Methionine and Methionine Analog Supplementation: Comparison of Bioavailability In Dairy Cows and Differential Utilization by Rumen Microbes in Batch Culture

THESIS

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Abstract

Methionine (Met) is one of the first limiting amino acids (AA) for milk and milk protein production in many dairy diets. Before dietary Met can be utilized by the cow, it is first exposed to microbial degradation in the rumen. Met that bypasses ruminal degradation, is absorbed in the hindgut, and is available for metabolism by the cow is termed ‘bioavailable’ Met. Met flow to the hindgut can be increased by adding supplemental Met to the diet or by supporting microbial protein synthesis (MPS) and subsequently increasing the flow of microbial protein (MCP) to the small intestine. The majority of protein, 50 to 80%, that reaches the hindgut of ruminants comes from MCP, whose AA composition closely resembles that of milk. Two studies were conducted to better understand the effect of Met supplementation on Met bioavailability and the impact on MPS in the rumen. In the first study, Se substitution for Met was used to estimate the change in the flux of methionine at the mammary gland. This method was used to compare the bioavailability of five Met treatments: control, DL-Met, Smartamine (SMA), 2-hydroxy-4-(methylthio)-butanoic acid (HMB), the isopropyl ester of HMB (HMBi), and an experimental rumen protected supplement (ERPM). Met supplements were fed to twenty Holstein cows as a top dressing that provided 18g of Met derivative/d, in a truncated Latin square experiment. In the second study, the effect of supplementation of Met, from Met and Met analogs, on the synthesis of Met de novo versus the incorporation of preformed Met into MCP was determined by dosing $^{15}$NH$_3$ and $^{13}$C-Met in batch cultures assigned to a randomized complete block experiment. Treatments consisted of
control, 0.097% L-Met, 0.097% D-Met, 0.125% HMBi, 0.098% HMB, 0.250% HMBi (2×HMBi), 0.063% HMBi + 0.049% DL-Met (HMBi+MET), and 0.098% HMB + 0.039% isopropanol (HMB+ISO) and treatments were inoculated with faunated or defaunated inocula. Unless otherwise specified, treatments within experiments were equal molar. The calculated metabolizable Met flow in the first study for cows supplemented with DL-Met, SMA, HMB, HMBi, and ERPM was 42.4, 47.0, 52.8, 48.7, and 50.3 g/d respectively. Metabolizable Met estimates were adjusted for reduced DMI with HMBi supplementation (20.4 kg/d versus 21.9 kg/d for control, $P < 0.01$). Mean milk production for all cows was 30.3 kg/d and was not affected by treatment. In the second experiment, both $^{13}$C and $^{15}$N enrichment of the pellet differed over time ($P < 0.001$). L-MET decreased ($P < 0.01$) $^{13}$C enrichment of bacterial Met compared with HMBi+MET and both had lower ($P < 0.03$) enrichment than all other treatments. D-MET tended to have lower enrichment than HMBi ($P = 0.16$) and 2×HMBi ($P = 0.12$). In order to make definitive comparisons regarding the synthesis of Met de novo versus incorporation from pre-formed (treatment) Met into MCP, free $^{13}$C-Met in the supernatant needs to be determined. It is possible that lower enrichment are due to dilution of labeled Met by an increased incorporation of Met into MCP provided from L-MET, HMBi+MET and D-MET compared to other treatments.
This thesis is dedicated….

to kindness, love, and compassion; whose qualities have inspired and encouraged me,
whether in the face of frustration or jubilation;

to perseverance and determination; whose never ending spirits have taught me to keep
pressing forward;

and to patience; whose attributes have encouraged me to do everything one step at a time.
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Major Field: Animal Sciences
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<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>amino acid</td>
</tr>
<tr>
<td>AAOX</td>
<td>amino acid oxidase</td>
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<tr>
<td>ADF</td>
<td>acid detergent fiber</td>
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<tr>
<td>APE</td>
<td>atom percent excess</td>
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<tr>
<td>BHMT</td>
<td>betaine-homocysteine methyltransferase</td>
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<tr>
<td>BCAA</td>
<td>branched chain amino acid</td>
</tr>
<tr>
<td>BCAT</td>
<td>branched chain amino acid transferase</td>
</tr>
<tr>
<td>BCFA</td>
<td>branched chain fatty acid</td>
</tr>
<tr>
<td>BPDS</td>
<td>binding protein-dependent systems</td>
</tr>
<tr>
<td>BUN</td>
<td>blood urea nitrogen</td>
</tr>
<tr>
<td>CBS</td>
<td>cystathionine β-synthase</td>
</tr>
<tr>
<td>CGL</td>
<td>cystathionine γ-lyase (also known as cystathionase)</td>
</tr>
<tr>
<td>CP</td>
<td>crude protein</td>
</tr>
<tr>
<td>D</td>
<td>defaunated</td>
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<tr>
<td>DM</td>
<td>dry matter</td>
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<tr>
<td>DMI</td>
<td>dry matter intake</td>
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<td>E</td>
<td>entry rate</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>EMPS</td>
<td>efficiency of microbial protein synthesis</td>
</tr>
<tr>
<td>ERPM</td>
<td>experimental rumen protected methionine supplement</td>
</tr>
<tr>
<td>F</td>
<td>faunated</td>
</tr>
<tr>
<td>GNMT</td>
<td>glycine $N$-methyltransferase</td>
</tr>
<tr>
<td>HADH</td>
<td>2-hydroxy acid dehydrogenase</td>
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<tr>
<td>HAO</td>
<td>$\alpha$-hydroxy acid oxidase</td>
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<tr>
<td>HMB</td>
<td>2-hydroxy-$4$-($methylthio$) butanoic acid</td>
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<td>HMBi</td>
<td>2-hydroxy-$4$-($methylthio$) butanoic acid isopropyl ester</td>
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<td>2-keto-$4$-($methylthio$) butanoic acid</td>
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<td>methionine adenosyltransferase</td>
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<tr>
<td>Met or MET</td>
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</tr>
<tr>
<td>MCP</td>
<td>microbial protein</td>
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<td>microbial protein synthesis</td>
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<td>MS</td>
<td>methionine synthase</td>
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<td>methionine sulfoxide reductase</td>
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<td>methylenetetrahydrofolate reductatse</td>
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<tr>
<td>MUN</td>
<td>milk urea nitrogen</td>
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<tr>
<td>N</td>
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</tr>
<tr>
<td>NDF</td>
<td>neutral detergent fiber</td>
</tr>
<tr>
<td>PG</td>
<td>peptidoglycan</td>
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<tr>
<td>r</td>
<td>entry rate of SeMet</td>
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<td>Description</td>
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<tr>
<td>RP-AA</td>
<td>rumen protected amino acid</td>
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<tr>
<td>RDP</td>
<td>rumen degraded protein</td>
</tr>
<tr>
<td>RUP</td>
<td>rumen undegraded protein</td>
</tr>
<tr>
<td>SA</td>
<td>specific activity</td>
</tr>
<tr>
<td>SAH</td>
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<td>SAHH</td>
<td>S-adenosylhomocysteine hydrolase</td>
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<td>S-adenosylmethionine</td>
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<tr>
<td>SeMet</td>
<td>selenomethionine</td>
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<tr>
<td>SMA</td>
<td>Smartamine</td>
</tr>
<tr>
<td>TMR</td>
<td>total mixed ration</td>
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<tr>
<td>VFA</td>
<td>volatile fatty acid</td>
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Chapter 1: Review of Literature

Introduction

Throughout the decade, the general public and special interest groups have become increasingly sensitive to environmental issues including those caused by agricultural pollution. Pressure on the agricultural community to decrease N pollution will continue in the future, and efforts should be made to preserve the image of agriculture. According to the Environmental Protection Agency (2005), animal feeding operations, which consist of approximately 238,000 working farms in the United States, generate an estimated 500 million tons of manure each year. Excessive N runoff contaminates aquatic ecosystems and leads to algae blooms and oxygen depletion, ultimately causing aquatic death. Manure N excretion increases atmospheric ammonia and N oxide emissions, which contributes to global pollution and global warming. Ammonia, produced mostly from the decomposition of urea excreted in urine, is a major byproduct of ruminant metabolism and can impact both soil and air quality. Ammonia is harmful to the ozone layer, contributes to acid deposition, and contaminates water systems.

Nitrogen losses result, in part, from a dietary imbalance of carbohydrate and protein or differences in the degradation rate of these nutrients and the subsequent
asynchrony in nutrient availability in the rumen (Stern et al., 1994). When energy requirements are met in dairy diets, increasing protein beyond the recommended protein level does not increase milk production, but increases manure N excretion (Wu and Satter, 2000; Olmos Colmenero and Broderick 2006). If more dietary crude protein is fed than required, the excess protein is broken down to ammonia N, absorbed, and metabolized to urea in the liver, which is recycled to the gut or excreted in urine (Broderick et al., 2009). Release of N in this manner is a waste of protein, which may account for up to 20% of protein fed to dairy cattle. Despite the economic loss associated with waste protein, VandeHaar and St-Pierre (2006) reported dairy farmers are less concerned about maximizing the efficiency of N usage and more concerned about the economic risk of underfeeding protein and potentially decreasing milk yields.

Inefficient conversion of dietary-N into animal protein may not be due to simple deficiencies in total amino acid (AA) supply, but to the profile of absorbed AA (Lobley and Milano, 1997). Balancing metabolizable AA supply can improve milk yield and milk components, and decrease dietary concentration of crude protein (Noftsger and St-Pierre, 2003). This can be accomplished while maintaining or even improving production while decreasing feed costs and N pollution into the environment. Research has focused on achieving a better understanding of AA utilization by rumen microbes and cows. The development of rumen protected AA (RP-AA) products has made it easier to balance metabolizable AA in dairy diets. However, rumen protection is accomplished using a variety of technologies, which differ in the amount of digestible AA provided to the cow. To appreciate these technologies, it is important to understand how or if these
products are directly utilized by rumen microbes, the strength of their rumen protection, the amount of metabolizable AA they contribute, and how they are utilized once they are absorbed. This review of literature will discuss these issues as they are related to methionine (Met), one of the first limiting AA in dairy cattle (Abe et al., 1997).

**Proteolysis in the Rumen**

The rate and extent of proteolysis in the rumen depends on a number of factors including solubility (type of protein), ruminal pH, dietary starch, and ruminal dilution rate. Soluble proteins are highly degradable because they are accessed more easily by microbial proteases (Bach et al., 2005). Protein chains containing disulfide bonds, Leu-containing peptides in the N-terminal group (Schwingel and Bates, 1996), and certain peptide bonds (Lys-Pro in comparison to Pro-Met dipeptides; Yang and Russel, 1992) are more resistant to degradation than other types of bonds. Proteolytic enzymes, which can either be cell-associated or extracellular, function best at a pH ranging from 5.5 to 7.0, and protein degradation is greatly reduced as the pH reaches the lower end of this range (Cardozo et al., 2000). Proteolytic bacteria are not as sensitive to pH shifts as cellulolytic bacteria. However, feed protein can often be encased by other nutrients, such as cellulose, which can interfere with protein digestion by rumen microbes (Assoumani et al., 1992). Therefore, maintaining a healthy population of cellulolytic bacteria can increase protein digestibility. In addition, ruminal dilution rate marginally affects overall protein degradation (Ørskov and McDonald, 1979).
Breakdown of soluble protein in the rumen has been attributed to a variety of microorganisms including bacteria (Wallace et al., 1997), protozoa (Forsberg et al., 1984; Lockwood et al., 1988), and anaerobic fungi (Wallace and Joblin, 1985). Rumen bacteria play the most important role, with the bacterial fraction exhibiting proteinase activity with 6 to 10 times greater specific activity than the protozoal fraction (Brock et al., 1982). A wide range of proteolytic microorganisms interact with each other to breakdown dietary protein to oligopeptides in the first step of protein breakdown in the rumen (Falconer and Wallace, 1998). Most ruminal bacteria possess protease activity except a few principally cellulolytic organisms (Wallace et al., 1997). Three bacterial species, S. bovis, R. amylophilus, and Prevotella spp. are known to be involved in the breakdown of oligopeptides to dipeptides (Wallace and McKain, 1991), of which the principal role of breakdown has been attributed to Prevotella spp. The breakdown of dipeptides and tripeptides to AAs by bacteria are largely attributed to the metalloenzyme containing species Megasphaera elsdenii, Prevotella spp., and Lachnospira multiparas (Wallace et al., 1996; Walker et al., 2005). In the last step of dietary protein breakdown, AAs are broken down into ammonia. The breakdown of AAs involves several species of bacteria and protozoa, and ruminal AA concentrations vary depending on the amount of rapidly degradable protein contained in the diet (Broderick and Wallace, 1988). Hyperammonia producing bacteria, although contributing to a small amount of the total bacterial population, significantly contribute to ammonia production. Proteinases intrinsic to plants contribute to proteolysis, the magnitude of which is small compared to that of bacteria (Zhu et al., 1999; Wallace et al., 2000). Protozoa also play a role in dipeptide
and AA breakdown and will be discussed later in more detail. Asao et al (1993) and Yanke et al (1993) found fungal cultures to contain a high metalloprotease activity, indicating their involvement in protein degradation, but their total contribution to proteolysis is minor compared to that of the entire microbial population.

Protozoa contribute to upwards of 40% (Russell and Rychlik, 2001) or 50% (Walker et al., 2005) of the microbial biomass (contribution of protozoa can vary greatly depending upon diet and can have a much smaller contribution; Firkins et al., 2007). Protozoa play a minor role in particulate degradation (Hino and Russell, 1987; Ushida et al., 1991) but, contrary to many bacteria, are capable of engulfing insoluble proteins (Walker et al., 2005). Protozoa predate rumen bacteria as their principal protein source and to a lesser extent utilize AA from RDP (Firkins et al., 2007). Predation of bacteria by protozoa recycles AA and peptides to the rumen (Hoover and Stokes, 1991), which helps support the growth of other rumen microbes (Bach et al., 2005). In vitro studies comparing defaunated and faunated media have shown that defaunation increases duodenal flow of dietary rumen undegraded dietary (RUP; smaller differences in RUP digestion can be found for diets with low quality forage) and bacterial protein (Jouany, 1996). The addition of a single species of protozoa (Isotricha ssp.) into defaunated rumens led to a decrease in NH$_3$ production, possibly through the incorporation of NH$_3$ into protein, the predation of hyperammonia-producing bacteria, or a combination of the two (Jouany, 1996).
Nitrogen Recycling

Ammonia is generally produced in the rumen faster than it can be reassembled into microbial protein, the excess of which is absorbed and transported to the liver (Morrison and Mackie, 1996). In a review of five studies, it was determined that 33% of hepatic urea is eliminated by the animal as urine and 67% is returned to the digestive tract (Lapierre and Lobley, 2001). Recycling of urea-N synthesized in the liver to the gut can provide a substantial contribution to available N, with the ability of increasing digestible N inflow from 50 to 60% in dairy cows (Lapierre and Lobley, 2001). The portion of blood urea N (BUN) that reaches the rumen can be converted to NH$_3$ and CO$_2$ by rumen microbial ureases of bacterial origin (Wallace et al., 1997). There is no urease activity associated with ciliate protozoa (Onodera et al., 1977) or fungi (Sakurada et al., 1994). Ammonia produced from the breakdown of dietary protein and from BUN recycled to the rumen can accumulate inside microbial cells (Russell and Strobel, 1987) where ruminal bacteria assimilate NH$_3$ into AA (Walker et al., 2005). The amount of microbial crude protein synthesized from NH$_3$-N varies and depends upon the NH$_3$-N: total N available to bacteria (Atasoglu et al., 1999).

AA Requirements of Microbes

Rumen microorganisms can synthesize AA from carbon skeletons and NH$_3$ and therefore do not technically have AA requirements (Kajikawa et al., 2002). Microbes can even synthesize the sulfur containing AAs cysteine and Met if sulfur is provided in the media (Van Soest, 1994). Sulfur requirements are expressed as a N to sulfur ratio (N:S).
Sulfur requirements of the host animal vary and are greater in fiber-producing animals (i.e., wool producers). Bacterial protein has an N:S ratio of 13:1. Although the AA pool in the rumen remains similar in cattle fed different diets (Ives et al., 2002), the pattern of AA synthesized by bacteria from carbon skeletons does vary (Bach et al., 2005). Validating that microbes have no AA requirements, Guliye et al. (2005) found that the efficiency of microbial protein synthesis (EMPS) does not decrease with any single AA deletion. Bacteria preferentially metabolize certain AA and preferentially incorporate others into microbial protein (Atasoglu et al., 2004).

Even though bacteria do not have AA requirements per se, they do respond to AA supplementation with improved efficiency of growth (Van Kessel and Russell, 1996; Cotta and Russell, 1982). AA supplementation also stimulates fibrolytic bacteria (Firkins et al., 2007). *Streptococcus bovis* grown in medium containing AA, waste less energy as heat, suggesting increased efficiency of energy and carbon use in the presence of AA (Russell and Strobel, 1993). Atasoglu et al. (2004) estimated that supplementation of a mixture of 20 AA increases growth rate by 46% and efficiency by 15%. To determine which AA would stimulate or inhibit bacterial growth, Kajikawa et al. (2002) sequentially added and then removed each of the 20 AA to cultures. In this study leucine, tyrosine, tryptophan, and glutamic acid were determined to be essential for improving bacterial growth whereas Met, phenylalanine, and valine were ‘sub-essential’. The addition of these seven ‘essential and sub-essential’ AA produced 21% and 25% of the total stimulatory effects found during the 20 AA mixture supplementation on growth rate and growth efficiency, respectively. Isoleucine, threonine, cysteine, phenylalanine, and
leucine supplementation at certain concentrations inhibited bacterial growth rate and efficiency by more than 10%. When researchers measured production of individual AA de novo by rumen microbes, with the exception of proline, the amount of Met derived from ammonia was lower than that of the other AA when peptides and amino acids were supplemented (Atasoglu et al., 1999). Other rumen microbes (*Prevotella ruminicola*, *Butyrivibrio fibrisolvens*, and *Selenomonas ruminantium*) are stimulated by cysteine (Cotta and Russell, 1982), a catabolite of Met.

**Microbial Protein Synthesis**

The majority of protein (50% to 80%) that reaches the small intestine of ruminants comes from rumen microbial protein (Storm and Ørskov, 1983). The AA composition of microbial protein closely resembles the AA composition of milk protein. Therefore, increasing the efficiency of microbial protein production should increase microbial protein supply to the cow with a balance of AA that can make milk production more efficient. Microbial protein synthesis (MPS; grams of crude protein or N synthesized) depends on a number of factors. Microbial growth in vitro has been shown to be dependent on the amount of energy available in the form of readily fermentable carbohydrates (Russell and Strobel, 1993). Supplementation of protein can increase microbial growth, but to a lesser extent. The amount of crude protein in the diet required to maximize MPS is not a fixed value and can vary depending on the fermentable energy content of the diet, the amount of dietary non-protein N, the extent of protein
degradation, the efficiency of rumen microbial growth, and the salivary N input into the
rumen (Satter et al., 1977).

Maximizing microbial growth and the amount of RDP that is captured by
microbes can improve the supply of AA to the small intestine and decrease N losses.
Asynchrony in energy and N availability to bacteria has traditionally been expected to
decrease the efficiency of MPS (EMPS; Stern and Hoover, 1979, where EMPS is defined
as the grams of microbial N per unit of rumen-available energy). If N content is too low,
fermentation becomes uncoupled to the production of ATP (Buttery, 1977), whereas if N
content is excessive, energy supply may become limiting. MPS can even occur when
urea is the sole N source, but EMPS can be limited by the absence of preformed AA.
Recycling of BUN to the rumen and the utilization of intracellular polysaccharide in
bacteria and protozoa can provide N and energy to microbes for MPS in between
feedings when dietary N is low (Firkins et al., 2007).

**Structure and Biological Function of Methionine**

Methionine is one of four sulfur containing AA, and with cysteine, is one of the
two sulfur containing AAs that are incorporated into proteins (Brosnan and Brosnan,
2006). The structures of the R-group in AAs contribute to the function of the AA and
how the AA will contribute to the function of the overall protein in which it resides. The
R-group of Met contains a methyl group covalently bonded to a sulfur atom. Met has
hydrophobic and electrophilic qualities which can be attributed to the terminal methyl
group and sulfur, respectively. (Brosnan et al., 2007). The characteristics of Met are
critical for its structure and metabolic function. Due to the hydrophobic nature of Met, more than two thirds of Met residues are buried inside the hydrophobic core of globular proteins (Brosnan and Brosnan, 2006). Met must bind to initiator tRNA in order for the preinitiation complex to form during the beginning of protein synthesis in eukaryotes (N-formylmethionine in bacteria) and it is believed that the hydrophobic nature of Met is essential for this phenomenon (Drabkin et al., 1998). S-Adenosylmethionine (SAM), a compound that is derived from Met, has a vast role in biochemical processes, a quality that is attributed to the strong electrophilic character of the carbons that are adjacent to the positively charged sulfur atom in both compounds (Fontecave, et al., 2004).

Methionine is an indispensible AA and it has many metabolic functions. Met is required for protein synthesis, is a precursor of cysteine, and is a methyl donor for several methylation reactions (Fukagawa, 2006; Levine et. al., 1996). In addition, homocysteine is a metabolic intermediate in Met metabolism. Although two thirds of Met residues are buried inside proteins, a third of Met residues in proteins are located on the protein surface. Met residues on protein surfaces are exposed to reactive oxygen species and can be oxidized into sulfoxides (Levine et. al., 1996). In some instances, Met residues on protein surfaces appear to become oxidized as a means to protect an enzyme’s catalytic site from reactive oxidative species. The oxidized Met residues are then converted back to Met via the enzyme methionine sulfoxide reductase (MSR). The impairment of MSR is associated with age related diseases (Moskovitz, 2005). In methionine’s most important biological role, Met is converted to SAM (also known as
AdoMet), a widely used enzyme substrate, second only to ATP in the number of biochemical processes for which it is involved (Cantoni, 1975).

**S-Adenosylmethionine**

Methylation processes are involved in a wide variety of cellular functions. The methylation of a protein’s amino- or carboxy-terminal end can cause changes in steric orientation, overall protein charge, and hydrophobicity; thus changing the overall function of the methylated protein (Chiang et al., 1996). SAM is the major biological methyl-donor in essential methylation reactions that occur in all living organisms (Cantoni, 1975; Chiang et al., 1996). Reactions catalyzed by methyltransferases, enzymes responsible for methylation reactions, include: the modification of macromolecules such as DNA and RNA; the inactivation of neurotransmitters such as epinephrine; and the methylation of hormones, signal-transduction systems, and phospholipids, as well as a number of important enzymes (Fontecave, et al., 2004; Loenen, 2006). Methyltransferases are also involved in the biosynthesis of small molecules such as phosphatidylcholine, creatine, and epinephrine; and the detoxification of xenobiotics (Brosnan et al., 2007). Heat shock proteins, which are responsible for maintaining the structural integrity of other cellular proteins, are methylated in response to environmental stress (Lanino et al., 1992). In addition, methylated DNA-binding proteins function as transcription factors and have the ability to regulate transcription (Prendergast and Ziff, 1991). The study of the human genome has indicated that class-1 SAM-dependent methyltransferases account for 0.6-1.6% of all genes (Katz et al., 2003). This
corresponds to 300 class-1 SAM methyltransferases (Brosnan et al., 2007). SAM is also a source of methylene groups, amino groups, ribosyl groups, and aminopropyl groups (Fontecave et al., 2004). SAM can also be decarboxylated to dSAM, which lacks a carboxyl group. Decarboxylated SAM can no longer function as a methyl donor, but instead donates aminopropyl groups for the synthesis of polyamines, such as spermine and spermidine, which are involved in cellular metabolism (Stipanuk, 2004). In humans, 85% of all methylation reactions and 48% of Met metabolism occurs in the liver (Finkelstein, 1990; Mato et al, 2002).

Methionine Metabolism

Methionine metabolism can be divided into three categories: transmethylation, remethylation, and transsulfuration. The transmethylation pathway regulates bodily Met levels and provides SAM for essential methylation reactions (Finkelstein, 2006). Transmethylation (Figure 1) involves the activation of Met to SAM by the constitutive enzymes known as methionine adenosyltransferases (MAT I in the liver and MAT II in other tissues). The methyl group in SAM is then transferred by various methyltransferases or a SAM-dependent, constitutive enzyme, glycine N-methyltransferase (GNMT), forming S-adenosylhomocysteine (SAH; Finkelstein, 2006). GNMT has a high $K_m$ (defined as the substrate concentration at $\frac{1}{2}$ of the maximum velocity of an enzyme) for SAM and is virtually insensitive to inhibition by SAH. When the diet contains excess Met, GNMT catalyzes the methylation of glycine by SAM to form sarcosine and SAH (Yeo and Wagner, 1994). Glycine can be regenerated from
sarcosine in mitochondria (Brosnan et al., 2007). Both GNMT and various methyltransferases can convert SAM to SAH, functioning to help maintain the proper SAM/SAH ratio (Heady and Kerr, 1973). The SAM/SAH ratio is thought to be important in methylation reactions (Cantoni et al., 1978). Transmethylation finishes with the reversible hydrolysis of SAH to adenosine and homocysteine by S-adenosylhomocysteine hyrdolase (SAHH; Brosnan et al., 2000; Finkelstein, 2006). The enzymes involved in the transmethylation reactions have several properties in which they share, including a low $K_m$ for sulfur-containing substrates, regulation through inhibition of Met synthesis with increasing dietary levels of Met concentration, and inhibition by SAM.

Remethylation (Figure 1) is driven by the biological need for methyl groups. Met can be regenerated by the activity of the enzyme methionine synthase (MS), which remethylates homocysteine back to Met, thus conserving Met’s carbon skeleton. The coenzyme 5-methyltetrahydrofolate, (5-CH$_3$-THF), which is synthesized by the enzyme methylenetetrahydrofolate reductase (MTHFR), is the biologically active form of folic acid, and acts as the methyl donor for MS catalyzed reactions (Brosnan et al., 2007). MTHFR generates new labile methyl groups from the one-carbon pool, a response that protects the system from incidences when dietary intake of methyl groups is low. Folic acid can also inhibit GNMT, an action that links the de novo synthesis of methyl groups from the one carbon folate pool to the availability of methyl groups in the diet (Wagner et al., 1985). In the liver and to a lesser extent the kidneys (kidney involvement is species dependent), homocysteine is remethylated to Met by the enzyme betaine-homocysteine methyltransferase (BHMT). BHMT uses betaine as a methyl donor. Betaine can be
found in the diet or can be generated during choline catabolism (Mudd et al., 2007). BHTM has a low affinity for homocysteine and is inhibited by Met. Hepatic BHTM activity increases under conditions of Met restriction or decreases when Met is in excess (Finkelstein, 2006). Methionine is a limiting amino acid in eukaryotes and must be consumed in the diet to meet biological demands. If the diet is somewhat limiting in Met, the need for Met can be lessened by its regeneration from homocysteine via the enzyme methionine synthase. The need for remethylation reactions is inversely related to the dietary intake levels of labile methyl groups (Mudd and Pool, 1975).

The transsulfuration pathway (Figure 1) is restricted to certain tissues, including the liver, pancreas, kidney, and intestine and allows for the catabolism of excess methionine and homocysteine and for the synthesis of cysteine and cysteine derivatives. The constitutive enzymes involved in the transsulfuration pathway include MAT (MAT III in the liver and MAT II in other tissues); SAHH, a SAM dependent methyltransferase (also involved in transmethylation); cystathionine β-synthase (CBS); and cystathionine γ-lyase (CGL, also known as cystathionase; Finkelstein, 2006). GNMT (also involved in transmethylation) is also utilized because it has a capacity for SAM. The first three steps in transsulfuration are similar to the steps involved in transmethylation. MAT converts Met into SAM, GNMT converts SAM to SAH, and SAHH converts SAH to homocysteine. CBS then catalyzes the condensation of homocysteine with serine to produce cystathionine in the first step that is truly unique to transsulfuration. Cystathionine can then be cleaved via the enzyme cystathionine gamma-lyase (cystathionase) to
Figure 1. Methionine Metabolism: Transmethylation, Remethylation, and Transsulfuration Pathways.

Abbreviations: MAT (Methionine adenosyltransferase), GNMT (Glycine N-methyltransferase), SAHH (S-Adenosylhomocysteine hydrolase), CBS (Cystathionine β-synthase), CGL (Cystathionine γ-lyase), MTHFR (Methylenetetrahydrofolate reductase), MS (Methionine synthase), BHMT (Betaine-homocysteine methyltransferase), THF (Tetrahydrofolate), DMG (Dimethylglycine).

Adapted from Brosnan et al., 2007
form cysteine, α-ketobutyrate, and ammonia (cysteine synthesis from Met is irreversible). Cysteine can be destined for glutathione synthesis and the formation of derivatives including taurine, and sulfate (Beatty and Reed, 1980). Because the formation of cysteine by CBS is an irreversible reaction, cysteine production is a Met sink. The transsulfuration pathway is highly active in the liver, kidneys, pancreas, and small intestine, with very limited activity in other tissues. With the exception of SAHH, the enzymes involved in transsulfuration differ from transmethylation enzymes because they have a high $K_m$ for substrates, hepatic enzyme content is upregulated with increases in dietary Met, and SAM acts as a positive effector. The high $K_m$ MAT III is found only in the liver and allows additional SAM to be synthesized when Met concentrations are high.

Methionine metabolism is dependent upon many enzymes and coenzymes. Therefore, for animals to properly metabolize Met, the diet must contain proper concentrations of the essential vitamins and minerals. More specifically, adequate intake of vitamins B2, B6, B9, and B12 are required to allow proper Met metabolism. 5-methyltetrahydrofolate, a derivative of folic acid (vitamin B9), is the source of methyl groups for MS. The prosthetic group in MS is derived from cobalamin (vitamin B12). 5-methyltetrahydrofolate synthesis is catalyzed by an enzyme that contains riboflavin (vitamin B2) as a cofactor. Two enzymes involved in the conversion of homocysteine to cysteine have pyridoxal phosphate (vitamin B6 derived) as their prosthetic groups (Brosnan et al., 2007). If a diet contains all necessary AA and is limiting in B-vitamins,
Met metabolism becomes strained. Because rumen microbes can synthesize B vitamins, cattle are usually able to meet B vitamin requirements when microbes wash out of the rumen and reach the small intestine (NRC, 2001).

**Methionine Metabolism in Microbes**

Most microorganisms can synthesize Met from homoserine and inorganic sulfur (Ravanel et al., 1998). Two pathways exist by which microorganisms can produce Met; the transsulfuration pathway, which uses cysteine as the sulfur source and goes through cystathionine as an intermediate, and the direct sulfydrylation pathway which bypasses cystathionine and uses inorganic sulfur as a direct sulfur source. Generally microorganisms utilize only one of these pathways to synthesize Met. For example, *Escherichia coli* (Sekowska et al., 2000) are known to utilize the transsulfuration pathway, and *Saccharomyces cerevisiae* (Thomas and Surdin-Kerjan. 1997) and *Corynebacterium glutamicum* (Kim et al., 2001) utilize the direct sulfydrylation pathway. However, some microorganisms have been found to utilize both pathways (*C. glutamicum*; Lee and Hwang. 2003), which combined with other Met regulatory mechanisms may be explained by the evolutionary history responsible for the divergence of these microorganisms.

Biosynthesis of Met in the transsulfuration pathway (Figure 2) begins when homoserine is acylated by homologous *metA* enzymes to either *O*-acetylhomoserine by homoserine acyltransferase, (*Bacillus subtilis*; Brush and Paulus. 1971) or to *O*-succinylhomoserine by homoserine succinyltransferase, (*E. coli*; Greene. 1994). Homocysteine is formed from *O*-acetylhomoserine or *O*-succinylhomoserine and cysteine in a two-step reaction.
catalyzed by cystathionine γ-synthase, *metB* and cystathionine β-lyase, *metC* in which cystathionine is an intermediate. In the direct sulfhydration pathway, cystathionine formation is bypassed and homocysteine is directly produced from *O*-acetylhomoserine by *O*-acetylhomoserine sulfydrylase, *metY*, using sulfide as a direct sulfur donor (Hwang et al., 2002). The last step in the production of Met utilizes methionine synthase, an enzyme that has been conserved among all organisms because without it the regeneration of the methyl group of SAM would not be possible (Ravanel et al., 1998). There are two types of methionine synthases that are capable of catalyzing the reaction that turns homocysteine into Met; the *metH* protein requires the coenzyme B<sub>12</sub> and is 100-fold faster than the reaction catalyzed by *metE*, a B<sub>12</sub>-independent enzyme (Greene. 1994). SAM can be synthesized from Met and ATP by *S*-adenosylmethionine synthase, *metK* (Rodionov et al., 2004).

Although protozoa in the rumen receive the majority of their AA from bacterial consumption, Met synthesis by protozoa has also been investigated. Or-Rashid et al (2001) studied the in vitro biosynthesis of Met from homocysteine, cystathionine, and homoserine plus cysteine by rumen bacteria, protozoa, and a mixture of both bacteria and protozoa. Disappearance of Met precursors occurred in all three media, indicating a synthesis of Met. Met production was highest in the bacteria medium, followed by mixed media and lastly the protozoa medium. The incorporation of Met into microbial cells followed the same pattern, with no significant incorporation found for protozoa cells, which liberated Met into the medium.
Figure 2. Methionine Synthesis by Bacteria: Direct Sulphydrylation and Transsulfuration Pathways.

Adapted from Hwang et al., 2002
Regulation of Methionine Metabolism

The enzymes involved in the transport and biosynthesis of Met are fairly conserved among bacteria; however, the regulation of Met metabolism varies among organisms (Sperandio et al., 2007). Gram-positive bacteria regulate Met metabolism using riboswitches and S-and T-box structures, whereas gram-negative bacteria utilize the transcriptional factors MetJ and MetR (Greene, 1996). A riboswitch is a genetic system in which an RNA structural transition in the 5’ region of the transcript (leader region) controls a downstream coding region in response to a regulatory signal (such as a metabolite) independent of an accessory protein or ribosome (Tucker and Breaker, 2005; Vitreschak et al., 2004). Riboswitches control gene expression through termination of transcription, inhibition of translation initiation, and regulation of RNA processing (Winkler and Breaker, 2005). High specificity to a particular regulator signal by riboswitches allows cells to modify gene expression in response to small changes in the environment.

Three riboswitch classes in bacteria are known to respond to concentrations of SAM; the S-box, $S_{MK}$-box, and SAM-II riboswitches (Tomšič et al., 2007). The S-box system controls a family of genes (most of which are directly involved in sulfur metabolism and the biosynthesis of cysteine, Met and SAM). This system is characterized by a highly conserved regulatory leader sequence (Grundy and Henkin, 1998) and is primarily found in Firmicutes (Grundy and Henkin, 2003; Rodionov et al., 2004; Tucker and Breaker, 2005). S-box gene expression is regulated by premature termination of gene transcription (Tomšič et al., 2007). Regulation of gene expression
through translation initiation occurs in a rare class of S-box elements in which SAM-dependent RNA structural rearrangement results in sequestration of the Shine-Dalgarno sequence (a sequence upstream of AUG in prokaryotes that aids recruitment of the ribosome to mRNA; Henkin and Grundy, 2006). The $S_{MK}$-box riboswitch system is found in the leader region of MetK (SAM synthetase) genes in lactic acid bacteria; translation initiation is regulated by SAM-dependent inhibition of ribosomal binding to mRNA (Fuchs et al., 2006; Fuchs et al., 2007). SAM-II riboswitches are also SAM-dependent; the mechanism of regulation is not fully understood (Corbino et al., 2005).

Grundy and Henkin (2003) discuss that approximately 60 transcription units from a variety of gram-positive bacteria are members of the S-box regulon. The leader sequence of these genes includes a transcription terminator, competing antiterminator, and the S-box, which functions as an anti-antiterminator (Nudler and Mirinov, 2004). Overexpression of SAM synthase \textit{in vivo}, shuts down Met synthesis in \textit{B. subtilis}, indicating that SAM is an effector molecule for Met biosynthesis (Yocum et al., 1996). When SAM binds to the S-box, a downstream hairpin forms and prematurely terminates transcription (Rodionov et al., 2004).

Other gram positive bacteria have a control system involving premature transcription T-boxes, which regulate expression of aminoacyl-tRNA synthetases and genes responsible for amino acid biosynthesis and transport (Grundy and Henkin, 2003). The T-box sequence has a ‘specifier codon’ that interacts with the anticodon of uncharged effector tRNA and binds to promote formation of an antiterminator structure (Rodionov et al., 2004). Rodionov et al. (2004) found 20 Met specific T-boxes in
Lactobacillales. T-box regulation in Bacillales and Clostridiales is used for methionyl tRNA synthetases (Vitreschak et al., 2008). In Bacillales, most amino acid aminoacyl tRNA synthetases exhibit T-box regulation, and Firmicutes use T-boxes to regulate branched-chain and aromatic aminoacyl tRNA synthetases (Vitreschak et al., 2008).

Gram-negative bacteria such as *E. coli* and *Salmonella enterica* regulate Met metabolism using the transcriptional regulators MetJ and MetR (Greene, 1996). The MetJ repressor in *E. coli* belongs to a class of DNA-binding proteins that interact with DNA bases via β-strand pairs (Somers and Phillips, 1992). MetJ is believed to bind seven operators (Old et al., 1991) located in the 5’ region of genes involved in Met biosynthesis, including the MetJ gene (Saint-Girons et al., 1984). The MetJ gene codes for a regulatory protein, which, when combined with a derivative of Met, represses the expression of the Met regulon. Met related genes in *E. coli* are negatively regulated by the MetJ repressor, with the metabolite SAM functioning as a corepressor (Saint-Girons et al., 1984). Some Met genes fall under the regulation of MetR, a LysR-type transcriptional regulator with homocysteine functioning as a coeffector (Mares et al., 1992).

**Branched Chain Amino Acids**

Branched chain amino acid transferase (BCAT), an enzyme involved in the methionine salvage pathway, has been conserved in many bacteria, and is known to transaminate KMB into Met, preferentially using branched chain amino acids (BCAA; isoleucine, leucine, and valine) as amino donors (variations of this enzyme exist in
bacteria; Sekowska et al., 2004). Yvon et al. (2007) found that BCAT transcription was repressed in the presence of free amino acids, especially isoleucine (isoleucine is also the regulator of the branched chain AA biosynthetic operon) in *Lactococcus lactis* subsp. *cremoris*. According to these researchers, Met also seemed to be involved in BCAT regulation, but the mechanism by which AA regulate BCAT remains unclear. More recently, Mäder et al. (2004) found that BCAT is regulated by the global transcriptional regulator CodY in *Bacillus subtilis*. More research needs to be conducted to further understand BCAT regulation in rumen bacteria.

Ruminal micro-organisms can use BCAA for protein synthesis or they can degrade BCAA to produce branched chain fatty acids (BCFA). Carbon skeletons of BCFA can be incorporated into the lipid and protein fractions of cells. The BCAAs valine, leucine, and isoleucine can be deaminated into the BCFA isobutyrate, isovalerate, and 2-methylbutyrate, which are important growth factors for several cellulolytic rumen bacteria (Allison and Peel, 1971). The BCFA isovalerate and isobutyrate are required by *Ruminococci flavefaciens*. Allison et al. (1962) concluded that *R. flavefaciens* require these BCFA because it possesses a limited ability to utilize exogenous BCAA or to synthesize the isopropyl group found in BCAA and in other cell wall components. Other rumen bacteria can use BCFA for the synthesis of long chain fatty acids.
Methionine Requirements of Dairy Cattle

Dairy cattle require significant dietary protein to meet the demands of maintenance, lactation, reproduction and growth. Lysine and Met are essential AA and have been identified as the first two limiting AA for growth (Abe et al., 1997), milk production (King et al., 1991; Schwab et al., 1992), and milk protein production (Schwab et al., 1976) in diets from which corn provides most of dietary RUP. Met and lysine are also the first limiting AA in cows fed legume forages, corn silage, corn grain, and soybean meal based diets in early and mid lactation (Schwab et al., 1976; Rulquin et al., 1993; NRC, 2001). Met is considered to be one of the most limiting AA in animals from which we demand significant levels of protein synthesis (Südekum et al., 2004), including wool-producing sheep and lactating dairy cows. Met is a limiting AA in diets with small amounts of corn, high forage diets, or when the majority of supplemental RUP is provided by soybean-derived products, animal-derived products, or a combination of both (Robert et al., 1994; Armentano et al., 1997; Rulquin and Delaby, 1997).

Amino acids that reach the hindgut of ruminants are supplied by sources including microbial protein, dietary feeds including AA that bypass microbial breakdown in the rumen, and endogenous secretions into the G.I. tract (Chalupa, 1975). It is estimated that Met must contribute 2.5% of total AA digested in the small intestine in order to meet requirements (Graulet et al., 2005). Met that is absorbed from the intestines and becomes available to cells for metabolism is known as metabolizable Met. Increasing the postruminal supply of limiting AA has been shown to increase wool production and N retention in sheep and cattle (Schelling et al., 1973), demonstrating an opportunity to
overcome AA deficiencies. Several strategies have been developed in an attempt to
influence the AA profile of duodenal digesta, including altering microbial protein
synthesis, feeding RUP and feeding RP-Met products that avoid microbial degradation in
the rumen (Bateman et al., 1999).

**Rumen Protected Methionine Supplements**

Rumen-protected products aim at providing limiting AA in a form that is resistant
to degradation by rumen microbes but is still available for absorption in the hindgut.
There are currently a number of commercial RP-Met products available on the market,
many of which utilize different technologies that allow them to resist microbial
breakdown in the rumen. Popular commercially available Met products include DL-Met,
Smartamine, 2-Hydroxy-4-(Methylthio)-Butanoic Acid (HMB), and the isopropyl ester of
HMB (HMBi). Of these products, Smartamine, HMB, and HMBi have some rumen
protected. (There are other commercial RP-Met products available but because they were
not highlighted in our research studies, they will not be discussed in this literature
review.) The extent of rumen protection of RP-Met products vary, but bacteria can
incorporate Met derived from these into microbial protein regardless of precursor (DL-
Met, HMB, or HMB esters such as HMBi; Patterson and Kung, 1988). The assorted RP-
Met products and the technologies they utilize result in various production responses,
including increases in milk production (Noftsger et al., 2005), milk protein percent, milk
protein yield (Rulquin et al., 2006; Noftsger et al., 2005), and lactose production
(Noftsger et al., 2005; St-Pierre and Sylvester, 2005). Milk yield and milk component
characteristics do not always change in response to increased dietary Met because other factors may be first limiting (Berthiaume et al., 2001), and stage of lactation can also influence detectible differences (NRC, 2001).

One type of rumen protected technology involves the coating of DL-Met with a polymer that is resistant to ruminal degradation. Smartamine (SMA) is a polymer-protected supplement containing DL-Met (50:50 enantiomeric mixture, minimum of 75% DL-Met; Adisseo, Inc., Antony France) that is lipid/pH-sensitive. The surface coating of stearic acid and poly (2-vinylpyridine-co-styrene), is designed to prevent Met degradation in the rumen but to be soluble in the acidic abomasum, allowing for the release of Met and absorption in the hindgut (Schwab and Ordway, 2003). Of all RP-Met products, SMA has the highest protection from microbial degradation (Rulquin et al., 2006) and appears to have the greatest efficacy as a source of metabolizable Met. In situ studies demonstrate a ruminal stability of SMA that averages 90% and intestinal disappearance estimates of 98% (Robert and Williams, 1997). The bioavailability of SMA in dairy cows has been estimated to be between 75 to 97% with digestibility tests (Robert and Williams, 1997) and 75% with blood tests (Rulquin and Kowalczyk, 2003).

Another method of rumen protection is the development of Met analogs in which a chemical blocking group is added to the α-amino group of Met or the acyl group is modified. The methionine hydroxyl analog supplement known as 2-hydroxy-4-(methylthio)-butanoic acid (HMB) is a popular Met analog in which the α-amino group is substituted with a hydroxyl group. HMB is more resistant to ruminal degradation than free Met and its production is less expensive than that of DL-Met protected supplements.
such as SMA (Wilson et al., 2008). Estimated bypass rates of HMB range from 99% (Jones et al., 1988) to 6% (Noftsger et al., 2005), and HMB supplementation has a moderate effect on plasma Met concentrations (Koenig et al., 1999; Koenig et al., 2002). The effect of HMB supplementation on production parameters in dairy cattle varies and may be due to factors previously mentioned. HMB supplementation has increased milk yield and milk fat percent (Lundquist et al., 1983; NRC, 2001) and improved performance of growing beef cattle (Venable et al., 2005). In other studies, HMB caused no change in protein yield and marginal increases in milk production (St-Pierre and Sylvester; 2005). Thus, contrary to SMA, HMB may not effectively replace metabolizable met for milk protein synthesis (Graulet et al., 2005).

Several esters of HMB have been developed in an attempt to increase its rumen protectiveness. Comparing microbial degradation of several HMB esters, the isopropyl ester of HMB (HMBi) was estimated to have 60% protection, but methyl, ethyl, butyl, and cyclohexyl esters were more readily hydrolyzed to HMB in the rumen (Robert et al., 2001). The ability of HMBi to ward off hydrolysis suggests that isopropyl esters present a steric hindrance of bacterial esterases. The bioavailability of HMBi is between 40 and 58%, based on blood kinetics (Robert et al., 2001; Robert et al., 2002) and milk true protein as indices (Schwab et al., 2001). Bioavailability determined by blood Met concentration was 53 and 74% for HMBi and SMA (Graulet et al., 2005). These measurements indicate bioavailability of HMBi to be 71% of that from SMA, whereas other research has indicated that HMB bioavailability is 50% that of SMA (Robert et al., 2001). Sylvester et al. (2003) saw an increase in milk, milk yield, and milk protein
production while reducing N excreted in manure following supplementation of HMBi compared to HMB.

**Comparing RP-Met Products: Metabolizable Methionine**

Accurately quantifying the amount of metabolizable Met supplied by Met supplements has obvious economic consequences in dairy production. Researchers have developed various methods to estimate or measure effects of Met supplementation on metabolizable Met supply. The AA supply that is available to fulfill requirements has been determined by estimating the AA that reaches and disappears from the duodenum using duodenal and ileal cannulas (Berthiaume et al., 2001). However, cannulation studies are expensive, result in high experimental error, and are subject to marker problems (Faichney, 2006), limiting the practicality and value of this method. Other studies investigating differences in metabolizable Met from supplements did so measuring changes in the concentration of Met in plasma (Blum et al., 1999; Südekum et al., 2004). However, Met concentrations in plasma only allow qualitative comparisons of metabolizable Met, and concentrations in plasma Met do not change (Casper and Schingoethe, 1988) when the supply of metabolizable Met changes. Furthermore, there are differences in the transport of Met and Met supplements in the brush border membrane in chick intestinal epithelium (Maenz and Engele-Schaan, 1996). If Met supplements are absorbed and metabolized at different rates in cows, they show at different times post feeding, thus making plasma Met concentrations difficult to compare accurately. The discrepancies in the findings of research studies are likely because
plasma concentrations of methionine may change very little when RP-Met is fed because the entry rate of Met into the plasma pool is minute compared to the total body flux and because protein synthesis does not increase with the entry rate of Met (Casper and Schingoethe, 1988). Metabolizable Met has also been compared by measuring production responses such as milk yield and milk protein from Met supplements (Rulquin et al., 2006). Milk yield and milk characteristics do not always change in response to increased dietary Met because other factors may be first limiting (Berthiaume et al., 2001) and other factors such as lactation stage of the cow can influence detectible differences (NRC, 2001).

**Uptake/Transport Mechanisms for Met and Met Analogs**

Transportation of AA via uptake and excretion are important to prokaryotic cells, especially for an AA as widely used as Met. The final accumulation of an AA via uptake is dependent upon several factors, including the activity of the uptake system, the hydrophobicity of the AA, the permeability of the bacterium’s plasma membrane, and the external AA concentration (Driessen et al., 1987). Significant diffusion through the plasma membrane may occur for some hydrophobic AA, including aromatic and branched chain AA, but diffusion of hydrophilic AA is not significant. Bacteria have two mechanisms for AA uptake, the major mechanism, binding protein-dependent systems (BPDS) and other secondary systems (Krämer, 1994). BPDS generally have higher affinity for their ligands than do secondary systems, but there is no major difference in kinetics between the systems. BPDS have been identified in gram-positive as well as
gram-negative bacteria. Despite the fact that most bacteria have a $\text{H}^+$-ATPase as a primary ion pump, $\text{NA}^+$-coupled transport of AA is common in bacteria. The use of Na+ as a coupling ion seems to be an adaptation to the rumen environment.

Genes controlling enzymes involved in Met biosynthesis in gram-negative bacteria are regulated by the $\text{MetJ}$ repressor (Sekowska et al., 2000), and there is evidence that Met transport is regulated by the same repressor. Bacterial uptake of Met in $E. \text{coli}$ through the high-affinity Met transport system is encoded by the $\text{MetD}$ locus (Greene, 1994), which along with its orthologs found in a variety of bacteria, constitute a new family of the ABC superfamily (Zhang et al., 2003; Hullo et al., 2004). This new family is designated as the Met uptake transporter (MUT) family. $\text{MetD}$ mutants are unable to transport $\text{D-Met}$ or to utilize it as a Met source (Kadner and Watson, 1974). In a study by Zhang et al (2003), it was confirmed that $\text{L-Met}$ effectively competes for $\text{D-Met}$ transport while competition in the other direction is poor.

Amino acid transport in gram-negative bacteria has been more extensively studied than in gram-positive bacteria (Hullo et al., 2004). $E. \text{coli}$ have two transport systems for L-Met: one with high affinity ($K_m = 0.1 \ \mu\text{M}$) and the other with lower affinity ($K_m = 40 \ \mu\text{M}$; Kadner, 1974) but only one transport system for D-Met (Kadner, 1977). Hegedüs et al. (1995) compared bacterial activity (measured by optical densities of the media) when Met (L-Met, DL-Met, and other Met analogs) was supplemented to bacteria grown in Met-free media. The Met-free media did not stimulate bacterial growth; the L-Met treatment demonstrated the most activity, but the DL-Met treatment was less active. Although there was no significant difference between the L- and DL-Met treatments,
these results may indicate that the dissimilar transport kinetics of the isomers may be responsible for the differences in activity.

With few exceptions (the peptidoglycan layer in the cell wall for example; Pollegioni et al., 2008; Lam et al., 2009), unlike L-Met, D-Met is not readily incorporated into microbial protein. For D-AAAs to be incorporated into proteins inside of the cell, they must first be converted to the corresponding L-AA. Catabolism of D-AAAs is similar to that of their L-AA counterparts, with the difference being in the stereospecificity of the enzymes involved. D-AAAs are oxidatively deaminated to an α-keto acid and ammonia by FAD-containing flavoenzymes known as D-AA oxidases (D-AAO; Pollegioni et al., 2008). FADH$_2$ is then reoxidized by oxygen, producing hydrogen peroxide to regenerate D-AAO. Both D- and L-AA oxidases are highly stereospecific and are virtually inactive toward the corresponding AA isomer (Khoronenkova and Tishkov., 2008). In the case of D-Met (Figure 3), the stereoisomer is converted to 2-keto-4-(methylthio) butanoic acid (KMB) by D-AAO, and KMB is transaminated into L-Met. Next to D-proline, D-Met is one of the best known substrates for D-AAO (Hamilton, 1985). In industrial production of enantiomerically pure chiral amines, AA oxidases are used to produce α-keto acid intermediates which are then transaminased to produce the desired AA enantiomer (Truppo et al., 2009).

Bacteria are capable of synthesizing D-AA for certain purposes, some of which (D-Ala and D-Glu) are important components of the peptidoglycan (PG) layer in the cell wall (Pollegioni et al., 2008). Recently Lam et al., (2009) found a racemase in the periplasm of *Vibrio cholerae* that produces D-Met and D-Leu. Addition of physiological
Figure 3. Methionine Bioconversion

Conversion of L-HMB, D-HMB, and D-Met to 2-keto-4-(methylthio) butanoic acid (KMB) and KMB into L-Met.
Adapted from Dibner. 2003
amounts of D-Met and D-Leu led to the reduction of the PG membrane in wild-type cells. Researchers concluded that D-AAs could down regulate PG synthesis in stationary phase cells and that D-AA production by *V. cholerae* can also down regulate PG synthesis of nearby cells, regardless of bacterial species, allowing cells to coordinate metabolism of the cell wall and cytoplasmic compartments with resource availability (Lam et al., 2009).

**Methionine and Methionine Analog Utilization in Eukaryotes**

In eukaryotes (specifically the chick) maximal rate of L-Met uptake across intestinal brush border vesicles is obtained under a Na\(^{+}\)-gradient, whereas DL-Met uptake occurs by energy- and Na\(^{+}\)-dependent mechanisms, energy-and Na\(^{+}\)-independent mechanisms, and by diffusion (Dibner et al., 1992). Each Met isomer has the ability to inhibit the uptake of the other isomer, but L-Met inhibition of D-Met uptake is more potent than the reverse (Brachet et al., 1987). D-Met is a noncompetitive inhibitor of L-Met uptake. Brachet and Puigserver (1989) discussed a maximal velocity for DL-Met uptake that is half of the maximal velocity of L-Met. Once absorbed, D-Met (from Smartamine or otherwise) is converted to L-Met, as discussed in the previous section. HMB transport is Na\(^{+}\)-dependent and distinct from the transport mechanisms of Met (Brachet and Puigserver, 1989), and HMB uptake is mediated by a nonstereospecific H\(^{+}\)-dependent system that functions to transport lactic acid and hydroxyl analogs of amino acids (Maenz et al., 1996).

Differences between the uptake of DL-Met and HMB have been investigated in several in vitro and in vivo studies, and HMB has uptake rates similar to or greater than
that of Met. The isotope dilution technique was used by Richards et al. (2005) to compare the uptake of HMB and Met in jejunum and ileum samples collected from birds using physiological concentrations of AA. Absorption of both metabolites was greater in the jejunum than the ileum, regardless of concentration or incubation time. At 20 mM, HMB uptake in both tissues was greater than that of DL-Met, regardless of incubation time. As AA concentration dropped, the differences in uptake of metabolites became less significant (p = 0.06 for 10 mM at 5 min), and no difference was found at concentrations of 0.1, 0.5, or 2.0 mM between metabolites.

D- and L- HMB are incorporated into protein in poultry liver as L-Met. Conversion of HMB occurs in two reactions (Figure 3). In the first step of HMB conversion, the enzyme that oxidizes the α-hydroxyl group of HMB to KMB varies depending upon whether the substrate is D-or L-HMB. L-HMB is the substrate of peroxisomal L-α-hydroxy acid oxidase (L-HAO). L-HAO is stereospecific and exhibits very little activity toward D-HMB (reactions containing 2.5 μmol D-HMB yielded 1.95 milliunits of activity compared to 12.68 milliunites toward L-HMB; Dibner and Knight, 1984). D-HMB is converted to 2-keto-4-(methylthio) butanoic acid by mitochondrial D-2-hydroxy acid dehydrogenase (D-HADH). In the second reaction, transamination of KMB yields L-HMB. Dibner and Knight (1984) detected significant quantities of D-HADH in organs, including the small and large intestine, liver, kidney, brain, and muscle tissues (collected from poultry), while major sites of D-HAO include the liver and the kidney. Dibner and Knight (1984) concluded that the kinetic mechanism behind the
similar rates of uptake between DL-HMB and DL-Met is the presence of two enzymes that simultaneously convert D- and L-HMB into L-Met.

In order to be utilized as an AA source, HMBi must be hydrolyzed to HMB and isopropanol, and HMB is subsequently converted to KMB and L-Met (as described above). Isopropanol can be absorbed by the digestive tract and is converted to acetone via the enzyme alcohol dehydrogenase in the liver (Bruss and Lopez, 2000). Met concentrations of blood serum significantly increase soon after rumen dosing of HMBi, which has led researchers to believe that HMBi may be transported across rumen epithelium. To study this hypothesis, Breves et al. (2010) incubated sections of harvested, intact rumen wall tissue with HMBi and measured HMBi and HMB concentration of serosal buffer. Based on these measurements, they concluded that only 10% of supplemented HMBi passes through the epithelium and enters the blood as HMB. This low epithelial transfer rate and the fact that no esterase activity has been documented in rumen epithelium suggests that absorption of HMBi through the rumen wall cannot solely explain the surge of HMB in blood serum after supplementation.
Introduction

Dairy cattle require significant dietary protein to meet the demands of maintenance, lactation, reproduction and growth. Lysine and methionine (Met) have been identified as the first two limiting amino acids (AA) for growth (Abe et al., 1997), milk production (King et al., 1991; Schwab et al., 1992) and milk protein production (Schwab et al., 1976) in diets where corn provides most of dietary rumen undegraded protein (RUP). Met is the first limiting AA for lactating cows (Robert et al., 1994; Armentano et al., 1997; Rulquin and Delaby, 1997) for diets with smaller amounts of corn, high forage diets, or when the majority of supplemental RUP was provided by soybean derived products, animal derived products, or a combination of both.

Methionine that is absorbed from the intestines and becomes available to cells for metabolism is known as metabolizable Met. Increasing the supply of metabolizable Met to the dairy cow usually requires the addition of expensive feed ingredients to the diet, such as fishmeal or rumen protected Met supplements (RP-Met). The assorted RP-Met products and the technologies they utilize, result in various production responses. If producers could accurately determine the effect of dietary Met supplements on
metabolizable Met supply, and the subsequent impact on cow performance, accurate economic comparisons between feedstuffs and supplements would be possible.

Recently, Weiss and St-Pierre (2009) developed a method to estimate the supply of metabolizable Met in the mammary Met pool based on changes in the concentration of Se in milk when Met sources are fed. In the 2009 study, dietary Selenomethionine (SeMet) was used to label the Met pool with SeMet and changes in the concentration of Se (a proxy of SeMet) in milk was used to determine the change in flux of methionine at the mammary gland in response to dietary changes of the AA (Weiss and St-Pierre, 2009). Feeding SeMet and supplemental Met for an extended period of time results in uniform labeling of SeMet in all pools of Met. If both Met and SeMet are fed at a constant rate for an extended period, the entry rate of Met into the Met pools is proportional to the entry rate of SeMet into the same pool. Also, the specific activity (SA) of SeMet in milk (concentration of SeMet/concentration of Met) is proportional to the SA of Met absorbed by the small intestine. The milk Se marker method does not require expensive cannulation surgeries, timely blood sampling, and is independent of lactation stage.

We hypothesized that we could use the Se-marker method to determine the relative bioavailability of five different methionine supplements. The supplements investigated were DL-Methionine, Smartamine (SMA), 2-hydroxy-4-(methylthio)-butanoic acid (HMB), the isopropyl ester of HMB (HMBi), and ERPM, an experimental rumen protected Met source consisting of DL-Met coated with phospholipids (Suriksh Impex, Adyar, Chennai, India).
Considerations

An important assumption to the selenomethionine (SeMet) marker method is that SeMet is transported and metabolized identically to Met. Changing the selenium source from selenite to SeMet, significantly changes the Se concentration of milk (Schrauzer, 2000). SeMet supplementation, however, has little to no effect on body weight gain, milk yield, or milk composition (fat and protein; Calamari et al., 2010). SeMet is absorbed in intestinal tissue through the AA transporter system predominantly by the Na\(^+\)-dependent neutral AA transporter (Wolffram et al., 1989; Schrauzer, 2000). Studies have confirmed the analogous transport of Met and SeMet (Wolffram et al., 1989; Vendeland et al., 1994; Leblondel et al., 2001) and demonstrated similar kinetics (Michaelis constant and maximal transport velocity) of Met and SeMet absorption (Wolffram et al., 1989; Nickel et al., 2009). Once SeMet is available in the body L-SeMet is readily incorporated into primary protein structures (Bakke et al., 2010), and can be released from proteins as needed for metabolic processes (Schrauzer, 2000).

Materials and Methods

Cow Selection and Management

Twenty Holstein cows were selected from the Waterman Dairy Facility at The Ohio State University and were moved to a tie stall barn. Cows were fed a total mixed ration (TMR) containing 0.3 mg of Se from Se-Met/kg of dietary DM (DM; Table 1). This base diet was fed as a control to all cows for a period of 10 days prior to treatment assignment. Only cows free of mastitis and Staphylococcus brevis infection history were
selected to participate. Cows were randomly assigned to stalls, and fresh water was supplied ad libitum. Cows were milked twice daily (0500 and 1700 h), and individual milk yield was recorded at each milking.

Experimental Design

The study was conducted as a replicated, truncated Latin square design (two blocks, each containing two, 14 d periods; Figure 4.). The study was conducted over a total of 58 d, extending from May 6 to July 4, 2009. All cows received the same basal diet, which differed only in the type of top-dress methionine supplement provided. Following the 10-d Se-yeast adjustment period, cows were randomly assigned to one of six methionine treatments (4 cows per treatment per block). The treatments included: (1) no supplementation (CON) (2) DL-methionine (MET), (3) HMB, (4) HMBi, (5) SMA, or (6) ERPM.

Feed Preparation and Analysis

TMR was prepared once daily using a TMR wagon and offered to all cows after each milking at approximately 0530 h and 1800 h (Table 1). The required amount of supplemented Met per treatment was calculated to be equi-molar, and each supplement was mixed with soyhulls to create 500 g/d topdressing to provide 18 g/d of MET equivalent. Immediately after each feeding, approximately 250 g of the assigned top-dress was poured onto the TMR and mixed by hand into the feed. Total mixed ration refusals were removed and weighed daily before feeding at approximately 1800 h. Individual feed intakes were calculated on a daily basis and were used to adjust the amount of feed offered per cow to maintain refusals of 6 to 10% during the first 10 d of
each period. Beginning on d 11 and going through the collection period (d 12 to 14 of each period), 95% of the average daily feed intake for the first 10 days was offered at each feeding to maximize supplement consumption.

Daily samples were taken of the TMR. Four TMR samples were randomly selected from each period and composited for analysis. They were dried in a forced-air oven for a minimum of 24 h at 55° C and ground to pass through a 2-mm screen of a Wiley mill (Arthur H. Thomas, Philadelphia, PA). Samples were analyzed for DM (105° C oven for a minimum of 8 hr), crude protein (Kjeldahl N; Foss 220 Kjeltec Auto Distillation), NDF, ADF (Ankom 200 Fiber Analyzer; Ankom Technology, Fairport NY), ash, and Se analysis (described below; Table 2: Chemical composition of TMR). TMR and top-dress samples were analyzed for Se content using a fluorometric method as described by Weiss and St-Pierre (2009).

**Milk Sampling**

Milk was sampled from a.m. and p.m. milking on days 12 to 14 of each period. Milk was collected into the dairy barn milking samplers and immediately transferred into acid washed bottles once milking was completed. All milk samples were refrigerated at the farm after milking and were transferred to the laboratory refrigerator and stored until analyses or shipment. Milk samples (p.m. and a.m.) were composited by day (50 g/d) and composites for all 3 sample days were added together to reach a final weight of 150g/period. Three 2.0 mL aliquots for each sample were placed into acid washed tubes and frozen at minus 10°C for future Se analysis.
Aliquots of milk sampled from each milking during the collection period were analyzed for fat, protein, lactose, solids, somatic cell count and milk urea nitrogen (MUN; DHI Cooperation, INC., Powell, OH). MUN was determined by a diacetyl monoxime assay on a Skalar SAN Plus segmented flow analyzer (Skalar, Inc., Norcross, GA). A portion of the 3-day milk composite was used to determine Kjeldahl N (same as above). At the time of Se analysis, the triplicate 2.0-mL tubes of milk were thawed and analyzed as described previously. Milk samples collected during the 10 d adjustment period prior to the start of the experiment were also analyzed for Se to validate the expected change in Se milk concentration following dietary inclusion of Se-yeast.

Calculations

A method was developed by Weiss and St-Pierre (2009) that measures the dilution of milk Se concentration (from Se-Met) to determine the change in the flux of methionine at the mammary gland when Met is supplemented in the diet. Entry rate of SeMet into the mammary gland is solely dependent upon the amount of SeMet consumed. However, in ruminants, the entry rate of Met into the mammary gland depends upon the amount of Met supplied by digestible RUP in addition to the extent of microbial protein synthesis and digestion. If the amount of Met consumption increases (such as for animals provided supplemental Met), then the flow of Met into the Met pools also increases, and the proportion of Met in the pool that is contributed from SeMet and the SA of SeMet, is diluted. We can quantify this dilution to determine the initial entry rate of Met for cows on treatment diets.
The entry rate (E) of Met can be calculated as follows (Shipley and Clark, 1972):

\[ E = \frac{r}{SA} \]  

[1]

where \( r \) = entry rate of SeMet (mg of SeMet/d) and \( SA \) = specific activity of a pool (mg of Se-met/g of Met).

Although the quantity of supplemented Met is known, we do not know the extent of microbial protein synthesis. Therefore, the actual entry rate of SeMet (\( r \)) when Se-yeast is fed is unknown. However, the method devised by Weiss and St-Pierre (2009) is designed to calculate the relative entry rate of Met following Met supplementation without requiring the actual entry rate of SeMet. In this method, cows must be fed at least 2 different diets with the same concentration of SeMet (\( r \) is the same for both treatments). For example, cows can be fed both a control and a treatment diet. The entry rates of Met when cows are fed control diets (\( E_C \)) or the treatment diet (\( E_T \)) are, respectively:

\[ E_C = \frac{r}{SA_C} \]  

[2]
\[ E_T = \frac{r}{SA_T} \]  

[3]

where \( r \) = entry rate of Se-Met and \( SA_C \) and \( SA_T = SA \) (Se-Met/Met) in milk from cows fed the control diet or treatment diet, respectively.

Equations [2] and [3] can be combined and rearranged to:

\[ E_T = E_C \times \left( \frac{SA_C}{SA_T} \right). \]  

[4]

The relative bioavailability of each treatment equals the proportional change in Met entry rate for each cow when on the control relative to the same cow on treatment. This equation can be used to determine the unknown entry rate of Met on the treatment diet.
from the entry rate of Met on the control diet. The entry rate of Met for animals on the control diet \( (E_C) \) was estimated from the NRC model (NRC, 2001). The entry rate for animals on the treatment diet can be estimated by multiplying \( E_C \) by the ratio of SA for control and treatment cows (Equation 4).

As in the previous study (Weiss and St-Pierre, 2009), milk Se concentration was used as a proxy for Se-met concentration and milk N was used as a proxy for milk Met concentration, which results in the final equations:

\[
E_T = E_C \times \left\{ \frac{Se_C}{Met_C} \right\} \div \left\{ \frac{Se_T}{Met_T} \right\} \quad [5a] \\
E_T = E_C \times \left\{ \frac{Se_C}{N_C} \right\} \div \left\{ \frac{Se_T}{N_T} \right\} \quad [5b]
\]

where \( E_C \) and \( E_T \) = entry rates of Met for cows fed control or treatment diets. Milk concentration of Se, Met, and N from cows fed control and treatment diets are designated as \( Se_C \) and \( Se_T \), \( Met_C \) and \( Met_T \), and \( N_C \) and \( N_T \), respectively.

**Statistical Analysis**

All data were analyzed using the Proc Mixed procedure of SAS (SAS Institute, 2004), using the following model:

\[
Y_{ijkl} = \mu + c_i + P_j + T_k + PT_{jk} + e_{ijk}
\]

where:

- \( Y_{ijkl} \) is the dependent, continuous variable,
- \( \mu \) is the overall population mean,
- \( c_i \) is the random effect of the ith cow (19 df) and \( \sim N(0, \sigma^2_c) \)
- \( P_j \) is the fixed effect of the jth period (3 df),
- \( e_{ijk} \) is the residual error term.

[43]
$T_k$ is the fixed effect of the $k$th treatment (5 df),

$PT_{jk}$ is the fixed effect of the $j$th Period × $k$th Treatment (15 df),

$e_{ijk}$ is the residual error, assumed independent and $\sim N(0, \sigma^2_e)$, (37 df).

The SA for each treatment $[(\text{Milk Se/ Milk N control}) / (\text{Milk Se/Milk N treatment})]$ was calculated so that each cow would serve as her own control. Calculating SA values in this manner reduces the number of treatments from 6 to 5 (no CON) and reduces the periods to 2 (4 weeks each). SA data were analyzed using the following model:

$$ Y_{ijkl} = \mu + c_i + P_j + T_k + e_{ijk} $$

where:

$Y_{ijkl}$ is the dependent, continuous variable,

$\mu$ is the overall population mean,

$c_i$ is the random effect of the $i$th cow (19 df) and $\sim N(0, \sigma^2_e)$

$P_j$ is the fixed effect of the $j$th period (1 df),

$T_k$ is the fixed effect of the $k$th treatment (4 df),

$e_{ijk}$ is the residual error, assumed independent and $\sim N(0, \sigma^2_e)$, (39 df).

**Results**

*Feed Analysis*

Total mixed ration samples were composited within each period and analyzed for DM, crude protein (determined using the Kjeldahl method), NDF, ADF, and ash, and SE using the method described above (Table 2). Based on analysis of the TMR, Se content
averaged 0.227 g/kg DM throughout the trial, despite an inclusion of 0.3 g/kg DM. The 0.073 g/kg DM difference between expected and observed Se content may be due to sampling, because total TMR samples were analyzed and not individual feed components.

*Milk Composition and Cow Performance Analysis*

The concentration of Se in milk increases soon after SeMet is supplemented and reaches a steady state within 7 d (Heard et al., 2007). Before the experiment started and 10 d after the diet was changed from sodium selenite to SeMet, milk samples were taken and analyzed to verify that milk Se concentration was in the expected range. The concentration of Se in milk 10 d after the Se source was changed to SeMet was 0.054 mg/kg, which is similar to values found during SeMet supplementation by Weiss and St-Pierre (2009). Milk Se concentration for SMA and HMBi were significantly lower than MET and CON (Table 3). SMA milk also tended to have a lower Se concentration than HMB (P = 0.09). Milk Se for ERPM tended to be lower than MET (P = 0.10) and CON (P = 0.11). No other significant differences were found among treatments for milk Se concentration. No significant differences were found for milk nitrogen among the treatments (Table 3).

HMBi treatment resulted in significantly lower DMI than MET, SMA, and CON and tended to be lower than HMB (P = 0.14) and ERPM (P = 0.12; Table 3). No other significant differences were found for DMI. Statistical analysis of milk yield only found trends for differences among treatments. HMBi tended to have greater milk yield than HMBi (P = 0.11), ERPM (P = 0.06), and CON (P = 0.14). MET also tended to have
greater milk yield than ERPM ($P = 0.09$). Percent milk fat for HMB was greater than MET and tended to be greater than SMA ($P = 0.11$) and CON ($P = 0.06$). HMBi also tended to have greater percent milk fat than CON ($P = 0.10$). Percent milk protein was greater for SMA than for MET ($P = 0.051$) and CON. No other significant differences were found among treatments for milk protein percent. The treatments did not significantly differ for SCC, percent milk lactose, percent milk solids, or MUN, which averaged 204 somatic cells per mL, 4.71%, 5.61%, and 12.7 mg/dL across treatments (data not shown).

*Calculated Entry Rate of Methionine*

The mean specific activity of milk for SMA and HMBi was lower than of both CON and MET, and HMB and ERPM tended to be lower than MET ($P = 0.13$ and 0.15 respectively; Table 3). The relative bioavailability (relative change in the flow of Met at the mammary gland) of MET, HMB, SMA, HMBi, and ERPM was 0.92, 1.03, 1.15, 1.14, and 1.10 respectively ($SEM = 0.06$; Figure 5). Relative bioavailability values have no units ($SA_{control}/SA_{treatment}$) and can also be expressed as a percent of the SA of CON.

When expressed as a percent of CON, the SA of MET is 92% of the SA of CON, and HMB, SMA, HMBi, and ERPM are 103%, 115%, 114%, and 110% of the SA of CON respectively. The relative bioavailability for SMA and HMBi were significantly greater than MET, whereas ERPM tended to be greater than MET ($p = 0.07$). No other significant differences were found for the relative bioavailability of treatments.

The entry rate of Met for CON was determined by entering the base diet and the mean DMI into the NRC nutritional model and getting an estimated value for daily
digestible Met flow. Based on the NRC model, the entry rate of digestible Met for CON cows was 45.8 g/d. The relative entry rate of Met for treatment cows is estimated using equation [4]. For example, if $E_C = 45.8$ g/d, $SA_C = 12.2$ µg/g and $SA_T$ is the SA for each treatment, in the case of MET:

$$E_{MET} = 45.8 \text{ g/d} \times \left( \frac{12.2 \text{ µg/g}}{12.8 \text{ µg/g}} \right),$$

$$E_{MET} = 45.8 \text{ g/d} \times (0.925),$$

$$E_{MET} = 42.4 \text{ g/d}$$

Using this method, the estimated entry rate of Met for all treatments is 42.4 g/d (MET), 47.0 g/d (HMB), 52.8 g/d (SMA), 48.7 g/d (HMBi), and 50.3 g/d (ERPM; Figure 6). Due to the decreased DMI for cows on the HMBi treatment, the basal entry rate of Met was adjusted to 42.6 g/d ($E_C$ is 42.6 g/d instead of 45.8 g/d when calculating $E_{HMBi}$).

**Discussion**

Overall, the flow of digestible Met was lower for the non-rumen protected product (MET) than for the rumen protected products, which was expected and indicates that these treatments provide some protection against degradation in the rumen. SMA resulted in the greatest flow of digestible Met to the duodenum (52.8 g/d), which shows that it has a higher bioavailability than all other treatments studied in this trial. Previous studies using different methods to determine the bioavailability of Met supplements consistently find higher bioavailability estimates for Smartamine when compared to dl-Met and the rumen protected supplements HMB and HMBi (Rulquin et al., 2006; Graulet et al., 2005; Robert et al., 2001). The relative entry rate of Met for the HMBi treatment
was expected to be more than the calculated 45.2 g/d and could have been higher if not for decreased DMI. Although Met flow had to be adjusted for the drop in DMI, HMBi still had greater Met flow than MET and the rumen protected products HMB and ERPM. Previous research has also found HMBi to be more bioavailable than HMB. Because ERPM is an experimental product and has not been intensively studied, it is not possible to compare the difference in the relative bioavailability of the Met supplements found in this study to the comparisons found using other measures of bioavailability. However, this experimental RP-Met product does show potential because it resulted in a slightly greater Met flow than HMB.

A number of researchers have compared milk yield and milk protein from cows fed different Met supplements. The ability to find differences in bioavailability based solely on these parameters can be challenging because other nutrients can be first limiting and the presence of differences can depend upon the lactation status of cows utilized in the study (NRC, 2001; Socha et al., 2008). Although we were able to find lower SA for SMA and HMB compared to MET and CON, no significant differences were found for milk yield, or milk N, and differences were only found for SMA when compared to MET and CON for percent milk protein. In other words, the Se-marker method is more sensitive than monitoring milk or milk protein yield. Furthermore, the Se-Marker method is relatively straight forward and does not depend on cannulation surgeries. Overall, compared to other methods, the Se-Marker method shows promise for comparing the supply of metabolizable Met provided by Met supplements.
Conclusions

The relative bioavailabilities of Smartamine, HMBi, and the experimental RP-Met product (ERPM) were all significantly higher than that of the non-rumen protected dl-Met treatment, which corresponds to increased flow of digestible Met to the cow. Digestible Met flow for the HMBi treatment had to be adjusted for a decrease in DMI. Overall, Smartamine resulted in the greatest flow of digestible Met to the duodenum (52.8 g/d), which shows that it has a higher bioavailability than other supplements. The experimental RP-Met product (ERPM) resulted in the next greatest digestible Met flow, with HMBi and HMB delivering a smaller amount of digestible Met. Digestible Met flow (compared by relative bioavailability data) for HMB, was not significantly different from MET. Future research utilizing more cows (per treatment) should be conducted utilizing the Se-Marker method to further compare the bioavailability of these products.
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<th>Ingredient</th>
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<tr>
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Table 1. Nutrient Composition of the TMR
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</tr>
<tr>
<td>P 1</td>
<td>Control  Control  Met 3  Met 3</td>
<td>P 1</td>
<td>Control  Control  Met 3  Met 3</td>
</tr>
<tr>
<td>P 2</td>
<td>Met 3   Met 3   Control  Control</td>
<td>P 2</td>
<td>Met 3   Met 3   Control  Control</td>
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<tr>
<td></td>
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<td></td>
<td>Cow 5   Cow 9   Cow 3   Cow 20</td>
</tr>
<tr>
<td>P 1</td>
<td>Control  Control  Met 4  Met 4</td>
<td>P 1</td>
<td>Control  Control  Met 4  Met 4</td>
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<tr>
<td>P 2</td>
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<td>P 2</td>
<td>Met 4   Met 4   Control  Control</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>Cow 6   Cow 10  Cow 4   Cow 15</td>
</tr>
<tr>
<td>P 1</td>
<td>Control  Control  Met 5  Met 5</td>
<td>P 1</td>
<td>Control  Control  Met 5  Met 5</td>
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<tr>
<td>P 2</td>
<td>Met 5   Met 5   Control  Control</td>
<td>P 2</td>
<td>Met 5   Met 5   Control  Control</td>
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Figure 4. Treatment Structure and Experimental Design
Table 2. Chemical Composition of the TMR
Chemical composition was determined experimentally.
<table>
<thead>
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<th></th>
<th>CON</th>
<th>MET</th>
<th>HMB</th>
<th>SMA</th>
<th>HMBi</th>
<th>ERPM</th>
<th>SEM</th>
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<tr>
<td>DMI (kg/d)</td>
<td>21.9^a</td>
<td>21.8^a</td>
<td>21.4^ab</td>
<td>22.2^a</td>
<td>20.4^b</td>
<td>21.4^ab</td>
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<td>Milk (kg/d)</td>
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<td>31.1</td>
<td>31.3</td>
<td>30.4</td>
<td>29.6</td>
<td>29.2</td>
<td>1.15</td>
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<td>Milk Se (mg/kg)</td>
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<td>0.062^a</td>
<td>0.058^ab</td>
<td>0.053^b</td>
<td>0.055^b</td>
<td>0.057^ab</td>
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<td>Milk N (g/kg)*</td>
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<td>4.81</td>
<td>4.98</td>
<td>4.90</td>
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<td>4.89</td>
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<td>Milk Se/Milk N (ug/g)</td>
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<td>12.8^a</td>
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<td>Milk Fat (%)</td>
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<tr>
<td>Milk Protein (%)</td>
<td>3.20^a</td>
<td>3.18^ab</td>
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</table>

Table 3. Effect of Methionine Treatments on DMI, Milk Production, and Milk Components

Superscripts indicate significance of $P < 0.05$.

* Milk N determined using the Kjeldahl method.
Figure 5. Effect of Treatment on Relative Bioavailability of Methionine

Bioavailability = \( \frac{SA_{\text{control}}}{SA_{\text{treatment}}} \) (within cow)

Superscripts indicate significance of \( P < 0.05 \).

Figure 6. Effect of Treatment on Digestible Methionine Flow to the Duodenum

Met flow is calculated by multiplying the relative bioavailability (Figure 5) by the Met flow contributed from the basal diet (CON; 45.8 g/d). HMBi Met flow was adjusted to account for lower DMI.
Chapter 3: Differential Incorporation of Methionine Supplied from D- and L-Methionine, 2-Hydroxy-4-(methylthio)-butanoic acid (HMB), and the Isopropyl Ester of HMB (HMBi) into Rumen Microbial Protein in Batch Culture

Introduction

Methionine is an important nutrient for both eukaryotic and prokaryotic growth. While it is important to understand how Met supplementation affects the cow as a whole, it is also important to understand how Met impacts microbial growth in the rumen. Similar to the cow, bacteria use methionine for incorporation into proteins. However, bacteria can also use methionine as a source of carbon and nitrogen. Amino acids and peptides stimulate rumen bacteria by mechanisms that are not fully understood (Walker et al., 2005). Therefore, the function of Met in the rumen goes beyond simple use as an AA, and Met supplementation could positively affect microbial growth and increase efficiency of microbial protein synthesis (EMPS).

The majority of protein (50% to 80%) that reaches the small intestine of ruminants comes from rumen microbial protein (MCP; Storm and Ørskov, 1983). The AA composition of MCP closely resembles the AA composition of milk. Therefore, increasing EMPS and subsequently increasing MCP flow to the duodenum could increase metabolizable protein supply to the cow with a balance of AA that can make milk
production more efficient. Supplementation with HMBi has been shown to increase milk production and milk protein (St-Pierre and Sylvester, 2005). These impacts on cow performance may be due to increased Met supply to the cow from increased EMPS.

Original Continuous Culture Study

In a study by Fowler et al, (2010), researchers dosed HMBi to dual flow continuous culture fermenter systems and determined the effect of HMBi supplementation on EMPS. Using a 4 × 4 Latin square design, researchers provided fermenters with the following treatments: no supplement, 0.11% HMBi, 0.097% DL-Met, or 0.055% HMBi + 0.048% DL-Met (as a percent of diet). Supplementation amounts were influenced by Noftsger et al., 2003 and were intended to supply an equi-molar amount of Met or Met precursors. Prior to the study they hypothesized that the delayed conversion of HMBi to L-Met would provide a more steady supply of Met to microbes for protein synthesis, which would consequently increase EMPS. Contrary to what was expected, results showed that HMBi supplementation had no effect on MCP synthesis. They also found no differences in ADF (acid detergent fiber) or true organic matter digestibility, although NDF (neutral detergent fiber; \( P = 0.04 \)) and hemicellulose digestibilities \( (P = 0.01) \) decreased linearly in response to HMBi replacing DL-Met supplementation. Concentration of NH\(_3\)-N was affected linearly \( (P = 0.07) \) and quadratically \( (P = 0.08) \) as HMBi decreased NH\(_3\)-N concentration compared to Met or HMBi + Met treatments. As HMBi replaced Met, the percentage of bacterial N derived from NH\(_3\)-N increased linearly \( (P = 0.02) \). Total VFA production, including branched chain AA decreased \( (P = 0.02) \) with increased substitution of HMBi for Met whereas
isovalerate ($P = 0.08$) and valerate ($P = 0.01$) production increased, despite the lack of change in the total digestibility of organic matter, suggesting a redirection of de novo AA synthesis when HMBi was dosed. In addition to these findings, $^{13}$C labeling kinetics demonstrated that HMBi supplementation increased the accumulation of free Met in the Met pool.

Although the conversion of DL-HMBi into L-Met should cause incorporation of NH$_3$ (HMBi does not contain N), while conversion of D-Met should cause no net change in NH$_3$, this alone probably does not account for the large difference seen in NH$_3$-N concentrations or bacterial N derived from NH$_3$-N, between the treatments. Increased synthesis of AA de novo by rumen microbes with HMBi supplementation may be necessary to counter that HMBi is a less available Met source compared to DL-Met.

**Considerations**

A number of theories may explain why HMBi supplementation led to the findings in the continuous culture study. We hypothesized that HMBi supplementation led to increased synthesis of AA de novo by microbes, accounting for the increase in free Met. The buildup of Met in the Met pool could also be explained by the differential metabolism of D vs L stereoisomers of metabolites (HMBi, HMB, and Met). Decreased utilization of Met derived by DL-HMBi compared to that of DL-Met may be because HMBi conversion into L-Met involves multiple steps. Another possible way to explain the findings: the isopropanol cleaved from HMBi during hydrolysis into HMB may reach a concentration that affects microbial cell wall fluidity and subsequently alters transport
kinetics of additional metabolites (HMBi, HMB, Met, etc) into microbial cells. A better comparison between HMBi, HMB, Met and Met derivatives and their effect on the incorporation of $^{13}$C from labeled methionine into microbial protein would provide a better understanding of the findings from our previous study and a better understanding of how these products are utilized in the rumen.

**Metabolism of Methionine, HMBi, and HMB Stereoisomers**

There are differences in the uptake of D- L-Met stereoisomers in prokaryotic organisms. It would not be unreasonable to assume that differences in transportation kinetics also exist between stereoisomers of DL-HMBi and DL-HMB produced from the hydrolysis of HMBi to HMB and isopropanol. In eukaryotes, D-HMB and L-HMB oxidation is catalyzed by mitochondrial D-HADH and peroxisomal L-HAO, respectively (research in chickens; Dibner and Knight, 1984). Lactic acid bacteria used to produce essential aromatic properties in cheese production have the ability to convert KMB to $\alpha$-keto-$\gamma$-methylthiohydroxybutyric acid (Hanniffy et al., 2009); a derivative of HMB, but less is known about the conversion of HMB to KMB in rumen bacteria. It is known that D-AA pools exist in a variety of bacteria and the pool size varies depending upon physiological and nutritional conditions (Bhattacharyya and Banerjee, 1974). Differences in the transportation kinetics of enzymes involved in the transport of DL-Met (and those involved in DL-HMBi transport) may lead to a higher concentration of D-Met in extracellular Met pools. The buildup of free Met found during the study (Fowler et al., 2010) may be due to a buildup of D-Met, especially if HMBi or HMB is preferentially converted to a D-isomer intermediate by rumen bacteria.
**Branched Chain Amino Acids**

Because 100% of DL-HMB (produced from HMBi) must be shuttled through a KMB intermediate, in contrast to 50% of DL-Met, more BCAA would be consumed by BCAT with HMBi supplementation when compared to that of Met (if equal molar amounts of supplement were used as Met). This could result in a disruption in the normal balance of one or more BCAA and could explain why isoleucine concentrations were lower during HMBi supplementation in the study by Fowler et al. (2010). The BCAA isoleucine is synthesized from threonine, both of which are produced from aspartate, which also is a precursor for lysine and methionine biosynthesis. Since this is a branched pathway, changing the concentration of one metabolite (isoleucine used by BCAT for the transamination of KMB, for example), could ultimately affect the concentration of other AAs in the pathway. However, the decreased BCAA concentrations could be somewhat abated since high SAM concentrations (synthesized from Met produced from the transamination of KMB) increase the activity of threonine synthase, the enzyme that produces threonine from homoserine (Azevedo et al., 2006).

**Isopropanol and Cell Membranes**

Alcohols and lipophylic agents interact with the lipid bilayer in cells (Sheetz and Singer, 1974) and have been shown to directly affect membrane fluidity (Grisham and Barnett. 1973; Hui and Barton, 1973). Changes in membrane fluidity can affect the activation energy of membrane-associated enzymes (McMurchie and Raison, 1978) which can in turn affect the transportation kinetics of metabolites (such as methionine) entering the cell. Increased cell membrane fluidity is also associated with leakage of
cellular contents and cell death in bacteria (Ezeji et al., 2010). Very small concentrations of methanol (1 to 2%) have been shown to detach bacteria from feed particles (Trabalza-Marinucci et al., 2006). Because HMBi is cleaved to HMB and isopropanol, it is possible that hydrolyzed isopropanol affected bacterial membranes in a similar manner.

**Continuous vs. Batch Culture Fermentation**

Although protozoa only contribute 11% of total crude protein flow to the small intestine (Shabi et al., 2000), they play an important role in protein and carbohydrate digestion (Bach et al., 2005; Williams and Withers, 1991). The continuous culture study (Fowler et al., 2010) had a substantially lower concentration of protozoa compared to the rumen. Because methionine supplementation affects protozoal growth rates and protozoa play an important role in nutrient metabolism, their absence in continuous culture studies could lead to inaccurate estimates of bacterial methionine utilization.

We hypothesized that D-Met, HMB, and HMBi supplementation would increase the synthesis of Met de novo and decrease incorporation of preformed Met into microbial N when compared to L-Met supplementation. The objective of this study was to determine the incorporation of Met from Met and Met analog supplements, into MCP by dosing $^{13}$C-Met and $^{15}$NH$_3$ to microbes in batch culture assigned to a randomized complete block experiment with 2 blocks.
Materials & Methods

Batch culture fermentation was used to determine the difference in the incorporation of Met into microbial protein when different Met and Met analog sources were supplemented to rumen microorganisms in a randomized complete block experiment. Treatments consisted of (1) an unsupplemented control (CON), (2) 0.097% L-Met, (3) 0.097% D-Met, (4) 0.125% HMBi, (5) 0.098% HMB, (6) 0.250% HMBi (2×HMBi), (7) 0.063% HMBi + 0.049% DL-Met (HMBi+MET), and (8) 0.098% HMB + 0.039% isopropanol (HMB+ISO). Unless otherwise specified, these treatments were provided in a presumed 50:50 mixture of D and L stereoisomers. CON, HMBi, and HMBi+MET treatments were designed to be similar to those in the original continuous culture study (Fowler et al., 2010). With the exception of CON, 2×HMBi, and HMBi+MET treatments, the inclusion rate for all treatments was designed to be equal molar and were included as a percent of the diet. Inclusion rates for HMBi+MET are ½ of the inclusion rates for the same substances in the other treatments except for the 2×HMBi inclusion rate, which was chosen assuming 50% escape of HMBi. In order to understand the results from the previous continuous culture study (containing defaunated inoculum) but to be applicable to rumen microbes in the cow (faunated), we dosed treatments with each faunated (F) and defaunated (D) inocula in an 8×2 factorial arrangement of treatments (Figure 7).

Faunated and D inocula were prepared from rumen fluid using continuous culture fermenters. Continuous cultures fermenters were strictly used to wash protozoa out of D inoculum and no dietary treatment was provided during continuous culture fermentation.
Faunated inoculum was also prepared with continuous culture fermenters so that differences between inocula other than the presence or absence of protozoa could be kept to a minimum. Faunated and D inocula were dosed to batch culture tubes containing feed, labels, and treatments. In order to determine Met incorporation over time, measurements were taken at four time points consisting of 0, 2, 8, and 24 hours (Figure 7). Cultures were fed a single dose of 0.50 g of a 50:50 ground alfalfa and concentrate mixture (Table 4). The entire experiment was replicated in a second block with fresh inocula.

**Inoculating Fermenters**

On the days of inoculation, 2.5 L of rumen fluid were collected from each of two Holstein cows (5 L total) and filtered through two layers of cheesecloth. The rumen fluid was transported to the laboratory in an insulated container at 39˚C. In the lab, the fluid was filtered through four layers of cheesecloth and mixed together to form a homogenous mixture. The fluid was added to dual flow continuous culture fermenters modeled after the system described by Hoover et al. 1976. The fermenters were fed 20 g of a 50:50 mixture of alfalfa and concentrate pellets immediately after inoculation and an additional 20 g of feed approximately 7 h later. At inoculation and for 1.5 days after inoculation, clarified rumen fluid (strained and autoclaved rumen fluid) was mixed in the buffer with a final concentration of 10% concentration of clarified rumen fluid, to provide growth factors that would help establish the microbial population in the fermenters. The dilution rate for both fermenters was maintained by continual infusion of buffer, modified from
Weller and Pilgrim (1974), set to dilute 7% of the total fermenter vessel volume per hour. The solids dilution rate was set to 5% per hour.

Fermenters settings were selected and maintained to create F and D inocula that would later be used in batch culture fermentation. In the first fermenter, the agitation paddles were set to 50 RPM, and the protozoa were retained using a compound filter that consisted of a 50 micron filter wrapped in layers of nylon cotton. In the second fermenter, the agitation paddles were set to 160 RPM, and the protozoa were effluxed using a wire mesh filter with a pore size of 300 microns (Karnati et al., 2009).

Fermenter Maintenance

Beginning the day after inoculation, fermenters were fed 40 g of feed (50:50 mixture of alfalfa and concentrate pellets; Figure 4) once daily at approximately 9:00 am each day. Fermenters were fed at approximately 7:30 am the day of batch culture inoculation. Dilution rates were checked at 9:00 am each day by individually weighing the fluid in the solid and liquid overflow collection vessels. The temperatures of the fermenters were checked throughout the day and were maintained at 39˚C. Buffer pH was maintained around 6.5 by bubbling CO₂ directly into the buffer tank and by constantly mixing the buffer solution. During the second replication, pH of the fermenters started to go above 7.0 prior to the daily feeding, therefore, CO₂ lines were bubbled directly into the fermenter liquid (beginning on day 3 of incubation). Liquid filtration lines (tubing) were frequently inspected to minimize and amend blockages. Filters in both fermenters were changed three times daily. The liquid removal pump was
temporarily shut off and the RPM in each sample was increased to 200 RPM once daily to homogenize the fermenter contents for sampling. Samples were examined under the microscope to monitor progress of defaunation. After defaunation was accomplished in the appropriate fermenter, cultures were incubated for an additional day prior to batch culture inoculation. Total incubation length was 8 days for the first replication and 7 days for the second.

Preliminary Studies: Inoculation Preparation

Two preliminary studies were conducted to determine the best preparation method for the inoculum. In the first preliminary study, batch culture fermentation was conducted for 8 and 24 h using inocula prepared with 2 or 8 layers of cheesecloth to determine the effectiveness of feed particle removal and NDF digestibility. After incubation, tubes were immediately submerged into an ice bath for 10 minutes to halt fermentation. Neutral detergent fiber (NDF; Ankom 200 Fiber Analyzer; Ankom Technology, Fairport NY) values were measured to determine which preparation method yielded the most fermentation (used to indicate microbial activity). It was determined that straining through 8 layers of cheesecloth did not significantly remove large feed particles, but instead decreased NDF digestibility. In the second study, inocula containing fermenter liquid (each faunated and defaunated) + no media solution (1:1, no dilution) and fermenter liquid + media solution (1:2, dilution) were compared by monitoring pH at 0, 2, 8, and 24 hours. The pH of the tubes decreased overtime and dropped below 5.6 for tubes prepared with inoculum lacking media solution, while the pH of batch culture liquid containing media solution (1:2, dilution) remained around 6.2.
The additional buffering capacity of media solution in a 1:2 dilution of fermenter liquid was necessary to maintain the pH of the batch culture liquid at a level that would prevent cell death. Decreased fermentation was found when comparing digestible NDF of batch culture samples prepared using the two inocula. However, a decrease in digestibility caused from diluting the microorganisms with added medium was a necessary sacrifice to increase buffering capacity of the inoculum.

*Batch Culture Inoculation*

A total of 0.5 grams of feed (0.25 g of each crushed alfalfa pellets and crushed concentrate pellets) were weighed into 198 batch culture tubes prior to the day of inoculation. The morning of the inoculation, treatment solutions were made by mixing the supplements with dH₂O to make homogenous stock solutions, which were dosed to the appropriate tubes. In addition to the treatments, the tubes were also dosed with 50 μg/tube of \(^{13}\text{C-}\text{Met}\) (0.10 molar % of Met) and 0.118 mg/tube of \(^{15}\text{NH}_4\text{SO}_4\). Fermenter vessels were sampled prior to inoculation to record the density of protozoa in each inoculum. These steps were repeated in the second replication (block).

Fermenter liquid from each vessel was poured with cold media solution in a 1:2 dilution (no additional cysteine was added to media solution) through two layers of cheesecloth into two separate vessels and warmed in a hot water bath to 39°C while bubbling with CO₂. While continually mixing by hand, 30 mL of the proper F or D inoculum was added to the assigned batch culture tubes. The tubes were immediately capped with one-way rubber stoppers and swirled. One mL of 6 N HCl was added to the 0 hour tubes, which were vortexed to bring the pH below 2 to stop fermentation. Tubes
were refrigerated until further processing. Other tubes were kept at 39°C and occasionally swirled to redistribute settled feed particles. At the end of each incubation time point, fermentation was halted as were the 0 hour samples.

**Measurements and Lab Analysis**

One of the replicate tubes from each treatment/time point/inocula was processed for $^{15}$NAN and total NAN, $^{15}$NH$_3$, NH$_3$, and TCA (trichloroacetic acid) soluble N. These samples were spun at 20,000 × g and the supernatant was removed. The pH of the precipitates were increased to > 9.0 using drops of NaOH, and precipitates were dried at 55°C for a minimum of 4 days. The dried precipitates were then weighed, and ground via mortar and pestle, and a 5 mg aliquot was sealed in an aluminum capsule. The supernatant from these tubes was filtered through Whatman # 1 filter paper and 0.1 mL of the supernatant was used for NH$_3$ determination (colorimetric method of Chaney and Marbach, 1962). A 20-mL aliquote was mixed with 5 mL of 50% TCA (10% final concentration of TCA) and used for $^{15}$N H$_3$ analysis (Hristov et al., 2001) and TCA soluble N (Griswold et al., 2003). Diffusion and precipitate samples were sent to Pennsylvania State University to be analyzed for $^{15}$NH$_3$ and $^{15}$NAN and total NAN analyses using gas mass spectrometry.

The other two replicate tubes from each treatment/time point/inocula were processed for bacterial Met, $^{13}$C enrichment of bacterial Met, and $^{13}$C enrichment of free Met (supernatant). These tubes were spun at 500 × g at -4°C to remove large feed particles and protozoa and again spun at 20,000 × g at -4°C to form a bacterial pellet.
Bacterial pellets were washed three times with 0.9% saline solution (saline was used in lieu of distilled H₂O to preserve the small bacterial pellet), and freeze dried. The supernatant was removed after 20,000 × g, and the freeze dried pellets were stored at -10°C and shipped on ice to the University of Maryland where gas chromatography - mass spectrometry was used to measure bacterial Met, 13C enrichment of bacterial Met, and 13C enrichment of free Met.

Samples were taken of the alfalfa and concentrate pellets that were used to feed the continuous culture fermenters during the protozoal washout period and to prepare the ground feeds provided during the batch culture fermentation. Samples of pelleted alfalfa and concentrate stocks were analyzed for NDF (using α-amylase), ADF (Ankom 200 Fiber Analyzer; Ankom Technology, Fairport NY), CP (Kjeldahl N; Foss, 220 Kjeltec Auto Distillation), and DM (105°C oven overnight). On the day of the inoculation, samples of fermenter vessel liquids were taken, fixed in formalin, and counted to document protozoal washout from the vessels.

**Statistical Analysis**

All data were analyzed as a randomized complete block with 2 × 8 factorial arrangement of treatments using the Proc Mixed procedure of SAS (SAS Institute, 2004), according to the following model:

\[ Y_{ijkl} = \mu + b_i + j + T_k + H_l + IT_{ik} + IH_{jl} + TH_{kl} + ITH_{ijkl} + e_{ijkl} \]

where:
$Y_{ijk}$ is the dependent, continuous variable,

$\mu$ is the overall population mean,

$b_i$ is the random effect of the $i$th block (1 df)

$i_j$ is the fixed effect of the $j$th inoculum (1 df),

$T_k$ is the fixed effect of the $k$th treatment (7 df),

$H_l$ is the fixed effect of the $l$th hour (3 df),

$IT_{jk}$ is the fixed effect of the $j$th inoculum $\times$ $k$th treatment (7 df),

$IH_{jl}$ is the fixed effect of the $j$th inoculum $\times$ $l$th hour (3 df),

$TH_{kl}$ is the fixed effect of the $k$th treatment $\times$ $l$th hour (21 df),

$ITH_{jkl}$ is the fixed effect of the $j$th inoculum $\times$ $k$th treatment $\times$ $l$th hour (21 df),

$e_{ijkl}$ is the residual error, assumed independent and $\sim N(0, \sigma^2_e)$, (63 df).

TCA soluble N, peptides, and NH$_3$ data were analyzed with a covariate so that the dependent variables could be adjusted for the presence of the substances in blank tubes. Significant interactions were determined with pairwise comparisons of least square means using Fisher’s Protected Least-Significant Difference.

**Results and Discussion**

**Feed Analysis**

The TMR was fed to cultures during both continuous culture and batch culture fermentation in a 50:50 mixture of alfalfa and concentrate. NDF, ADF, and CP were
determined experimentally, while RDP and RUP were calculated from a feed library (NRC, 2001; Table 5).

**Protozoal Counts**

Defaunation progress was monitored during continuous culture fermentation, prior to inoculation of batch culture tubes. Samples of inocula collected the morning of batch culture inoculation, contained an average of 23,740 protozoal cells per mL in F inocula, while D inocula contained an average of 2,670 protozoal cells per mL, indicating a reduction of total protozoa. Smaller protozoa such as *Entodinium caudatum*, which were the predominant protozoal species present in the defaunated inocula, are more difficult to remove from continuous culture fermenters and their removal may not have been guaranteed with an extended defaunation period.

**Soluble Nitrogen**

Total soluble N and NH₃ can be measured directly, but peptide concentration is determined by difference. These measurements are a net value from feed degradation, microbial incorporation and cell lysis (dependent and independent of protozoal predation). Ammonia concentrations allow crude prediction of the efficiency of conversion of dietary N into microbial N (Firkins et al., 2007). Adding labeled NH₃ allows better prediction of de novo AA production (transfer of $^{15}$NH₃ to microbial NAN) or direct uptake of AA (no transfer).
Mean total soluble N (Figure 8) and mean NH₃ concentration (Figure 9), averaged across treatments, varied by inocula at 2 h and 8 h (2 way interaction; \( P < 0.04 \)), but not at 0 h or 24 h. Mean \(^{15}\)N enrichment of NH₃-N, averaged across treatments and inocula, decreased over time (main effect; \( P < 0.001 \)) as microbes incorporated more \(^{15}\)NH₃ into microbial N than was returned to the supernatant. Further demonstrating incorporation of \(^{15}\)NH₃ into microbial N, the mean \(^{15}\)N enrichment of NAN, averaged across treatments and inocula, increased over time (main effect, \( P < 0.001 \); Figure 10).

Mean peptide concentration gradually declined during incubation (main effect of time, \( P < 0.001 \)), but concentrations at 8 h and 24 h varied by inocula and treatment (3 way interaction, \( P < 0.02 \); Table 6). However, the difference in peptide concentration within these time points were not consistent among inocula and inferences regarding treatment effect on peptide concentration are hard to make. Furthermore, peptide concentration alone is not an accurate indicator of EMPS. There were significant differences within treatments between F and D inocula, verifying that protozoa had an effect on protein degradation and N recycling at these times.

The decline of soluble N concentration indicates that microbes incorporated more soluble N into microbial N than was returned to the supernatant from proteolysis. An NH₃ concentration of at least 5 mg/dL is assumed to be required for optimal microbial protein synthesis but the optimum value varies (Firkins et al., 2007). Batch culture incubation is a closed system and does not have the added protection of BUN recycling; therefore, AA-N becomes increasingly important to ensure microbes have adequate dietary N for MPS. Based off of current knowledge, the NH₃ concentrations during this
experiment (values above) should have been adequate to support MPS. Defaunation usually decreases NH₃ concentrations, which should subsequently lead to an increase in EMPS and the efficiency in which microbes utilize BUN in vivo (Firkins et al., 2007). Decreased NH₃ concentrations in D cultures in this study can be an indicator of increased EMPS or increased use of NH₃ than preformed AA.

*Insoluble Nitrogen*

Non ammonia nitrogen (NAN) found in the precipitate consists of feed N and microbial N. Microbes break feed proteins into peptides, AA, and NH₃, thereby solubilizing feed N. Nitrogen that is added to the precipitate during incubation is therefore of microbial origin. Without quantifying the size of the bacterial pellet or the exact degradation rate of feed protein, it is not possible to definitively know the exact proportion of NAN in the precipitate that is of feed or microbial origin once fermentation has begun. Incorporation of NH₃ into NAN can occur quickly and enrichment of NAN (above background) may occur in 0-h tubes. On the contrary, the incorporation of feed N into microbial protein should be minimal at 0 h of incubation. Therefore, the amount of feed that contributes to NAN in the precipitate should be similar to what was initially provided in the tube prior to incubation. Dosing labeled NH₃ and Met into each tube allows the mathematical determination of the total amount of NAN that was derived from labeled NH₃ or labeled Met. If microbes only incorporated preformed AA into microbial protein, the presence of labeled N in the precipitate would be at background levels. An increased proportion of ^15^NAN to NAN in the precipitate relative to that of another tube.
(or treatment) indicates that microbes in the first tube relied more heavily on de novo AA synthesis rather than incorporating preformed AA into microbial protein.

Mean total NAN and mean total NAN derived from $^{15}$NH$_3$, averaged across treatments and inocula, differed by time (main effect; $P < 0.001$). The mean total NAN increased from 0 to 2 h, remained constant from 2 to and 8 h, and declined from 8 h to 24 h (Figure 11). Total NAN data reflects a net accumulation of NAN from an increase of microbial N relative to decreased feed N at 2 and 8 h. The interaction of inocula by time for mean total NAN derived from $^{15}$NH$_3$ tended to be significant ($P = 0.14$), and inocula means differed only at 2 h, with that of F inocula being 1.11 ($\pm 0.14$) mg/tube and D being 1.46 ($\pm 0.14$) mg/tube (data not shown), which may be due to an increase in bacterial N accumulation due to decreased protozoal predation at 2 h.

$^{13}$C Methionine

Preformed Met incorporated into microbial protein can come from $^{13}$C -Met or Met (treatments or feed). As with $^{15}$NH$_3$, $^{13}$C -Met can be used to monitor the incorporation of free Met from the supernatant into microbial protein. If microbes did not incorporate any labeled Met into microbial protein, $^{13}$C concentration of the bacterial pellet would remain at background levels (no enrichment). Therefore, $^{13}$C-Met present in the bacterial pellet above background levels is due to the direct incorporation of Met into microbial N. Met supplied from treatments or the breakdown of dietary N will dilute labeled Met in the pellet as it is incorporated into microbial protein. Enrichment is a function of total Met, which can be of dietary or microbial origin, so it is important to
measure either the amount of microbial N or the amount of free Met remaining in the supernatant, to calculate flux measurements.

Mass spectroscopy analysis of the supernatant was unable to detect free Met. Hydrolysis of methionine in HCl requires the addition of a stabilizer to prevent the degradation of methionine (Fountoulakis and Lahm, 1998). No stabilizer was added to tubes when 1 mL of 6N HCl was added to each tube to halt fermentation during processing. Therefore, we speculated that free Met degraded when samples were stored after acidification, while samples were processed. Without knowing $^{13}$C enrichment of free Met, the amount of total NAN (pellet) that came from $^{13}$C-Met cannot be determined. Without these data, comparisons of treatment effects on synthesis of Met de novo and incorporation of preformed Met into microbial N are difficult to make. Analysis of the bacterial pellet was successful. Because Met incorporation into microbial N should be minimal at 0 hours, and to conserve resources, 0 hour samples were not analyzed for $^{13}$C enrichment. No interactions were found for mean $^{13}$C enrichment of bacterial Met. whereas enrichment varied by time ($P < 0.001$; Figure 12) and treatment ($P < 0.001$; Figure 13). Mean $^{13}$C enrichment of bacterial Met tended to vary by inocula (main effect; $P = 0.15$), with enrichment of F tubes ($1.14 \pm 0.13$ APE) being slightly lower than D tubes ($1.19 \pm 0.13$ APE), indicating that protozoa may have an effect on the incorporation of preformed Met into microbial protein.

Enrichment of bacterial Met varies by treatments and may be due to differential incorporation of preformed Met in comparison to de novo synthesis of Met among treatments. If this assumption is correct, L-Met supplementation may result in decreased
13C enrichment of bacteria compared to other treatments and may be because L-Met is more readily incorporated into microbial N than the other Met and Met precursors supplemented in vitro. If differences in 13C enrichment are due to dilution of the label in microbial Met, these data may indicate that the treatments influenced incorporation of preformed Met into bacterial protein. Dilution of the label by L-Met in the L-MET and HMBi+MET treatment is consistent with data found in the original study (Fowler et al., 2010). If the differences in 13C enrichment were due to dilution of the label, we could also conclude that isopropanol had little effect on Met incorporation, because 13C enrichment of bacteria did not differ between HMB+ISO and the HMB treatments. Because the 13C enrichment of free Met could not be determined, comparisons of treatment effects on the synthesis of Met de novo versus the incorporation of preformed Met into microbial protein could not be made. Future efforts should be made to determine the total size of the bacterial pellet so that 13C enrichment data can be expressed as a percent of total bacteria.

**Conclusion**

Because microbes incorporated NH3 into NAN similarly across all treatments and 13C enrichment of free Met could not be determined, comparisons of treatment effect on the synthesis of Met de novo versus incorporation of preformed Met into microbial protein cannot be made. If treatment differences in 13C enrichment of bacterial Met are due to dilution of the label in the fluid precursor pool, it is possible that microbes were able to utilize L-MET more than they utilized D-MET, HMB or HMBi and that D-MET
HMB, and HMBi supplementation increased synthesis of Met de novo compared to L-Met. Isopropanol had little effect on the rate of Met (from HMB) incorporation into microbial N, since the bacterial $^{13}$C enrichment was similar between HMB+ISO and HMB treatments. Efforts should be made to determine the amount of microbial N present in the pellet so that the percent of $^{13}$C in bacterial protein can be determined and treatment comparisons can be made.
Following Arrows: Labeled Met and $^{15}\text{N}_2\text{H}_4\text{SO}_4$ were supplied to all tubes. All tubes were assigned to one of eight treatments: CON, L-MET, D-MET, HMB, HMBi, $2 \times$ HMBi, HMBi + MET, and HMBi + ISO. Tubes within each treatment were inoculated with either F (faunated) or D (defaunated) inocula. Within each treatment × inocula, individual tubes were incubated for 0, 2, 8, and 24 hours.

Figure 7: Batch Culture Treatment Structure

- Label Inclusion
  - $0.118 \text{ mg } (^{15}\text{N}_2\text{H}_4)\text{SO}_4$
  - $50 \mu\text{g } ^{13}\text{C}-\text{Methionine}$

Time series were taken for all 16 batches. The rest are omitted for clarity.
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<thead>
<tr>
<th>Ingredient</th>
<th>% of DM (total diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfalfa pellets</td>
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<tr>
<td>Pelleted concentrate</td>
<td>50.00</td>
</tr>
<tr>
<td>Corn grain, ground</td>
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<tr>
<td>Soybean hulls</td>
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<td>Distillers dry grains and solubles</td>
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<tr>
<td>Blood meal, ring-dried</td>
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<td>CaPO4</td>
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<td>Limestone</td>
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<tr>
<td>MgO</td>
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<tr>
<td>Trace mineralized Salt</td>
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<tr>
<td>Vit A Premix</td>
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<tr>
<td>Vit D Premix</td>
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<tr>
<td>Vit E Premix</td>
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Table 4. Ingredients of Culture Diets
Table 5. Nutrient Composition of Feed

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<tr>
<th>Nutrient</th>
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<td>CP$^1$</td>
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<td>RUP$^2$</td>
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$^1$ Measured experimentally

$^2$ Calculated from a feed library (NRC, 2001)
Figure 8. Inocula Effect on Total Soluble N Concentration
Mean total Soluble N (TCA) concentration (averaged across treatments) differed between inocula at 2 hours ($P < 0.04$) and 8 hours ($P < 0.003$), but did not differ at 0 or 24 hours.

Figure 9. Inocula Effect on NH$_3$ Concentration
Mean NH$_3$ concentration (averaged across treatments) differed between inocula at 2 hours ($P < 0.001$) and 8 hours ($P < 0.02$), but did not differ between inocula at 0 or 24 hours.
### Table 6. Methionine Treatment and Inocula Effect on Peptide Concentration

Treatment by inocula interaction was not significant at 0 or 2 h, but was significant at 8 and 24 h ($P < 0.03$). Superscripts indicate significance of $P < 0.05$. 

<table>
<thead>
<tr>
<th>Treatment</th>
<th>H</th>
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<th>L-MET</th>
<th>D-MET</th>
<th>HMBi</th>
<th>HMB</th>
<th>2×HMB</th>
<th>HMBi + MET</th>
<th>HMB + ISO</th>
<th>SEM</th>
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<tbody>
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Superscripts indicate significance of $P < 0.05$.
Figure 10. Effect of Time on $^{15}$N Enrichment of NH$_3$N and NAN

Mean $^{15}$N enrichment of NH$_3$ (averaged across treatments) decreased over time (main effect; $P < 0.001$), while mean $^{15}$N enrichment of NH$_3$ (averaged across treatments) increased from 0 to 8 h and was constant from 8 to 24 h (main effect; $P < 0.001$).

Figure 11. Effect of Time on Total NAN and NAN Derived From NH$_3$

Total NAN (averaged across treatments) increased from 0 to 8 h and decreased from 8 to 24 h (main effect; $P < 0.001$). NAN derived from NH$_3$ increased from 2 to 8 hours and was constant from 8 to 24 h (main effect; $P < 0.001$).
Figure 12. Effect of Time on $^{13}$C Enrichment of Bacterial Methionine

Mean $^{13}$C enrichment of bacterial methionine (averaged across treatments) changed over time (main effect; $P < 0.001$). Enrichment at 2 h = 1.18 ($\pm$ 0.13) APE, 8 h = 1.34 ($\pm$ 0.13) APE, 24 h = 0.97 ($\pm$ 0.13) APE.
Figure 13. Effect of Methionine Treatment on $^{13}$C Enrichment of Bacterial Methionine

Mean $^{13}$C enrichment of bacteria (averaged across inocula and time) differed by treatments (main effect; $P < 0.001$). Mean $^{13}$C enrichment of L-MET (0.74 ± 0.13 APE) was lower that of HMBi+MET (1.03 ± 0.13 APE), both of which were lower than all other treatments. Mean $^{13}$C enrichment of D-MET (1.19 ± 0.13 APE) tended to be lower than HMBi (1.29 ± 0.013 APE; $P = 0.16$) and 2×HMBi (1.30 ± 0.13 APE; $P < 0.12$).
References


