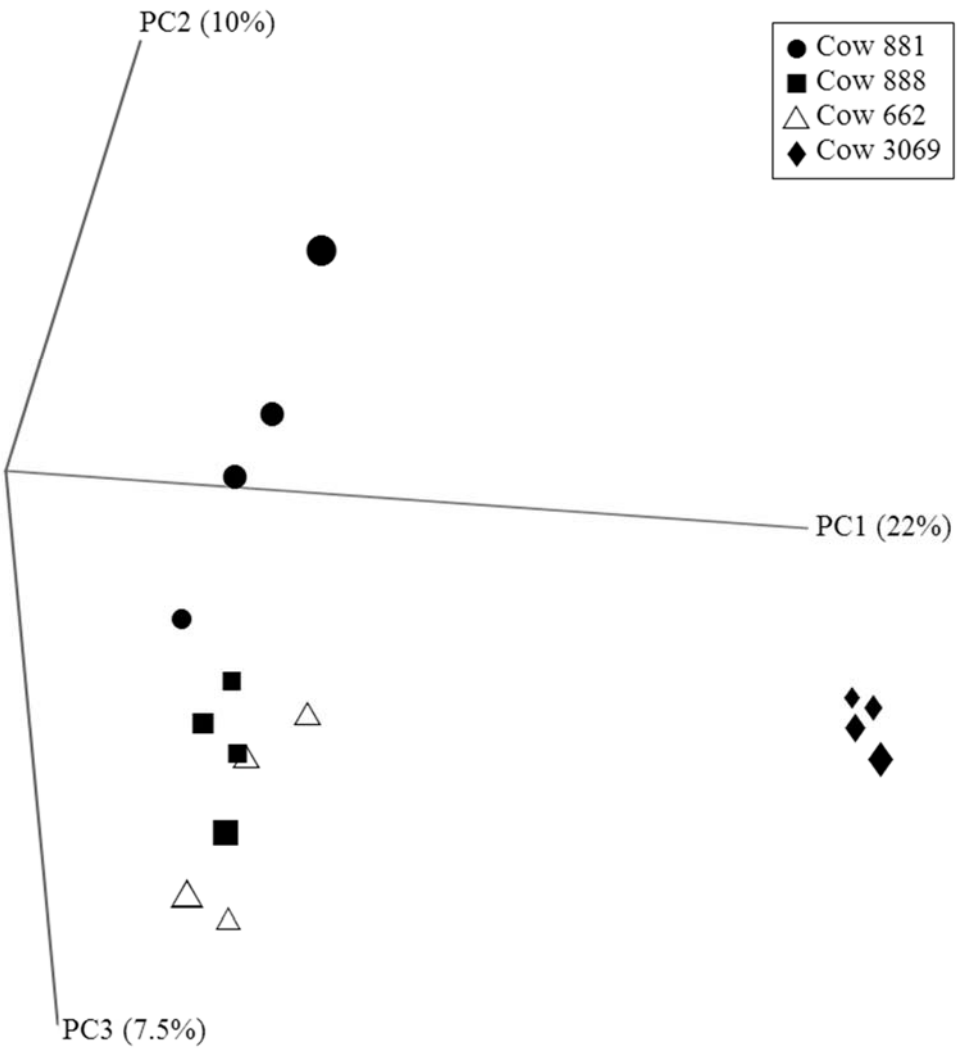


Figure 3.19 Principal coordinate analysis generated by bioinformatic analysis of microbial DNA extracted from rumen digesta. Each cow is represented by a different symbol, four for each day of collection. Distance between symbols represents dissimilarity of microbial community structure.

GENERAL SUMMARY AND CONCLUSIONS

Research into factors affecting the microbial community is a challenging yet exciting frontier in ruminant nutrition. As feed additives such as HMB may have the potential to influence microbiome structure and function, our understanding of these microorganisms and their interactions must become increasingly comprehensive in order to reap the desired results in animal productivity and health.

The use of microbial markers is an essential tool for estimating MCP yield at the duodenum. Real-time PCR allows for the targeting specific groups of microorganisms, quantifying them in terms of relative abundance based on their level of amplification. A recently developed approach, DNA markers have the potential to accurately estimated abundance of MCP, however the technique demands careful and precise lab work; for example, small errors in pipetting can be compounded exponentially during amplification. Several recommendations for future research using DNA as microbial markers have surfaced over the course of the study. First, it is necessary to accurately attain the initial mass of sample that is used for DNA extraction. In this study, the volume of initial sample was errantly recorded instead of mass, leading to complications in the calculations. Having an initial mass allows one to calculate exactly how much DNA was harvested from extraction. It may be beneficial to use the same mass for each sample going into the DNA extraction step for consistency. Second, the DNA extraction method used in this technique should be standardized, as some extraction kit protocols, such as the one used in this study (PowerMag Soil DNA Isolation Kit, Mo Bio, Carlsbad, CA), call for the transfer of an aliquot of the sample after centrifugation. This step results in a reduction of total DNA yielded from the original mass of the sample, and therefore

must be accounted for in the calculations. Consequently, it may be advisable to adopt a DNA extraction protocol in which the total DNA yielded is representative of the original mass of sample used in order to reduce the possibility of experimental error introduced by these transferring steps. Third, the current technique utilizes Ct values in order to calculate abundance. It may be possible to revise this equation, quantifying amplification in terms of number of copies rather than Ct. This could allow for more accurate estimates of abundance, given that Ct is an indirect measure of amplification. Fourth, a liquid handling machine such as the epMotion M5073 (Eppendorf, Hamburg, Germany, Appendix Figure 5.10) should be incorporated into the DNA extraction process and RT-PCR procedures whenever possible. These types of instruments can be accurate (Spaulding et al., 2007) in the pipetting and transferring of samples and reagents, and therefore would reduce human error introduced which can compound the variability in observations, especially after amplification. Fourth, targeting the 16s rRNA gene to quantify abundance of bacterial DNA could hinder the accuracy of the technique, as some species of bacteria contain multiple copes if the 16s gene (Dahllöf et al., 2000). Dahllöf et al. (2000) suggested the gene encoding for the RNA polymerase beta subunit (rpoB) as an alternative, as it is believed that the gene exists as a single copy in bacteria. Fifth, as the technique targets bacteria and protozoa only in isolated pellets, a portion microbial nitrogen may be misrepresented as the contributions of archaea and fungi are ignored. This limitation could be overcome by developing additional primers and probes to target archaea and fungi in either pellet.

Integral to estimating MCP flow is the isolation of a microbial pellet from rumen digesta. One important consideration is the dislodging of particle associated bacteria

from the solid portion of rumen digesta prior to isolation via differential centrifugation, which was demonstrated in Experiment 1. In order to achieve this, Experiment 2 used a common method where strained rumen solids were combined with MacDougal's buffer, shaken in a capped jar, and were then strained through cheesecloth. In future studies, it may be useful to blend rumen solids with buffer in a commercial blender prior to the second straining, as was described in Experiment 1. It is apparent that the blender is more efficient in violently agitating the feed particles than shaking by hand, however, there is a concern that microbial cells may become prematurely lysed before isolation and DNA extraction, potentially leading to degradation of the microbial DNA prior to further analyses. A comparative study of the two techniques measuring the abundance of microbial DNA isolated using either method would be beneficial to future research, so that the rumen bacterial community is most accurately represented. In addition, it is imperative to ensure that enough microbial sample is isolated in this step, especially when multiple microbial markers will be used to estimate MCP. In Experiment 2, 250 mL centrifuge bottles were used to strain and shake the solid portion of rumen digesta to dislodge particle associated bacteria. In future research, if the hand shaking method is utilized, it would be advisable to use a larger screw capped container, such as a mason jar. Although microbial pellets were successfully harvested from the 250 mL bottles, we had to be very conservative with the amount of sample used in each analysis, especially after lyophilization, as rumen bacterial and protozoal isolates are inherently low in DM. Using a larger container for the shaking step would allow for a greater volume of digesta to be processed, resulting in larger microbial pellets after differential centrifugation.

Bioinformatics, the interpretation of high throughput sequencing data, is an emerging science which is not yet completely understood, especially through the lens of ruminant nutrition. Bioinformatics attempts to make sense of voluminous amounts of data generated and present the information in a useful way—leaps and bounds in this technology have been made in the last several years, especially with the continual technological advances in processing power and analytical tools. One of those tools used in Experiment 2, the MaAsLin (Multivariate Association with Linear Models) analysis, was able to draw associations of nutritional metadata with OTUs, of which were subsequently identified as several specific microorganisms. This information provides a solid foundation for future research, especially in those instances where associations of microorganisms were made with useful nutritional measurements, such as rumen ammonia concentration or fiber digestibility. In future research, there may be the potential for these microorganisms to be isolated and cultured, allowing for more in depth analysis of their role in the rumen. From an applied perspective, these microorganisms could potentially be incorporated into probiotics or targeted with prebiotics, perhaps enhancing their effects in the rumen. Another result of the MaAsLin analysis revealed huge differences in the rumen microbiome of one of our experimental cows, which was further confirmed by principal coordinate analysis generated by QIIME. We hypothesized that this may have either been due to her recent introduction into our research herd, and/or the result of cannulation surgery and subsequent drugs administered shortly before the commencement of the experiment. In order to explore these questions, future research could analyze and compare the rumen microbiome of animals from differing farms. If rumen microbiomes were significantly different between farms, it

would also beg the question as to whether or not a cow's community structure would conform similarly to those animals on a different farm should she be moved. Additionally, the microbiome of animals undergoing cannulation surgery could be analyzed before and shortly after the procedure. This would be extremely useful to planning experiments involving the microbiome where cows must be cannulated prior to experimentation, as there may be a certain period in which the microbial community must re-stabalize.

The effects of HMB on rumen microbial activity and milk production and composition we observed were mixed relative to what has been observed previously in literature. This work contributes to the fact that HMB does have a stimulatory effect on the rumen microbial community, however the mechanism and extent of these effects has yet to be determined. As HMB has been shown to increase milk fat yield (Patton et al., 1970; Holter et al., 1972; Huber et al., 1984; Zanton et al., 2014), its continued inclusion in dairy diets is advisable, however further investigation into its effects in the rumen is warranted in order to optimize its utilization as a supplement. It may be possible to enhance the effects of HMB in the rumen by feeding it alongside probiotics or prebiotics; perhaps certain genera of bacteria, such as nitrate reducers (Moreno-Vivián et al., 1999), may be able to replace they hydroxyl group of HMB with an amino group, rendering methionine.

In conclusion, there are many opportunities to advance research in the area of the role of the microbial community in ruminant nutrition. With increasing availability of access to improved technology, techniques, and tools, our understanding of these

symbiotes will undoubtedly improve, making way for myriads of advancements in ration formulation, productivity, animal health, and much more.

APPENDIX I

EXPERIMENT 1 DIET ACCORDING TO THE CPM DAIRY RATION ANALYZER (2000)

CPM-Dairy

CNCPS Evaluation

Farm: UNL Dairy Research Unit	BW: 1500 lb	DIM: 100
Ration: Experiment 1 Ration	BCS: 3.00	Milk: 80.00 lb
Ration By: Kononoff & Ramirez	Growth: 0.62 lb/d	Fat: 3.50 %
Organization: University of Nebraska-Lincoln	Lact#: 3	TP: 3.10 %

Cost ($)	0.10	IOF ($)	-0.10			Ingredient	DM (lb/d)
DMI (lb/d)	50.4	Model	49.8	% Model	101.2	Corn Silage	15.854
ME Bal (mCal)	2.2	CP (%)	17.9	NDF (%)	29.2	CornGrainGrndMed	11.567
MP Bal (g)	94.7	RUP (% CP)	35.7	ForageNDF (% NDF)	67.3	Alfalfa Hay	9.283
NP / MP (%)	61.6	LCFA (%)	2.1	ForageNDF (% DM)	19.7	SoybeanML47.5Solv	7.453
BactMP (% MP)	54.2	EE (%)	2.7	peNDF (%)	19.4	SoybeanHullsGrnd	4.000
Rumen N Balance				Lignin (%)	3.1	Soy Pass	1.014
Pept (g)	90	Pept & NH3 (g)	98	NFC (%)	44.0	CalciumCarbonate	0.450
% rqd	141	% rqd	125	Sil Acids (%)	2.7	SodiumBicarbonate	0.330
Amino Acid Balance				Sugar (%)	4.4	CalciumPhosDi	0.150
Met (g)	6.7	Lys (g)	32.2	Starch (%)	20.3	SaltNaCl	0.110
Met (% rqd)	114	Lys (% rqd)	121	Sol Fiber (%)	16.6	MagOx	0.090
Met (% mp)	2.00	Lys (% mp)	6.89	Lys:Met	3.45:1	Trace Premix	0.060
						Vitamin Premix	0.060
Possible production due to ME and MP						Total	50.421

	Milk(lb)	Fat (%)	TP (%)	Milk(lb)	Fat (%)	TP (%)
Trg:	80.0	3.50	3.10	80.0	3.50	3.10
	Yield Constant			Composition Constant		
ME:	80.0	n/a	n/a	84.5	3.50	n/a
MP:	80.0	n/a	3.27	84.4	3.50	3.10
Adjustments based on Rulquin AA Ratios:						
	80.0	n/a	-0.03	-0.7	3.50	3.10

n/a - Equations not available

Ration DM (%)	60.91	Forage (% DM)	49.85

Diet Summary:

Ingredient	AF lb/d	DM lb/d	% AF	% DM
Corn Silage	43.98	15.85	53.13	31.44
CornGrainGrndMed	13.14	11.57	15.88	22.94
Alfalfa Hay	10.60	9.28	12.80	18.41
SoybeanML47.5Solv	8.28	7.45	10.00	14.78
SoybeanHullsGrnd	4.40	4.00	5.31	7.93
Soy Pass	1.12	1.01	1.36	2.01
CalciumCarbonate	0.45	0.45	0.55	0.89
SodiumBicarbonate	0.33	0.33	0.40	0.65
CalciumPhosDi	0.15	0.15	0.18	0.30
SaltNaCl	0.11	0.11	0.13	0.22
MagOx	0.09	0.09	0.11	0.18
Trace Premix	0.06	0.06	0.08	0.12
Vitamin Premix	0.06	0.06	0.08	0.12
Total	82.78	50.42		

Macro Nutrients		
Nutrient	DM	AF
Dry Matter (%)	100.00	60.91
Forage (%)	49.85	29.90
Crude Prot (%)	17.91	10.91
RUP (%CP)	35.66	35.66
RDP (%CP)	64.34	64.34
RDP (%)	11.52	7.02
Sol Prot (%CP)	26.16	26.16
ME (mCal/lb)	1.21	0.74
NEl (mCal/lb)	0.78	0.47
Nem (mCal/lb)	0.78	0.47
NEg (mCal/lb)	0.51	0.31
ADF (%)	19.97	12.16
NDF (%)	29.23	17.80
For NDF (%NDF)	67.31	41.00
Forage NDF (%)	19.67	11.98
peNDF (%)	19.37	11.80
Lignin (%)	3.08	1.88
NFC (%)	44.01	26.80
Sil Acids (%)	2.72	1.65
Sugar (%)	4.43	2.70
Starch (%)	20.29	12.36
Sol Fiber (%)	16.58	10.10
EE Total (%)	2.66	1.62
EE 1 (%)	2.66	1.62
EE 2 (%)	0.00	0.00
EE 3 (%)	0.00	0.00
LCFA Total (%)	2.08	1.26
Ash (%)	8.10	4.93
Cost ($/d)	0.10	0.10
Cost ($T)	3.93	2.39

Minerals and Vitamins		
Nutrient	DM	AF
Dry Matter (%)	100.00	60.91
Calcium (%)	0.87	0.53
Phosphorus (%)	0.40	0.24
Magnesium (%)	0.29	0.17
Potassium (%)	1.61	0.98
Sulfur (%)	0.22	0.13
Sodium (%)	0.28	0.17
Chlorine (%)	0.36	0.22
Iron (ppm)	206.45	125.74
Zinc (ppm)	75.06	45.72
Copper (ppm)	22.32	13.60
Manganese (ppm)	55.92	34.06
Selenium (ppm)	0.34	0.21
Cobalt (ppm)	0.64	0.39
Iodine (ppm)	1.04	0.63
Vitamin A (KIU/lb)	3.11	1.90
Vitamin D (KIU/lb)	0.79	0.48
Vitamin E (IU/lb)	25.04	15.25
DCAD1 (meq/100g)	29.71	18.09
DCAD2 (meq/100g)	33.40	20.35
Cost ($/d)	0.10	0.10
Cost ($T)	3.93	2.39

APPENDIX II

LOW AND HIGH MP DIETS ACCORDING TO THE DAIRY NRC (2001) MODEL

Low MP:

Summary Report

Animal Inputs

Animal Type : Lactating Cow
Age : 60 months
Body Weight : 624 kg
Milk Fat : 3.50%
Days In Milk : 100

Milk Production : 31.8 (kg/day)
Days Pregnant : 0
Breed : Holstein
Milk True Protein : 3.10%

Diet Nutrient Balances

Requirements	NEl (Mcal/day)	MP (g/day)	Ca (g/day)	P (g/day)	K (g/day)
Maintenance	10.0	787	20	23	158
Pregnancy	0.0	0	0	0	0
Lactation	22.2	1471	39	29	48
Growth	0.0	0	0	0	0
Total Required	32.2	2259	59	52	206
Total Supplied	33.2	2132	118*	59*	312*
Balance	1.1	-127	59	8	106

* Note that these mineral supplied values are total *absorbable* supplied.

Animal Performance

DMI - Actual : 22.0 (kg/day)
DMI - Predicted : 22.0 (kg/day)

NEl Allowable Milk : 33.3 (kg/day)
MP Allowable Milk : 29.1 (kg/day)

Milk Production : 31.8 (kg/day)

Days to gain one condition score : > 305

Daily Weight Change due to Reserves : 0.2 (kg/day)

Protein Values

RDP Required : 2123 (g/d)
RDP Supplied : 2276 (g/d)
RDP Balance : 152 (g/d)

RUP Required : 1278 (g/d)
RUP Supplied : 1115 (g/d)
RUP Balance : -163 (g/d)

MP - Bacterial : 1155 (g/d)
MP - RUP : 873 (g/d)
MP - Endogenous : 104 (g/d)

CP - Diet : 15.4 (%DM)
CP - RDP : 10.3 (%DM)
CP - RUP : 5.1 (%DM)

Diet Concentrations

NDF : 33.6 (%DM)
Forage NDF : 25.2 (%DM)
ADF : 22.0 (%DM)
NFC : 42.2 (%DM)
Undiscounted TDN : 68 (%DM)
ME : 2.40 (Mcal/kg DM)
NEl : 1.51 (Mcal/kg DM)
NEg : 1.00 (Mcal/kg DM)
Ca : 0.9 (%DM)
P : 0.4 (%DM)
Ether-Extract : 2.7 (%DM)
DCAD : 273 (mEQ/kg)

Target Diet Concentrations

NEl : 1.46 (Mcal/kg)
MP : 103 (g/kg)

Ca : 3 (g/kg)
P : 2 (g/kg)

Diet Summary

Feed Name	kg/day (Dry Matter)	kg/day (As-Fed)	% (Dry Matter)
Corn Silage, HMP	7.70	20.93	35.01
Alfalfa Hay	3.00	3.42	13.64
Brome Hay	2.00	2.37	9.09
Corn Grain, ground, dry	3.63	4.12	16.50
Soybean, Hulls	1.75	1.93	7.95
SBM	0.80	0.89	3.64
RF DDGS	1.25	1.39	5.68
Molasses, Sugarcane	0.90	1.21	4.09
Blood Meal, ring dried	0.10	0.11	0.45
Soypass	0.00	0.00	0.00
Limestone	0.20	0.20	0.92
Calcium Phosphate (Di-)	0.09	0.09	0.41
Megalac	0.04	0.04	0.16
Urea	0.16	0.16	0.75
Salt	0.14	0.14	0.64
Sodium Bicarbonate	0.14	0.14	0.64
Magnesium Oxide	0.05	0.05	0.23
UNL mineral PMX	0.02	0.02	0.10
UNL Vit PMX	0.02	0.02	0.10
Canola Meal, mech. Extract	0.00	0.00	0.00

Energy and Protein Supply

Feed Name	DMI	TDN	ME	NEl	NEg	CP	RUP	RDP	NDF	MCP
	(kg/day)	(g/day)	(Mcal/day)	(Mcal/day)	(Mcal/day)	(g/day)	(g/day)	(g/day)	(kg/day)	(g/day)
Corn Silage, HMP	7.7	5158	17.6	10.9	7.0	562	196	366	3.0	-
Alfalfa Hay	3.0	1718	6.2	3.8	2.2	597	111	486	1.2	-
Brome Hay	2.0	1122	3.8	2.3	1.2	216	92	124	1.4	-
Corn Grain, ground, dry	3.6	3220	11.5	7.4	5.4	330	152	178	0.3	-
Soybean, Hulls	1.7	1177	4.2	2.6	1.7	243	105	138	1.1	-
SBM	0.8	651	2.8	1.8	1.3	430	177	254	0.1	-
RF DDGS	1.3	960	3.7	2.4	1.7	400	199	201	0.4	-
Molasses, Sugarcane	0.9	728	2.5	1.6	1.1	52	9	43	0.0	-
Blood Meal, ring dried	0.1	76	0.4	0.2	0.2	95	73	22	0.0	-
Soypass	0.0	0	0.0	0.0	0.0	0	0	0	0.0	-
Limestone	0.2	0	0.0	0.0	0.0	0	0	0	0.0	-
Calcium Phosphate (Di-)	0.1	0	0.0	0.0	0.0	0	0	0	0.0	-
Megalac	0.0	59	0.2	0.2	0.1	0	0	0	0.0	-
Urea	0.2	0	0.0	0.0	0.0	464	0	464	0.0	-
Salt	0.1	0	0.0	0.0	0.0	0	0	0	0.0	-
Sodium Bicarbonate	0.1	0	0.0	0.0	0.0	0	0	0	0.0	-
Magnesium Oxide	0.1	0	0.0	0.0	0.0	0	0	0	0.0	-
UNL mineral PMX	0.0	0	0.0	0.0	0.0	0	0	0	0.0	-
UNL Vit PMX	0.0	0	0.0	0.0	0.0	0	0	0	0.0	-
Canola Meal, mech. Extract	0.0	0	0.0	0.0	0.0	0	0	0	0.0	-
Totals :	22.0	14870	52.9	33.2	22.0	3391	1115	2276	7.4	1805

Feed Name	ME	NEl	NEg	Kp
	(Mcal/kg)	(Mcal/kg)	(Mcal/kg)	(%/hr)
Corn Silage, HMP	2.28	1.41	0.90	5.22
Alfalfa Hay	2.05	1.25	0.74	4.69
Brome Hay	1.89	1.14	0.62	4.17
Corn Grain, ground, dry	3.18	2.05	1.48	6.91
Soybean, Hulls	2.39	1.49	0.98	6.91
SBM	3.47	2.25	1.65	6.91
RF DDGS	2.99	1.93	1.36	6.91
Molasses, Sugarcane	2.83	1.80	1.27	6.91
Blood Meal, ring dried	3.65	2.38	1.76	6.91

Soypass	3.48	2.26	1.66	6.91
Limestone	0.00	0.00	0.00	6.91
Calcium Phosphate (Di-)	0.00	0.00	0.00	6.91
Megalac	6.38	5.10	3.51	6.91
Urea	0.00	0.00	0.00	6.91
Salt	0.00	0.00	0.00	6.91
Sodium Bicarbonate	0.00	0.00	0.00	6.91
Magnesium Oxide	0.00	0.00	0.00	6.91
UNL mineral PMX	0.00	0.00	0.00	6.91
UNL Vit PMX	0.00	0.00	0.00	6.91
Canola Meal, mech. Extract	2.81	1.80	1.25	6.91

Increment over Maintenance : 3.4 X
Energy/Protein Discount Factor : 6.6%
Undiscounted TDN in Diet : 67.6%
Diet RUP Digestibility : 78.2%

Duodenal Amino Acid Supply

Total Dry Matter Intake : 22.0 kg
Total RUP : 1115 g/day
Total MCP : 1805 g/day

Amino Acid	Flow (g/day)	DIGAA Flow (g/day)	Percent of MP
Arginine	121	97	4.56%
Histidine	61	49	2.30%
Isoleucine	128	102	4.78%
Leucine	241	192	9.01%
Lysine	173	138	6.48%
Methionine	50	40	1.87%
Phenylalanine	134	107	5.01%
Threonine	131	104	4.88%
Valine	149	119	5.56%
Totals	1189	948	44.46%

Total Essential Amino Acids : 1192 (g/day)

High MP:

Summary Report

Animal Inputs

Animal Type : Lactating Cow
Age : 60 months
Body Weight : 624 kg
Milk Fat : 3.50%
Days In Milk : 100

Milk Production : 31.8 (kg/day)
Days Pregnant : 0
Breed : Holstein
Milk True Protein : 3.10%

Diet Nutrient Balances

Requirements	NEl (Mcal/day)	MP (g/day)	Ca (g/day)	P (g/day)	K (g/day)
Maintenance	10.0	785	20	23	158
Pregnancy	0.0	0	0	0	0
Lactation	22.2	1471	39	29	48
Growth	0.0	0	0	0	0
Total Required	32.2	2257	59	52	205
Total Supplied	33.5	2267	120*	62*	326*
Balance	1.3	11	61	10	121

* Note that these mineral supplied values are total *absorbable* supplied.

Animal Performance

DMI - Actual : 21.9 (kg/day)
DMI - Predicted : 22.0 (kg/day)

NEl Allowable Milk : 33.7 (kg/day)
MP Allowable Milk : 32.0 (kg/day)

Milk Production : 31.8 (kg/day)

Days to gain one condition score : > 305

Daily Weight Change due to Reserves : 0.3 (kg/day)

Protein Values

RDP Required : 2122 (g/d)
RDP Supplied : 2123 (g/d)
RDP Balance : 1 (g/d)

RUP Required : 1248 (g/d)
RUP Supplied : 1261 (g/d)
RUP Balance : 13 (g/d)

MP - Bacterial : 1155 (g/d)
MP - RUP : 1009 (g/d)
MP - Endogenous : 104 (g/d)

CP - Diet : 15.4 (%DM)
CP - RDP : 9.7 (%DM)
CP - RUP : 5.7 (%DM)

Diet Concentrations

NDF : 33.8 (%DM)
Forage NDF : 25.2 (%DM)
ADF : 22.2 (%DM)
NFC : 41.9 (%DM)
Undiscounted TDN : 68 (%DM)
ME : 2.43 (Mcal/kg DM)
NEl : 1.53 (Mcal/kg DM)
NEg : 1.01 (Mcal/kg DM)
Ca : 0.9 (%DM)
P : 0.4 (%DM)
Ether-Extract : 2.6 (%DM)
DCAD : 286 (mEQ/kg)

Target Diet Concentrations

NEl : 1.46 (Mcal/kg)
MP : 103 (g/kg)

Ca : 3 (g/kg)
P : 2 (g/kg)

Diet Summary

Feed Name	kg/day (Dry Matter)	kg/day (As-Fed)	% (Dry Matter)
Corn Silage, HMP	7.70	20.93	35.09
Alfalfa Hay	3.00	3.42	13.67
Brome Hay	2.00	2.37	9.11
Corn Grain, ground, dry	2.91	3.30	13.26
Soybean, Hulls	1.75	1.93	7.97
SBM	1.60	1.79	7.29
RF DDGS	1.25	1.39	5.70
Molasses, Sugarcane	0.90	1.21	4.10
Blood Meal, ring dried	0.10	0.11	0.45
Soypass	0.00	0.00	0.00
Limestone	0.20	0.20	0.92
Calcium Phosphate (Di-)	0.09	0.09	0.41
Megalac	0.04	0.04	0.17
Urea	0.03	0.03	0.15
Salt	0.14	0.14	0.64
Sodium Bicarbonate	0.14	0.14	0.64
Magnesium Oxide	0.05	0.05	0.23
UNL mineral PMX	0.02	0.02	0.10
UNL Vit PMX	0.02	0.02	0.10
Canola Meal, mech. Extract	0.00	0.00	0.00

Energy and Protein Supply

Feed Name	DMI	TDN	ME	NEl	NEg	CP	RUP	RDP	NDF	MCP
	(kg/day)	(g/day)	(Mcal/day)	(Mcal/day)	(Mcal/day)	(g/day)	(g/day)	(g/day)	(kg/day)	(g/day)
Corn Silage, HMP	7.7	5158	17.5	10.9	7.0	562	196	366	3.0	-
Alfalfa Hay	3.0	1718	6.1	3.7	2.2	597	111	486	1.2	-
Brome Hay	2.0	1122	3.8	2.3	1.2	216	92	124	1.4	-
Corn Grain, ground, dry	2.9	2581	9.2	6.0	4.3	265	122	143	0.3	-
Soybean, Hulls	1.7	1177	4.2	2.6	1.7	243	105	138	1.1	-
SBM	1.6	1302	5.6	3.6	2.6	861	353	508	0.2	-
RF DDGS	1.3	960	3.7	2.4	1.7	400	199	201	0.4	-
Molasses, Sugarcane	0.9	728	2.5	1.6	1.1	52	9	43	0.0	-
Blood Meal, ring dried	0.1	76	0.4	0.2	0.2	95	73	22	0.0	-
Soypass	0.0	0	0.0	0.0	0.0	0	0	0	0.0	-
Limestone	0.2	0	0.0	0.0	0.0	0	0	0	0.0	-
Calcium Phosphate (Di-)	0.1	0	0.0	0.0	0.0	0	0	0	0.0	-
Megalac	0.0	59	0.2	0.2	0.1	0	0	0	0.0	-
Urea	0.0	0	0.0	0.0	0.0	93	0	93	0.0	-
Salt	0.1	0	0.0	0.0	0.0	0	0	0	0.0	-
Sodium Bicarbonate	0.1	0	0.0	0.0	0.0	0	0	0	0.0	-
Magnesium Oxide	0.1	0	0.0	0.0	0.0	0	0	0	0.0	-
UNL mineral PMX	0.0	0	0.0	0.0	0.0	0	0	0	0.0	-
UNL Vit PMX	0.0	0	0.0	0.0	0.0	0	0	0	0.0	-
Canola Meal, mech. Extract	0.0	0	0.0	0.0	0.0	0	0	0	0.0	-
Totals :	21.9	14881	53.3	33.5	22.2	3385	1261	2123	7.4	1804

Feed Name	ME	NEl	NEg	Kp
	(Mcal/kg)	(Mcal/kg)	(Mcal/kg)	(%/hr)
Corn Silage, HMP	2.28	1.41	0.90	5.21
Alfalfa Hay	2.05	1.25	0.74	4.68
Brome Hay	1.89	1.14	0.62	4.17
Corn Grain, ground, dry	3.18	2.05	1.48	6.90
Soybean, Hulls	2.38	1.48	0.98	6.90
SBM	3.47	2.25	1.65	6.90
RF DDGS	2.99	1.92	1.36	6.90
Molasses, Sugarcane	2.82	1.79	1.27	6.90
Blood Meal, ring dried	3.65	2.37	1.76	6.90

Soypass	3.48	2.26	1.66	6.90
Limestone	0.00	0.00	0.00	6.90
Calcium Phosphate (Di-)	0.00	0.00	0.00	6.90
Megalac	6.37	5.10	3.50	6.90
Urea	0.00	0.00	0.00	6.90
Salt	0.00	0.00	0.00	6.90
Sodium Bicarbonate	0.00	0.00	0.00	6.90
Magnesium Oxide	0.00	0.00	0.00	6.90
UNL mineral PMX	0.00	0.00	0.00	6.90
UNL Vit PMX	0.00	0.00	0.00	6.90
Canola Meal, mech. Extract	2.80	1.79	1.25	6.90

Increment over Maintenance : 3.4 X
Energy/Protein Discount Factor : 6.8%
Undiscounted TDN in Diet : 67.8%
Diet RUP Digestibility : 80.0%

APPENDIX III

CALCULATION OF MCP FLOW USING DNA MARKERS

Calculation of "R" (ratio of abundance of target DNA per g of microbial protein) in isolated microbial pellets from rumen digesta:

1.) mg of DNA per g of dry microbial pellet is calculated:

- The concentration of DNA in ng/µl is multiplied by the volume of the sample resulting from DNA extraction (from microbial pellets) in order to attain total mass of DNA recovered in ng.
- The mass of DNA recovered is divided by the initial wet pellet sample weight used for DNA extraction in order to attain ng of DNA per g of wet pellet.
- ng of DNA/g of wet pellet is divided by 1,000,000 to attain mg of DNA per gram of wet pellet.
- The original pellet sample weight used for DNA extraction is multiplied by the % DM of the sample in order to attain the dry sample weight in g.
- The total DNA recovered in ng is divided by the sample dry weight in g in order to attain ng of DNA per g of dry pellet.
- ng DNA/g dry pellet is divided by 1,000,000 to attain mg of DNA/g of dry microbial pellet.

2.) The abundance of target DNA/ng of DNA is calculated:

- Resulting from three, 10-fold serial dilutions, the slope of the standard curve is calculated using the ng of DNA from the serial dilutions vs. their CTs resulting from RT-PCR.
- The efficiency of RT-PCR is calculated by 10^(-1/slope).
- The abundance of target DNA/ng of DNA is calculated by (1/efficiency^CT)/ng DNA.

3.) The abundance of target DNA/g of dry microbial pellet is calculated:

- The abundance of target DNA/ng of DNA is multiplied by 1,000,000 to attain the abundance of DNA/mg of DNA.
- The abundance of DNA/mg of DNA is multiplied by the mg of DNA/g of dry pellet to attain the abundance of target DNA/g of dry pellet.

4.) "R" is calculated (the ratio of the abundance of target DNA per g of microbial protein):

- The abundance of target DNA/g of dry pellet is divided by the percent crude protein in the microbial pellet in order to attain the abundance of target DNA/g of microbial protein.
- 1 is divided by the abundance of target DNA/g of microbial protein, so that 1 abundance equals "x" g of MCP.

Calculation of MCP flow in duodenal contents:

1.) The mg of DNA/g of duodenal dry matter is calculated:

- The concentration of DNA in ng/µl is multiplied by the volume of the sample resulting from DNA extraction (from duodenal contents) in order to attain total mass of DNA recovered in ng.
- The dry sample weight in g is calculated by multiplying the mass of the duodenal content sample by the percent dry matter of the sample.
- The total mass of DNA recovered is divided by the dry sample weight in order to attain ng of DNA/g of dry matter.
- The ng of DNA/g of DM is divided by 1,000,000 to attain the mg of DNA/g of DM.

2.) The abundance of target DNA per g of DM is calculated:

- The abundance of target DNA/ng of DNA is calculated by (1/(efficiency^CT)/ng of DNA used in RT-PCR (the efficiency is that which was calculated by serial dilutions of DNA from microbial pellets, while the CT is the result of RT-PCR performed on DNA from duodenal contents).
- The abundance of target DNA/ng of DNA is multiplied by 1,000,000 to attain the abundance of target DNA/mg of DNA.
- The abundance of target DNA per g of DM is calculated by multiplying the abundance of target DNA/mg of DNA by the mg of DNA/g of DM.

3.) The mg of MCP/g of duodenal DM is calculated:

- The abundance of target DNA/g of DM is multiplied by "R", where 1 abundance equals "x"g of MCP, in order to attain the g of MCP/g of duodenal content DM.
- g of MCP/g of duodenal fluid DM is multiplied by 1,000 to attain mg of MCP/g of duodenal content DM.

4.) The flow of g MCP/day is calculated:

- An estimate of duodenal DM flow in g/d is multiplied by the mg of MCP/g of duodenal content DM to attain the mg of MCP flow/d.
- mg of MCP flow/d is divided by 1,000 to attain the g of MCP/d.

APPENDIX IV

PROTOCOL FOR BIOINFORMATIC ANALYSIS OF SEQUENCING DATA

This protocol assumes bioinformatics are being conducted on a Mac computer with QIIME, Mothur, and Perl installed (this work can be conducted on PC as well, but requires slightly different commands/programs) with access to the necessary scripts. Some basic knowledge of navigating in Terminal is required for these analyses. Here is a good resource with some commands you will likely use:

http://guides.macrumors.com/Terminal

1.) Acquire sequencing data in a format ready for UPARSE

- First, raw sequencing data must be "quality controlled" to the standards of Dr. Fernando's lab. Typically, this will be done before the sequencing data is returned to you.
- After quality control, samples must be demultiplexed in QIIME. Basically, reads are organized according to what samples they came from. Therefore, this step requires a "mapping file", which is a text document made in excel detailing which sample belongs to which cow, period, treatment, etc. (You will want to work from an example when creating this file, as some extraneous data is required for the program to run).
    - Start QIIME my typing "macqiime" in the terminal and hitting enter. Navigate to the directory from which you will be working (the folder which contains your files).
    - Demultiplex using the command:

```
split_libraries.py –f test.fna –b variable_length
–l 0 –L 1000 –x –M 1 –o split_library_master/ -m
mappingfile.txt
```

- Remember, whatever you type after "-f" is your file containing your sequencing data. Whatever you type after the part of the command with a "-o" is what the resulting file will be called and/or where it will be saved. If no subfolder is indicated before a "/", the file will be created in whatever directory you are currently working from. The "-m" requires you to indicate your mapping—make sure it is in the same directory you are working from.

- Next, reads must be trimmed in Perl
  - Launch Perl by typing "perl" in the terminal and hitting enter.
  - Trim reads:

```
./min_max_length.pl –min=80 –max=177
-fasta=test.split.fna
```

  - In this case, "-fasta" indicates which file will be trimmed, i.e. the file resulting from the demultiplexing step (make sure you have the "min_max_length.pl" Perl script in your directory).

- Finally, reverse compliment your reads using Mothur
  - Launch Mothur by typing "mothur" and hitting enter (mothur.exe must be in your directory).

```
reverse.seqs(fasta=test.trimmed.fasta)
```

2.) Submit your file with the batch script to TUSKER for analysis in UPARSE

- The next steps require access to TUSKER, one of the University of Nebraska's super computers housed in the Holland Computing Center. Access to TUSKER requires permission from a professor with an account (Dr. Samodha Fernando) in order to register a new user. Additionally, a physical, USB "YubiKey" is required to logon and can be purchased from the Computing Center.
- Login to TUSKER:

```
ssh <username>@tusker.unl.edu
<password>
```

- Copy the USEARCH batch script into your user account "work" directory on TUSKER. The batch script is a series of commands designed to sort sequences, remove singletons, cluster OTUs, remove chimeras, align OTUs, and convert the file into a usable format for downstream analysis. The batch script is located on TUSKER at:

  /home/samodha/shared/Programs/usearch_batch_master.pbs

  Copy the batch script to your work directory on TUSKER at:

  /work/samodha/<username>

  - The batch script can be copied and pasted into your work directory using terminal commands, or alternatively, you can download free software like "Fetch" to help you navigate and transfer files on TUSKER graphically (recommended).

- Rename your file which resulted from reverse complementing to "test.trim.rc.fasta". This way, you will not have to change the commands in the batch script.
- Upload your file to your work directory in TUSKER:

```
scp –r ./<your local directory>
/test.trim.rc.fasta <username>
@tusker.unl.edu:work/
samodha/<username>
```

(Or alternatively, drag and drop the file into your work directory in Fetch.)

- Submit the job to TUSKER from your work directory:

```
qsub usearch_batch_master.pbs
```

  - Once the script is done running, all of the files you need for downstream analysis in QIIME will appear in your work directory.

3.) Analyze OTU data using QIIME pipeline

- Launch QIIME in the terminal by typing "macqiime" and hitting enter.
- Assign taxonomy to the OTUs:

```
assign_taxonomy.py –i test.otus2.fa –t
/macqiime/greengenes/gg_12_10_otus/taxonomy/97_ot
u_taxonomy.txt –r
/macqiime/greengenes/gg_12_10_otus/rep_set/97_otu
s.fasta –o test.otus2.fa.assign_gg_taxa/
```

  - This step will add names of specific taxonomic classifications to your OTUs.
- Open the resulting file in Excel, along with the "test.otu_table.txt" that came from the USEARCH batch script.

  - Add the taxa outputted to the OTU table by first sorting by the ID number—you should have the same number of OTUs as you do taxa names.
  - Label the column “taxonomy”.

- Convert the OTU table to the BIOM file format

```
convert_biom.py –i test.otu_table.txt –o
test.otu_table.biom --biom_table_type=”out
table” --process_obs_metadata taxonomy
```

- Remove *Cyanobacteria* from your OTU table: these data may have actually originated from plant material in your samples, therefore should not be included:

```
filter_taxa_from_otu_table.py –i
test.otu_table.biom –o
test.otu_table.taxa_filter.biom –n Cyanobacteria
```

- Sort your OTU table—note that the mapping file comes back into play here and that the output file is renamed to “rumen.sort.biom":

```
sort_otu_table.py –i test.otu_table.biom –m
mappingfile.txt –s Diet –o rumen.sort.biom
```

- Make an OTU heatmap:

```
make_otu_heatmap_html.py –i rumen.sort.biom –o
total_analysis/total.heatmap
```

- Make an OTU network (a network image can be created using the files generated in the free software “Cytoscape”. There are QIIME-specific tutorials for this online):

```
make_otu_network.py –i rumen.sort.biom –m
mappingfile.txt –o
total_analysis/total.summarize_taxa
```

- Create bar, area, and pie charts summarizing taxa information (two commands):

```
summarize_taxa.py –i rumen.sort.biom –L
2,3,4,5,6,7 –o
total_analysis/total.summarize_taxa
```

```
plot_taxa_summary.py –i
total_analysis/total.summarize_taxa/rumen.sort_L2
.txt,total_analysis/total.summarize_taxa/rumen.so
rt_L3.txt,total_analysis/total.summarize_taxa/rum
en.sort_L4.txt,total_analysis/total.summarize_tax
a/rumen.sort_L5.txt,total_analysis/total.summariz
e_taxa/rumen.sort_L6.txt,total_analysis/total.sum
marize_taxa/rumen.sort_L7.txt,total_analysis/tota
l.summarize_taxa/rumen.sort_L2.txt –l
Phylum,Class,Order,Family,Genus,Species –c
bar,area,pie –o total_analysis/total.taxa_plots
```

- Tables generated in this step representing relative percentages of taxa can be analyzed statistically in SAS—you can copy and paste using the "transpose" option into an Excel file to set up your SAS infile.

- The final commands require some additional files to be generated:
  - First, sequences must be aligned by submitting your original file from UPARSE (test.otus2.fa) to the RDP aligner at http://pyro.cme.msu.edu.
  - After completion of alignment, download your aligned file from RDP.
  - Some changes must be made to the file in a text editing program like "Text Wrangler" in order to make the file compatible with Mothur:
    - In Text Wrangler, select all (command + a), find (command + f), find ".", and replace all with "-".
    - Remove the last line of data (series of x) at the bottom of the file.
    - Add 10 letter "A"s after the ">" sign. This can be accomplished by find ">", and replace all with ">AAAAAAAAAA".
  - Change the filename to something like "test.otus2.aligned.fasta"

- The commands are run in Mothur:

```
dist.seqs(fasta=test.otus2.aligned.fasta,
processors=10, cutoff=.10)
```

  - This command generates a ".dist" file, a distance matrix of OTUs.

```
clearcut(phylip=test.otus2.aligned.dist)
```

  - This command generates a ".tre" file, a phylogenic tree which can be viewed in software like "FigTree". The ".tre" file is needed for the next series of commands.

- Launch QIIME in the terminal.
  - We need to see some stats on your ".biom" file for the next step using this command:

```
print_biom_table_summary.py –i rumen.sort.biom
```

    - Look for the part of the readout "Min:" followed by a number. This is the lowest of number of sequences in a sample. Make a note of this number, as you will need it as an input in the next command. For example, we will say 1437.

  - Generate plots of beta diversity:

```
beta_diversity_through_plots.py –i
rumen.sort.biom –e 1437 –m mappingfile.txt –p
qiime_parameters_working.txt –t
test.otus2.aligned.tre –c Diet –o
total_analysis/total.beta_diversity
```

    - Note that this command required a "-e" parameter input; this is the minimum number sequences from the previous step. Also note a "-p" (parameter) input was required. This is a file which can be located on the Mac computer in Samodha's lab office. You will

need to copy this file into the directory you are working from in order for this command to work. Additionally, "-t" for ".tre" was required; this is your ".tre" file you generated using the clearcut command in Mothur.

- Generate jackknifed plots of beta diversity:

```
jackknifed_beta_diversity.py –i rumen.sort.biom –
e 1437 –m mappingfile.txt –p
qiime_parameters_working.txt –t
test.otus2.aligned.tre –o
total_analysis/total.jackknifed_beta_diversity
```

  - The previous two commands will generate a series of files which can be very helpful in visualizing your sequencing data. Of these, there are principal coordinate analysis plots that can be viewed in either 2d or 3d. The 3d plots generated will include a Java applet called "KiNG" (Jar). "Jar" can be launched from within these files, and the PCoA ".jar" files can be loaded and viewed from there.

- The previous analyses was conducted on what we refer to as the "total" biom. In the next steps, we will define the "core" biom. The core biom refers to OTUs that are present in a certain proportion of samples (which you define). For example, say that I had 4 cows consuming each of 4 diets, resulting in 16 samples per diet, for a total of 64 total samples. If I wanted to define my core at around 94 % by diet type, I would first split by OTU table by diet type. In the next command, I would indicate "15", for 15/16 samples, or around 94 %. This means that to be considered a part of the core, a particular OTU must be present in 15 out of the 16

samples I have from cows consuming that particular diet. This makes the analysis more strict, which can be helpful in reducing extraneous information/noise from the analysis.

- In QIIME, split the OTU table based on which category in your mapping file that you want to define the core—in our case, this will be diet type:

```
split_otu_table.py –i rumen.sort.biom –m
mappingfile.txt –f Diet –o rumen.sort.split
```

  - In our example, this will have created 4 different files, one for each diet type, in the folder "rumen.sort.split". They will be named based on your diet names in the mapping file.

- The next commands will filter the OTUs from each diet type based on whether they are present in 15/16 samples, and create a new file for each in the folder "rumen.core.split":

```
filter_otus_from_otu_table.py –i
rumen.sort.split/rumen.sort_Diet1.biom –s 15 –o
rumen.core.split/Diet1.core.biom
```

```
filter_otus_from_otu_table.py –i
rumen.sort.split/rumen.sort_Diet2.biom –s 15 –o
rumen.core.split/Diet2.core.biom
```

```
filter_otus_from_otu_table.py –i
rumen.sort.split/rumen.sort_Diet3.biom –s 15 –o
rumen.core.split/Diet3.core.biom
```

```
filter_otus_from_otu_table.py –i
rumen.sort.split/rumen.sort_Diet4.biom –s 15 –o
rumen.core.split/Diet4.core.biom
```

- Next, merge the filtered OTU tables back together:

```
merge_otu_tables.py –i
rumen.core.split/Diet1.core.biom,rumen.core.split
/Diet2.core.biom,rumen.core.split/Diet3.core.biom
,rumen.core.split/Diet4.core.biom –o
rumen.core.biom
```

- Now that we have the core biom defined, we need to filter the OTUs from our OTU table that we do not want to include. First we need to convert our new core biom file to a text file:

```
convert_biom.py –i rumen.core.biom –o
rumen.core.txt –b
```

- Now, open the new "rumen.core.txt" file in Excel. Copy the first column (OTU IDs) and paste into a word file. Save the file as "core_keep.txt".
- The next command will filer the OTUs that we do not want to be a part of the core:

```
filter_otus_from_otu_table.py –i rumen.sort.biom
--negate_ids_to_exclude –e core_keep.txt –o
rumen.core.biom
```

  - Now that you have a ".biom" file to work from in which your core is defined. You may want to run the "print_biom_table_summary.py" command again on your new file to see how many sequences are left in the core compared to your "total" file.

- With the core defined, using the new "rumen.core.biom" file, you can now go back and create new heatmaps, OTU networks, bar, area, and pie charts, and PCoA plots with the commands described above.

4.) Analyze OTU data using multivariate association with linear models (MaAsLin) and nucleotide BLAST (Basic Local Alignment Search Tool)

- MaAsLin is a statistical tool which was designed by the Huttenhower Lab (Harvard School of Public Health, Boston, MA) to identify associations between the relative abundance of OTUs in sample and metadata. Along with BLAST, it can be useful in identifying specific microorganisms which may be associated with other meaningful observations in a study, such as milk yield or rumen ammonia.
- The MaAsLin tool can be used in an online interface at huttenhower.org/galaxy/
- In order to use MaAsLin, you must create a file in a format that it can read (an example file can be located on the website):
    - Open your OTU table in Excel.
    - Because MaAsLin reads OTUs in terms of relative abundance, you must total up each column of reads, then divide each cell (in the same column) by the total number of reads.
    - Rename the first row from "OTUid" to "sample"
    - For each sample number in the first row, rename to "sample1, sample2, sample 3, etc.".
    - The next rows should contain your metadata—the first cell in the row is the title (ex. "Rumen_Ammonia" or "Animal_ID"), while the next cells in the row contain data corresponding to the particular sample. Data can either be numerical or qualitative (ex. "143.7" or "Cow881").

    - Your OTU table (in relative abundance) should begin below the metadata, with each column of the first row being the taxonomy name (ex. "Bacteria|Firmicutes|Clostridia|Clostridiales|Leahspiraceae")
    - Save the file as ".txt", TAB delimited.
- Upload your file to the Huttenhower Lab website for analysis:
    - Visit the Huttenhower Lab website (huttenhower.org/galaxy/), and click on "Get Data" and "Upload File" in the left hand column.
    - From the "File Format:" drop-down menu, select "maaslin".
    - Click "Choose File" and select your ".txt" file you created.
    - Click "Execute".
- Run the MaAsLin analysis:
    - Navigate to the MaAsLin page by clicking "MaAsLin" in the left hand column.
    - From the first drop-down menu, you should be able to select the file that you just uploaded.
    - On the second drop-down menu, select the title of the last row of metadata in your file.
    - The next three text fields allow you to fine tune the statistical parameters of the analysis. Leave in the default values for now.
    - In the last drop-down menu, select "Two Files: Complete zipped results + Summary".
    - Click "Execute".

    - You should now see the analysis running in the right hand column of the page. After completion, you will be able to download the results. For any associations that were drawn with a *q*-value less than or equal to what was defined (0.05), a scattergram will be generated for numerical metadata, or a notched box plot for qualitative metadata, along with a tab-delimited file.

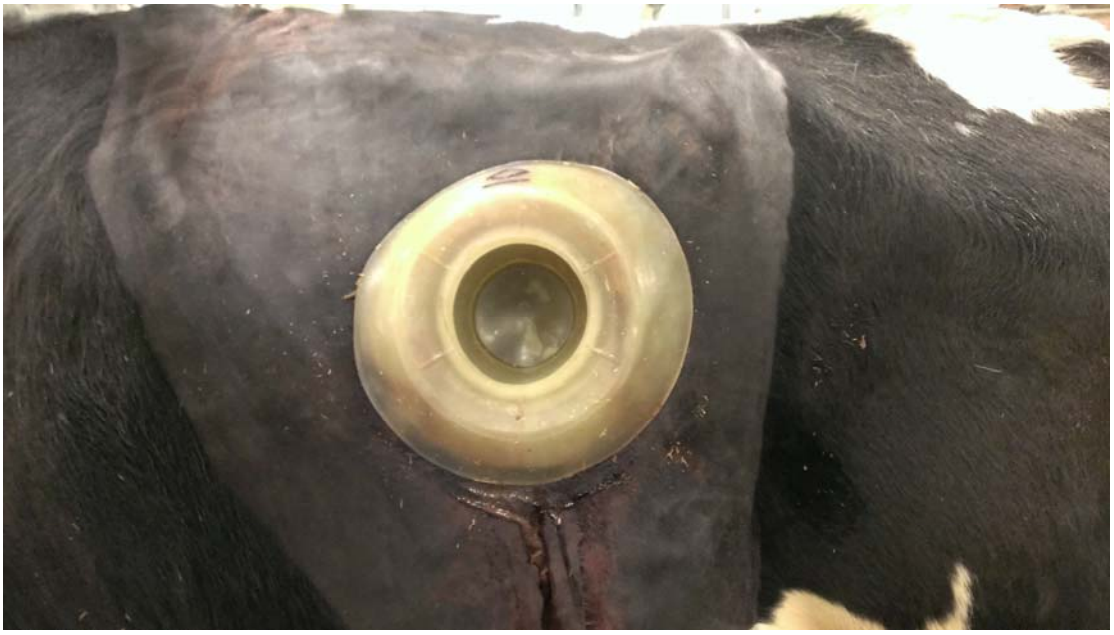
- BLAST OTUs that were significantly associated with metadata to give you a better idea of which specific microorganism may be involved:
    - Locate the ".fasta" file that resulted from the UPARSE batch script.
    - Open the ".fasta" file in a text-editing program.
    - Find (command+f) an OTU ID that was significantly associated with metadata.
    - Copy the sequence of the particular OTU.
    - Search "Nucleotide BLAST" on Google and click the first result.
    - Paste the sequence into the first text field ("Enter accession number(s), gi(s), or FASTA sequence(s)").
    - Under the "Choose Search Set" box, on the first "Database" drop-down menu, select "16s ribosomal RNA sequences (Bacteria and Archaea)".
    - Click "BLAST".
        - After searching the database, BLAST will present you with a list of the top matches for specific microorganisms along with a max score, total score, query cover %, E-value, and maximum identity %. The max score is a score of the best aligned sequence, while

the total score is the sum of scores of all aligned sequences. The query cover % represents the percentage of the sequence that overlaps with the potentially matching microorganism's sequence. The E-value represents the number of expected hits that occur by chance when searching the database; the smaller the E-value, the more significant the match. The identity % is the percent identity between the query and the hit in a nucleotide-to-nucleotide alignment (Agostino, 2013).

APPENDIX V

Appendix Figure 5.1 A 360° panoramic view of the UNL Dairy research barn where the trials were conducted.

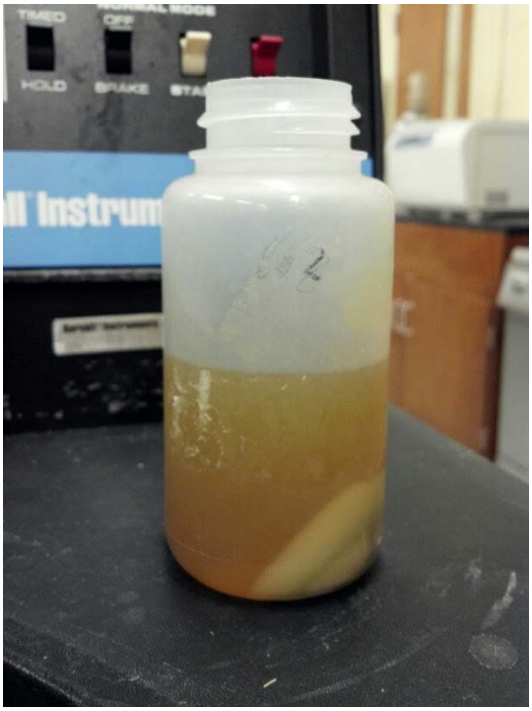
Appendix Figure 5.2 A load of green chop corn is dumped at the UNL Animal Science Complex in preparation for ensiling and eventual inclusion in experimental rations.



Appendix Figure 5.3 One of the rumen cannulae (Bar Diamond, Parma, ID) shortly after ruminal cannulation surgery in preparation for the experiment.

Appendix Figure 5.4 One of the duodenal cannulae (Bar Diamond, Parma, ID) shortly after duodenal cannulation surgery in preparation for the experiment.

Appendix Figure 5.5 Rumen digesta is strained for microbial isolation.



Appendix Figure 5.6 A microbial pellet is isolated from differential centrifugation of rumen fluid.

Appendix Figure 5.7 Microbial cells are physically lysed via bead beating during DNA extraction.

Appendix Figure 5.8 The Leco Nitrogen Analyzer (LECO Corporation, St. Joseph, MI) used for N quantification.

Appendix Figure 5.9 The MagMAX Express-96 Deep Well Magnetic Particle Processor (Applied Biosystems, Foster City, CA) used for DNA extraction in Experiment 2.

Appendix Figure 5.10 The epMotion M5073 (Eppendorf, Hamburg, Germany) liquid handling machine used in preparation for RT-PCR.

Appendix Figure 5.11 The Ion Torrent Personal Genome Machine (Life Technologies; Carlsbad, CA) used for sequencing of microbial DNA.

APPENDIX VI

DEFENSE SEMINAR SLIDES

Defense Seminar:

The Effects of HMTBa (2-Hydroxy-4-Methylthio-Butanoic Acid) Supplementation on Ruminal Microbial Crude Protein Synthesis and Community Structure in Dairy Cattle

Chad J. R. Jenkins
University of Nebraska-Lincoln
11-24-2014

Outline

- **Introduction**
  - Review of protein digestion in ruminants
  - Problem and approach
    - HMTBa
- **Experiment 1**
  - "Estimation of bacterial protein in rumen digesta using DNA markers"
  - Description of using DNA as a microbial marker
  - Results and implications
- **Experiment 2**
  - "Flow of microbial crude protein out of the rumen when dairy cattle are supplemented with HMTBa"
  - Materials and methods
  - Results and Discussion
- **Conclusion**
  - Summary and recommendations
  - Closing remarks

Introduction Protein Digestion in Ruminants:

- **Most protein entering the rumen is degraded by rumen microorganisms**
  - Converted to ammonia (Griffen et al., 1992)
  - Used as nitrogen source for microbial growth (Baldwin and Allison, 1983)
  - "Rumen degradable protein" (RDP)
- **Some proteins escape ruminal degradation by microorganisms**
  - Solubility in the rumen
    - Altered structural characteristic
    - Encapsulation
    - Chemical alteration
  - Rate of passage
  - "Rumen undegradable protein" (RUP)

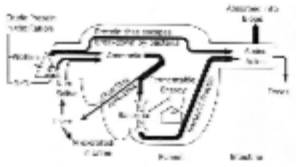
Introduction Protein Digestion in Ruminants:

- **Microbial protein**
  - Dead microbes flowing out of the rumen
  - "Microbial crude protein" (MCP)
- **Endogenous protein**
  - Sloughed epithelial cells
  - "Endogenous crude protein" (ECP)

Introduction Protein Digestion in Ruminants:

- **Metabolizable protein (MP)**
  - Protein available for absorption and utilization after the rumen
    - Demand increases during lactation
    - Efficiency of use for lactation is around 67 % (NRC, 2001)

Introduction Protein Digestion in Ruminants:

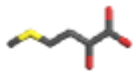


Introduction Problem and Approach:

- **Increased demand for MP during lactation**
  - Around 1,000 g/d dry - Around 2,500 g/d lactating (Block, 2010; Lee et al., 2012)
  - RUP is added to the diet: ex. animal [illegible]
- $$$ costly ingredients

Introduction Problem and Approach:

- **Maximize microbial efficiency with cost-effective inputs to support microbial activity**
  - Energy (carbohydrases→fiber)
  - N (protein)
  - Additives!
    - 2-Hydroxy-4-methylthio-Butanoic Acid (HMTBa)



Introduction HMTBa:

- 2-Hydroxy-4-Methylthio-Butanoic Acid (HMTBa)
  - Methionine analog (structurally similar)
  - Fed in swine and poultry industry as a substitute for methionine (Met)
    - In non-ruminants, converted to α-keto analog of methionine, transaminated to L-Met
  - Used in dairy industry for apparent effects on milk and milk fat yield (Zanton et al., 2014)
    - Ruminants possess enzymes for conversion of HMTBa to Met (Papas et al., 1984)
    - Studies suggest HMTBa is more resistant to rumen degradation than Met
      - 0 to 40 % rumen escape (Vázquez-Añón et al., 2001)
      - Minimal effects on blood Met concentration (Johnson et al., 1999)
      - Portion may be absorbed through rumen and omasal walls (McCollum et al., 2000)
    - Mainly utilized in the rumen—effects poorly understood
      - Effect on microbial activity?

Introduction HMTBa:

The effects of methionine analogue supplementation on rumen digestive conditions; the '+' represents a positive effect, '-' a negative effect, and '0' no effect

	Rumen Microbes		Digestibility		TPs			Rumen Measurements		
Study	MCP Yield	Protozoa Count	Fiber	Starch	Lactose	Propionate	Butyrate	Ammonia	pH	Yield
Rogers et al., 1979		+								
Rogers et al., 1979					+	-	+			
Oh et al., 1972	+		+	+				-		
Bull and Vandzant, 1972			+							
Lundgren et al., 1987					+	-	0			
Vazquez-Añón et al., 2006	+		0		0	0	0	-	0	
Vázquez et al., 2008	0	0	+	0	0	0	0	0	0	0

[1]Experiment was conducted in vivo
[2]Experiment was conducted in vitro

Introduction HMTBa:

- Questions to address:
  - How is microbial activity influenced in the rumen?
    - Is MCP increased post-ruminally?
  - Is microbial community structure influenced?
  - Are these effects altered under different dietary conditions (MP concentration)?

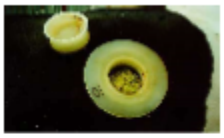
Experiment 1:

Estimation of bacterial protein in rumen digesta using DNA markers

C. J. R Jenkins, E. Castillo-Lopez, S. C. Fernando, and P. J. Kononoff

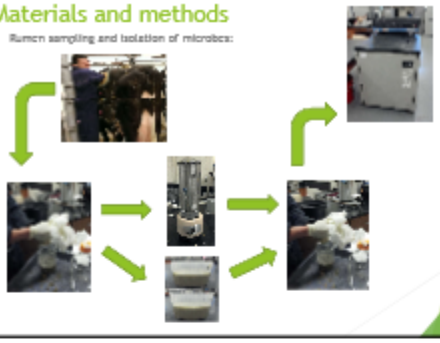
Introduction

- Studies involving evaluation of microbial community often involve sampling of rumen digesta for isolation of microbes
  - It is believed that greater proportions of bacteria are associated with rumen solids than fluid
    - Particle associated bacteria (PAB) vs. liquid associated bacteria (LAB)
  - Difference has never been measured using DNA as a microbial marker



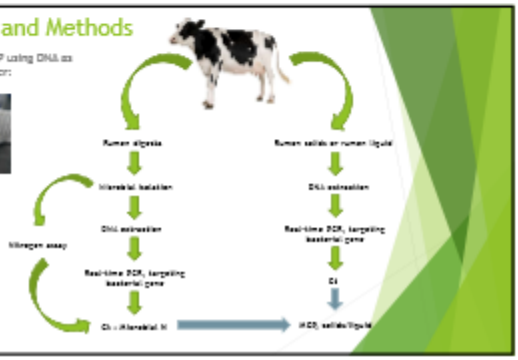
Materials and methods

Rumen sampling and isolation of microbes:



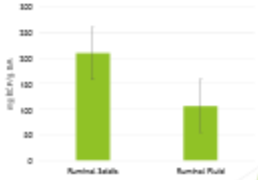
Materials and Methods

Estimation of MCP using DNA as a microbial marker:



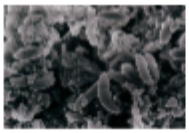
Results

- Bacterial crude protein concentration tended to be higher ($P = 0.09$) in the solid portion of rumen digesta than in the liquid portion
  - Solid: 109.5 ± 16.6 mg BCP/g DM
  - Liquid: 106.4 ± 43.6 mg BCP/g DM



Discussion

- Results emphasize the need to sample both the solid and liquid fractions
  - Avoid misrepresentation of bacterial community
- Future work/recommendations:
  - Make a second estimate of RCP using a different marker (purines)
  - DNA sequencing: evaluate community structure of FAB vs. LAB
  - Compare "blending" isolation method with traditional "shaking"



Experiment 2:

Flow of microbial crude protein out of the rumen when dairy cattle are supplemented with HMTBa

C. J. R. Jenkins, S. C. Fernando, C. L. Anderson, N. D. Mulligs, E. Castillo-Lopez and P. J. Hanzlicek

Introduction

- Commercial HMTBa product (Alimet, Novus International, St. Charles, MO), has been shown to have enhancing effects on rumen microbial community
  - Mechanism of utilization in rumen poorly characterized
  - How does it perform under different dietary conditions (concentration of MP)?
    - Flow of MCP to duodenum?
    - Rumen microbial activity?
  - Does it affect rumen microbial community composition?

alimet

Introduction

The effects of methionine analogue supplementation on rumen digestive conditions; the '+' represents a positive effect, '-' a negative effect, and '0' not affected

	Rumen Isolates			Digestibility		TVFA			Rumen Measurements		
Study	MCP Yield	Protozoa Count	Fiber Digest.	Starch Digest.	Acetate	Propionate	Butyrate	Ammonia	pH	Feed Intake
Rogers et al., 1979		+								
Rogers et al., 1979					+	-	+			
Oh et al., 1979	+		+	+				-		
Bull and Vandersall, 1973			+							
Lundquist et al., 1987					+	-	0			
Vazquez-Añón et al., 2006	+		0		0	0	0	-	0	
Vazquez-Añón et al., 2008	0	0	+	0	0	0	0	0	0	0

*Experiment was conducted in vivo
*Experiment was conducted in vitro

Introduction

- **Hypothesis:**
  - Rumen microbial activity will increase with HMTBa supplementation in combination with a diet high in MP
  - Microbial response lower or nonexistent with diet low in MP
  - Rumen microbial community structure will be altered by HMTBa supplementation and diet type

Materials and Methods

- **Factors of interest:**
  - Intake and nutrient digestibility
  - Lactation performance (milk yield and components)
  - Rumen conditions
    - pH, VFA and ammonia concentration
  - MCP flow to the duodenum
  - Microbial community structure

Materials and Methods Experimental Design:

- **Four multiparous, lactating Holstein cows fitted with ruminal and duodenal cannulae**
  - 4 × 4 Latin square design
  - 2 × 2 factorial arrangement of treatments
    - Control vs. HMB
    - Low MP vs. High MP
  - 28 d periods, 19 d of adaptation, 9 d of sample collection

Materials and Methods Treatments:

Ingredient and chemical composition of TMR fed during the experiment formulated to supply concentrations of metabolizable protein either deficient or in excess of requirements for lactating dairy cows[1]

Ingredient, % DM	Level of Metabolizable Protein	
	Low MP	High MP
Corn silage	19.0	19.1
Corn grain	19.0	19.1
alfalfa hay	12.7	12.7
Grass hay	6.35	6.35
Soybean hulls	7.94	7.95
Soybean meal DDGS[2]	8.48	8.50
Molasses cane	4.00	4.00
Soybean meal	3.49	7.50
Limestone	1.00	1.00
Urea	0.70	0.18
Sodium bicarbonate	0.80	0.80
Dicalcium phosphate	0.80	0.80
Blood meal	0.48	0.48
Magnesium oxide	0.22	0.22
Salt	0.21	0.21
Megalac	0.17	0.17
Mineral premix[3]	0.10	0.10
Vitamin premix[4]	0.10	0.10

Chemical composition	Level of Metabolizable Protein	
	Low MP	High MP
DM, %	62.6	65.4
CP, %	16.4	18.4
RUP, % CP	33.5	33.1
RDP, % CP	66.7	66.9
NDF, %	33.7	33.8
ADF, %	23.1	23.2
Starch, %	27.1	24.0
Ether extract, %	3.71	3.62
MP, kg/d	2.12	3.08
ME, Mcal/d[5]	33.8	33.8

[1]according to the CPM Dairy Ration analyzer (v3.0.8.1; Boucher et al., 2009)
[2]Dried distillers grains with solubles
[3]Formulated to provide 1.0 % Ca, 0.40 % P, 0.24 % Mg, and 1.2 % K.
[4]Formulated to supply approximately 132,000 IU of vitamin A, 24,000 IU of vitamin D3, and 800 IU of vitamin E/d in the ration.
[5]according to the dairy NRC (2001) model

Materials and Methods Treatments:

- **Diets were top dressed each morning prior to feeding**
  - 250 g of HMB or 250 g control
    - HMB top dress: ground corn utilized as a carrier
    - Control top dress: ground corn only
  - HMB – 0.114 % of diet DM (25 g)
- 15 g/d Cr_2O_3 dosed via rumen cannula

Materials and Methods Sampling:

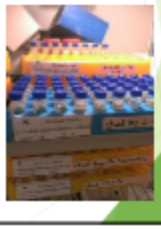
- **Rumen and duodenal digesta**
  - Every 4 h on d 23 - 26
  - Offset by 1 h each day
  - pH taken with handheld probe
- **Bacteria and protozoa**
  - Twice daily, d 25 - 26
- **Feces**
  - Every other rumen/duodenal timepoint
- **Feed**
  - TMR, ingredients, and orts, d 27 -28
- **Milk**
  - Twice daily, d 23 - 26
- **Rumen evacuation**
  - d 27, 4 h after feeding
  - d 28, 4 h before feeding

Materials and Methods Laboratory Analyses:

- **Feed, feces, rumen contents, duodenal contents**
  - All samples dried (60˚C) and ground through 1-mm Wiley mill
  - Subsamples of TMR, ingredients, and orts sent to external laboratory (Cumberland Valley Analytical Services, Hagerstown, MD)
    - Analyzed for DM, N, soluble protein, RDP, NDF, aNDF, ADFN, NDFN, lignin, starch, sugar, ether extract, ash, and minerals
  - Subsamples of feces, rumen and duodenal contents sent to external laboratory (Ansalab, Fulton, IL) for Cr_2O_3
  - Feed, orts, rumen contents, duodenal contents, and feces also analyzed at the UNL Ruminant Nutrition lab
    - DM, ash, N, NDF, starch, INDF and iADF (for digesta flow)

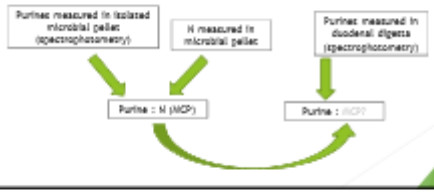
Materials and Methods Laboratory Analyses:

- **Rumen fluid**
  - Analyzed for ammonia using phenol-hypochlorite assay (Chaney and Edvard, 1962)
  - Analyzed for VFA using gas chromatography (Erwin et al., 1961)



Materials and Methods Laboratory Analyses:

- **Estimation of MCP using purines as a microbial marker (Zinn and Owens, 1986; Broderick and Merchen, 1992)**
  - Bacteria are high in nucleic acid, namely adenine and guanine (purines)
  - Feeds are typically low in adenine and guanine



Materials and Methods

Estimation of bacterial and protozoal CP using DNA as a microbial marker:

Materials and Methods Laboratory Analyses:

- **DNA sequencing and bioinformatics**
  - DNA extracted from rumen digesta at 0, 4, 12, and 16 h after feeding
  - Sequencing performed on the Ion Torrent Personal Genome Machine (Life Technologies, Carlsbad, CA) using V3 region of 16s rRNA
  - Reads analyzed using UPARSE (www.drive5.com/uparse) pipeline within Quantitative Insights Into Microbial Ecology (QIIME)
  - Analyses conducted remotely on one UNL's super computers, "Tusker" (Holland Computing Center), using a secure shell (SSH) client
    - Reads clustered into operational taxonomic units (OTUs): cluster of similar 16s rRNA sequences
  - Associations of OTUs and metadata were analyzed using multivariate association with linear models (MaAsLin, Harvard School of Public Health, Boston, MA)
    - OTU taxonomy identified using BLAST (Basic Local Alignment Search Tool, NCBI, Bethesda, MD)

Results and Discussion Milk and Intake:

Effects of feeding DDGS with Low and High MP diets on milk production and composition

	Treatment					P-value[1]		
	Low MP		High MP					
	Control	DDGS	Control	DDGS	SEM[2]	MP	DDGS	MP × DDGS
DMI, kg/d	22.8	22.7	24.1	24.0	1.10	0.45	0.94	0.93
Milk yield, kg/d	37.0	37.1	38.1	38.4	1.16	0.41	0.78	0.93
3.5 % FCM[3]	38.0	37.8	38.7	39.0	0.99	0.41	0.77	0.98
Fat, %	0.66	1.76	0.67	1.79	0.11	0.76	0.47	0.67
Fat yield, kg/d	1.02	0.97	1.04	1.04	0.09	0.34	0.79	0.97
Protein, %	3.04	3.02	3.05	3.02	0.09	0.94	0.57	0.97
Protein yield, kg/d	0.91	0.88	0.93	0.91	0.06	0.74	0.78	0.98
MUN, mg/dL	11.4	12.0	11.0	11.8	0.97	0.41	0.21	0.66

[1] P-values for contrasts of level of metabolizable protein and NPN supplementation
[2] The highest standard error of treatment means is shown
[3] 3.5 % Fat-corrected milk = (milk fat (kg) × 14.3713) + (milk yield (kg) × 0.4324)
MUN, Urea Nitrogen

Results and Discussion Milk and Intake:

Effects of feeding HMB with high or low MP diets on nutrient digestibility estimated using chromium oxide, iNDF or iADF as digesta flow markers

	Treatment					P-value[2]		
	Low MP		High MP					
	Control	HMB	Control	HMB	SEM[1]	MP	HMB	MP × HMB
Cr_2O_3 markers								
DM, %	69.8	71.7	64.0	68.2	1.48	0.07	0.27	0.66
N, %	69.8	69.0	61.7	64.8	2.20	0.08	0.21	0.82
NDF, %	58.8	60.2	48.7	47.2	2.82	0.11	0.49	0.87
OM, %	69.4	69.8	61.1	61.2	1.66	0.03	0.27	0.66
iNDF markers								
DM, %	69.8	69.0	67.0	68.1	1.80	0.01	0.84	0.21
N, %	69.8	69.2	64.0	66.8	1.80	0.01	0.80	0.69
NDF, %	68.2	69.1	58.1	61.7	2.48	0.01	0.77	0.06
OM, %	67.2	68.6	64.2	64.7	1.37	0.01	0.80	0.44
iADF markers								
DM, %	62.6	61.8	57.4	62.1	1.10	0.48	0.68	0.14
N, %	62.0	61.8	54.4	60.8	1.80	0.11	0.22	0.16
NDF, %	44.2	41.4	34.0	42.8	2.17	0.44	0.40	0.22
OM, %	63.4	63.2	57.7	63.7	1.44	0.41	0.68	0.14

[1]The highest standard error of treatment means is shown.
[2]P-values for contrasts of level of metabolizable protein and HMB supplementation

Results and Discussion Milk and Intake:

- In order to attain a concentration of MP deficient in Low MP diet, SBM was reduced and replaced with ground corn and urea
  - All nutrient digestibilities observed to be greater than High MP
- INDF was the only digesta flow marker to result in significant digestibility estimates
  - Estimations of digesta flow attained from the Cr_2O_3 markers may be less sensitive to contributions of digesta phases (Iparraguerre et al., 2007) as Cr_2O_3 does not associate with any phase as it flows through the digestive system (Hanchen, 1988)
  - INDF and iADF as risk for introducing variation, as procedure involves in situ bag incubation in the rumen—possibility for wash out

Results and Discussion Rumen characteristics:

Effects of feeding HMB with Low or High MP diets on rumen pH, gas1, and kinetics

	Treatment					P-value[2]		
	Low MP		High MP					
	Control	HMB	Control	HMB	SEM[3]	MP	HMB	MP × HMB
Rumen pH	6.80	6.84	6.87	6.78	0.16	0.08	<0.01	0.00
Rumen Temp, °C	10.6	10.1	10.3	10.4	1.28	0.08	0.41	0.83
Gas[1], L/h								
DM k_d	5.80	5.97	5.97	5.97	0.24	0.72	0.10	0.83
DM k_p	6.40	6.58	6.01	6.20	0.27	0.18	0.21	0.84
NDF k_d	5.76	5.80	5.68	5.84	0.21	0.24	0.82	0.71
NDF k_p	2.04	1.97	2.41	2.05	0.21	0.24	0.27	0.19

[2]P-values for contrasts of level of metabolizable protein and HMB supplementation
[3]The highest standard error of treatment means is shown
[1]Gas of passage (k_p) rate of digestion (k_d)

Results and Discussion Rumen characteristics:

- pH reduced from Low MP to High MP diets and with HMB supplementation
  - Effect of HMB on pH has not been previously observed
- Rate of digestion was reflective of digestibility estimates

Results and Discussion Rumen characteristics:

Effects of feeding HMB with Low or High MP diets on rumen ammonia and volatile fatty acids

	Treatment					P-value[1]		
	Low MP		High MP					
	Control	HMB	Control	HMB	SEM[2]	MP	HMB	MP × HMB
Rumen ammonia, mg/dl	17.0	17.2	16.0	17.2	0.88	0.16	0.06	0.30
Total VFA, (mM) VFA mol/100 mol	120	126	118	126	4.24	0.77	0.02	0.48
Acetate	62.1	61.6	62.0	62.2	1.24	0.22	<0.01	0.21
Propionate	22.0	28.1	21.6	24.8	1.22	0.62	<0.01	0.08
Isobutyrate	0.92	0.86	0.91	0.90	0.07	0.00	0.22	0.12
Butyrate	11.1	10.6	11.0	10.8	0.48	0.07	0.06	0.40
Isovalerate	0.06	1.07	1.04	1.00	0.12	0.00	0.00	0.71
Valerate	1.48	1.48	1.40	1.47	0.08	<0.01	0.22	0.22
A:P[3]	2.82	2.80	2.78	2.64	0.08	0.40	<0.01	0.04

[1]P-values for measures of level of metabolizable protein and HMB supplementation
[2]The highest standard error of treatment means is shown
[3]Ratio of acetate to propionate

Results and Discussion Rumen characteristics:

- **Rumen ammonia and VFA concentration results were surprising, as our observations were contrary to what has typically been observed in the past**
  - Blake et al. (1986) suggested that HMB supplementation may stimulate protein digestion, releasing more ammonia in the rumen
  - Most recent in vivo study found no effects of HMB on VFA concentration (Hofsager et al., 2005)
  - More investigation into the mechanism of these effects is required

Results and Discussion Duodenal Digesta N Flow:

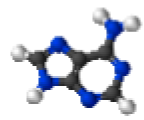
Effects of feeding HMB with Low and High MP diets on duodenal digesta and N flows estimated using purines and either chromium oxide, iNDF or iADF as digesta flow markers

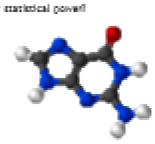
	Treatment					P-value[1]		
	Low MP		High MP					
	Control	HMB	Control	HMB	SEM[2]	MP	HMB	MP × HMB
Cr_2O_3 marker								
Duodenal Flow, kg DM/d	17.4	17.8	17.7	17.7	1.01	0.07	0.00	0.00
Duodenal N Flow, g DM/d	768	772	787	788	43.2	0.64	0.46	0.41
Bacterial N Flow, g DM/d	267	302	288	288	48.4	0.28	0.22	0.48
Bacterial N of total N Flow, %	48.4	20.7	47.2	42.2	4.27	0.78	0.18	0.47
iNDF marker								
Duodenal Flow, kg DM/d	16.7	16.7	16.0	16.8	1.18	0.70	0.28	0.20
Duodenal N Flow, g DM/d	687	702	678	772	42.8	0.40	0.27	0.41
Bacterial N Flow, g DM/d	242	270	218	288	44.2	0.71	0.67	0.17
Bacterial N of total N Flow, %	48.4	20.7	40.2	42.8	7.08	0.72	0.18	0.68
iADF marker								
Duodenal Flow, kg DM/d	20.4	21.4	18.4	21.8	1.88	0.88	0.28	0.42
Duodenal N Flow, g DM/d	611	684	820	882	61.8	0.72	0.28	0.40
Bacterial N Flow, g DM/d	480	248	282	408	48.2	0.80	0.42	0.22
Bacterial N of total N Flow, %	48.4	20.7	40.2	42.8	4.11	0.72	0.18	0.68

[1]P-values for measures of level of metabolizable protein and HMB supplementation
[2]The highest standard error of treatment means is shown

Results and Discussion Duodenal Digesta N Flow:

- **No effects of HMB on microbial protein flow were observed using purines as a microbial marker and any of the digesta flow markers**
  - Similar results as most recent in vivo study (Hofsager et al., 2005)
  - A meta-analysis of studies involving purines (Clark et al., 1992) found a range of estimates of purine concentration in mixed ruminal bacteria from 3.40 to 13.02 %
    - Overcome variation by increasing statistical power!





Results and Discussion Microbial DNA in Duodenal Fluid:

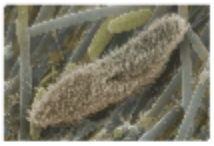
Effects of feeding HMB with Low or High MP diets on bacterial and protozoal DNA abundance in duodenal fluid.

	Treatment					P-Value[2]		
	Low MP		High MP					
	Control	HMB	Control	HMB	SEM[1]	MP	HMB	MP × HMB
Bacteria								
Ct[3]	19.0	19.4	18.8	19.4	0.20	0.46	0.10	0.22
Abundance, g DM[4]	2.26E+11	1.84E+11	2.34E+11	1.11E+11	0.32	0.43	0.41	0.34
Protozoa								
Ct[3]	28.1	28.0	28.2	28.1	0.09	0.61	0.08	0.46
Abundance, g DM	0.00	0.00	0.01	0.00	1.20	0.27	0.42	0.22

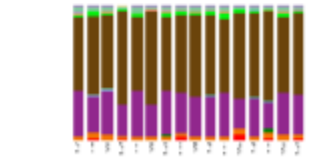
[1]Highest standard error of treatment means is shown.
[2]P-values for summary of level of MP and HMB supplementation.
[3]Cycle threshold resulting from RT-PCR targeting DNA encoding part of the bacterial 16S rRNA gene.
[4]Abundance of targeted DNA per gram of duodenal fluid DM; abundance = ((Efficiency^Ct))(Castillo-Lopez et al., 2016)
[5]Cycle threshold resulting from RT-PCR targeting DNA encoding part of the protozoal 18S rRNA gene

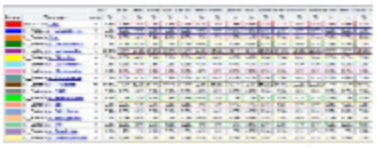
Results and Discussion Microbial DNA in Duodenal Fluid:

- Bacterial Ct observed were much higher than expected—microbial protein flow was not calculated
  - Needs to be revisited for comparison of observations made using purines
- An effect of HMB on protozoal Ct was observed
  - Ct was higher for protozoal target DNA in duodenal fluid of cows consuming HMB, suggesting less protozoal DNA was present



Results and Discussion Bioinformatics:



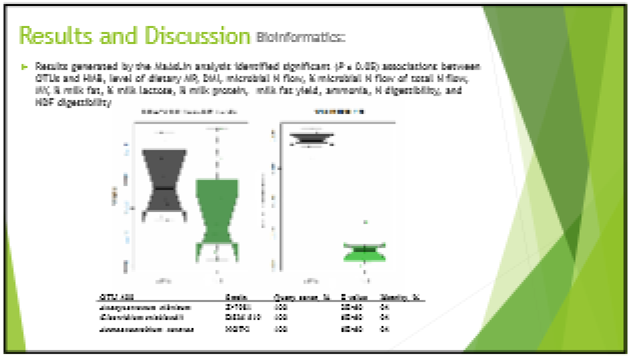


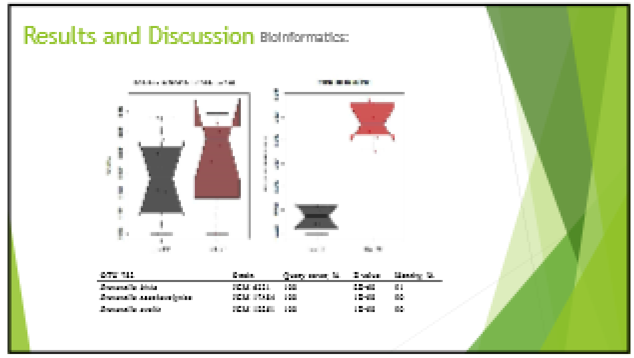
Results and Discussion Bioinformatics:

Effect of feeding HMB with Low and High MP diets on proportion of phyla of rumen bacterial populations relative to total number of reads recovered from ruminal digesta enriched from bioinformatics analysis of rare OTUs

	Treatment					P-Value[1]		
	Low MP		High MP					
	Control	HMB	Control	HMB	SEM[2]	MP	HMB	MP × HMB
Core analysis								
Firmicutes	61.4	60.1	60.2	60.0	0.76	0.42	0.77	0.84
Bacteroidetes	27.0	28.1	28.7	28.0	0.52	0.24	0.84	0.68
Other	1.70	1.18	0.68	1.18	0.33	0.28	0.08	0.22
Proteobacteria	1.61	1.70	1.71	1.61	0.48	0.97	0.70	0.17
TM7	1.80	1.82	1.68	1.10	0.17	0.24	0.24	0.78
Tenericutes	1.02	0.90	1.10	1.08	0.15	0.22	0.84	0.78
Actinobacteria	0.40	0.68	0.48	0.68	0.14	0.87	0.24	0.81
SR1	0.78	0.72	0.78	1.02	0.14	0.42	0.84	0.47
Spirochaetes	0.28	0.28	0.28	0.42	0.07	0.42	0.42	0.42
Fibrobacteres	0.24	0.42	0.22	0.28	0.04	0.78	0.04	0.21
Verrucomicrobia	0.04	0.04	0.10	0.04	0.05	1.00	0.12	0.12

[1]P-values for summary of level of metabolizable protein and HMB supplementation
[2]The highest standard error of treatment means is shown


Results and Discussion Bioinformatics: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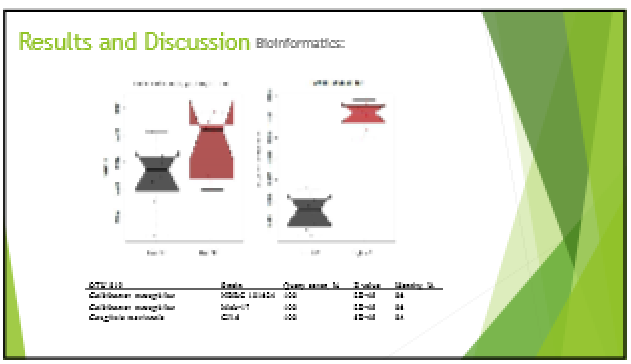
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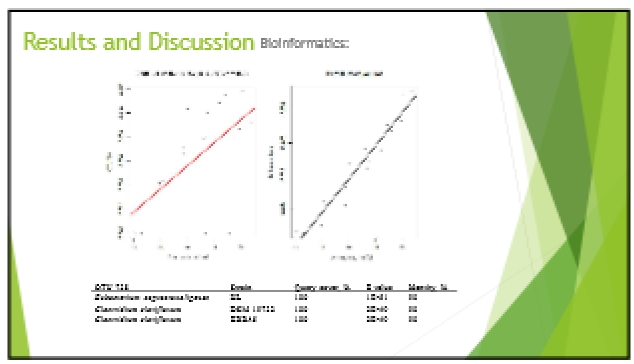
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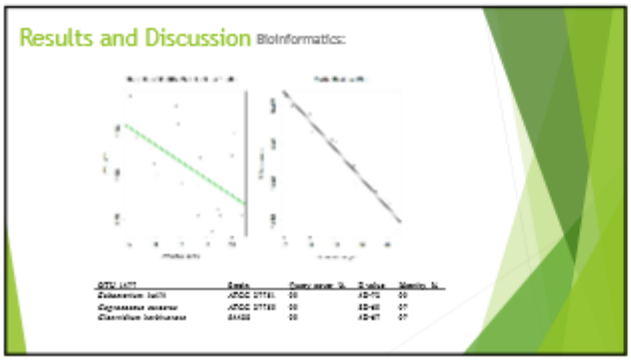
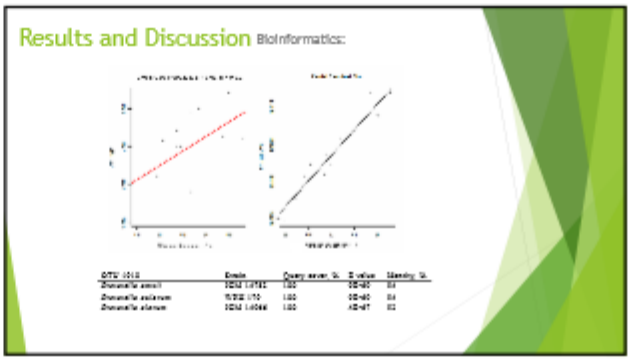
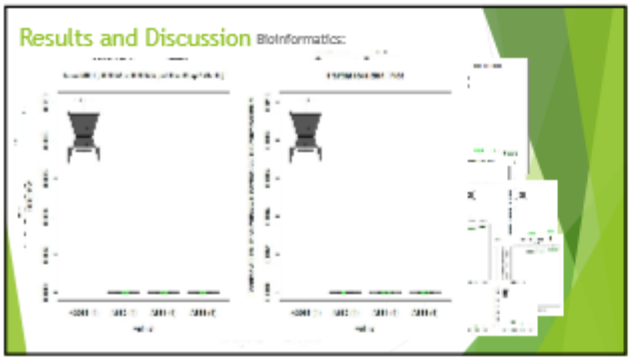
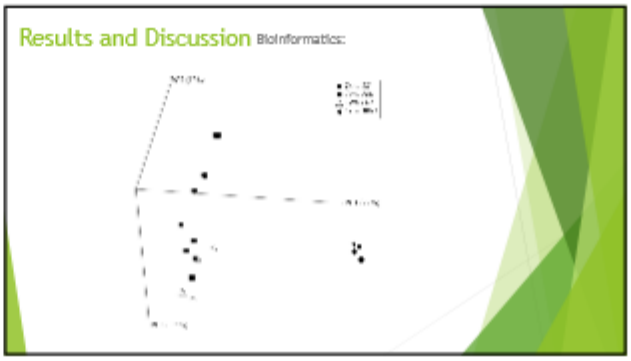

Results and Discussion Bioinformatics:

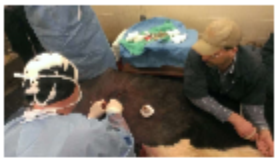

Results and Discussion Bioinformatics:


Results and Discussion Bioinformatics:


Results and Discussion Bioinformatics:

Results and Discussion Bioinformatics:

- Why was cow 3069's rumen microbial community structure so different than the other cows?
  - New to the dairy research barn?
  - Proximity of sampling to surgery?



Conclusion:

Summary, Recommendations, and Acknowledgements

Conclusion

- Level of dietary MP and HMB supplementation did not interact
- Observations of effects of HMB on rumen microbial activity were mixed relative to what has been previously observed
  - Observations indicate there is a stimulatory effect on rumen microorganisms
  - Further investigation into mechanisms is required
- We observed an effect of HMB on microbial community structure and associations of microorganisms and metadata
  - Good starting point for future work, i.e. isolation and culturing of particular microorganisms
- Continued inclusion in dairy diets is advisable (Zanton et al., 2014), however a better understanding of effects in the rumen would help nutritionists know how to best utilize it as a supplement

Acknowledgements Thank you!!

- Co-advisors:
  - Dr. Kononoff and Dr. Fernando
- Former and current graduate students:
  - Ezequiel Castillo-Lopez, Hugo Ramirez Ramirez, Henry Paz, Alison Foth, Gabe Garcia Gomez, Jana Harding
- Erin Marotz and Darren Stritzek
- Undergraduate employees:
  - Kiley Jilgra and Kellyn Saathoff
- Leah Jenkins