RUMINAL PROTECTION AND INTESTINAL AVAILABILITY OF RUMEN-PROTECTED METHIONINE AND LYSINE IN LACTATING DAIRY COWS

by

Sara Menchu

A thesis submitted in partial fulfillment
of requirements for the degree

of

MASTER OF SCIENCE

in

Animal, Dairy, and Veterinary Sciences

Approved:

_____ Jeffery O. Hall, D.V.M., Ph.D. Major Professor	_____ Jong-Su Eun, Ph.D. Committee Member
_____ Kerry A. Rood, M.S., D.V.M., M.P.H. Committee Member	_____ Richard S. Inouye, Ph.D. Vice Provost for Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

2019

ABSTRACT

Ruminal Protection and Intestinal Availability of Rumen-Protected Methionine and Lysine in Lactating Dairy Cows

by

Sara S. Menchu, Master of Science

Utah State University, 2019

Major Professor: Dr. Jeffery O. Hall
Department: Animal, Dairy, and Veterinary Sciences

Rumen protected Methionine (**MET**) and Lysine (**LYS**) are critical for milk protein synthesis in dairy cows. N-acetyl-L-methionine (**NALM**) is a **MET** derivative that consists of L-Met protected with an acetyl group that is attached to the α-amino group. N-acetyl-L-lysine (**NALL**) is a **LYS** derivative that is similarly protected. The objectives of these studies were to quantify the gastrointestinal availability of **NALM** and **NALL**. Three experiments were run as 3 × 3 Latin square using 3 second lactation Holstein cows that have been fitted with cannulas in the rumen and duodenum. The cows were fed diets containing the supplements for two weeks prior to each experiment so that the rumen microbes had time to adjust to the supplement. Each period consisted of 10 d of adaptation followed by 2 d of sampling. A dose of 0, 30, or 60 g of **NALM** was placed under the rumen mat at the time of feeding every day during experiment 1. The cows

were similarly supplied with 0, 60, or 120 g of **ƐNALL** during experiment 2. The cows were supplemented with 0 g, 120 g of **ƐNALL**, or 120 g of **diNALL** during experiment 3. On day one of sampling, a liquid marker (Co-EDTA) was also administered at the time of the protected **AA** administration. Blood, ruminal, and duodenal samples were taken at hours 0, 1, 3, 6, 9, 12, and 24 post-feeding. There were no differences for milk production, milk protein, milk fat, or **DMI** for **NALM** or either **NALL**. There were no differences for ruminal escape (69.1% and 46.2% respectively) and duodenal appearance (2.16% and 3.40% respectively). The ruminal escape of **ƐNALL** was not different between the 120 g dose (32.7%) and the 60 g dose (27.2%). Duodenal appearance was higher ($P < 0.01$) for the 60 g dose (2.86%) than for the 120 g dose (1.19%) of **ƐNALL**. Acetate, propionate, butyrate, and valerate were higher ($P < 0.01$) for the supplemented cows during experiment 1 with **NALL**. There were no differences between **ƐNALL** and **diNALL** for rumen escape, duodenal appearance, **VFA** production, or blood **LYS AUC**. Results of the experiment verify significant protection of the N-acetyl **MET** and **LYS** from rumen degradation.

(77 pages)

PUBLIC ABSTRACT

Ruminal Protection and Intestinal Availability of Rumen-Protected Methionine and Lysine in Lactating Dairy Cows

Sara S. Menchu, Master of Science

Rumen protected Methionine (**MET**) and Lysine (**LYS**) are critical for milk protein synthesis in dairy cows. N-acetyl-L-methionine (**NALM**) is a **MET** derivative that consists of L-Met protected with an acetyl group that is attached to the α-amino group. N-acetyl-L-lysine (**NALL**) is a **LYS** derivative that is similarly protected. The objectives of these studies were to quantify the gastrointestinal availability of **NALM** and **NALL**. Three experiments were run as 3 × 3 Latin square using 3 second lactation Holstein cows that have been fitted with cannulas in the rumen and duodenum. The cows were fed diets containing the supplements for two weeks prior to each experiment so that the rumen microbes had time to adjust to the supplement. Each period consisted of 10 d of adaptation followed by 2 d of sampling. A dose of 0, 30, or 60 g of **NALM** was placed under the rumen mat at the time of feeding every day during experiment 1. The cows were similarly supplied with 0, 60, or 120 g of **ƐNALL** during experiment 2. The cows were supplemented with 0 g, 120 g of **ƐNALL**, or 120 g of **diNALL** during experiment 3. On day one of sampling, a liquid marker (Co-EDTA) was also administered at the time of the protected **AA** administration. Blood, ruminal, and duodenal samples were taken at hours 0, 1, 3, 6, 9, 12, and 24 post-feeding. There were no differences for milk

production, milk protein, milk fat, or **DMI** for **NALM** or either **NALL**. There were no differences for ruminal escape (69.1% and 46.2% respectively) and duodenal appearance (2.16% and 3.40% respectively). The ruminal escape of **ƐNALL** was not different between the 120 g dose (32.7%) and the 60 g dose (27.2%). Duodenal appearance was higher ($P < 0.01$) for the 60 g dose (2.86%) than for the 120 g dose (1.19%) of **ƐNALL**. Acetate, propionate, butyrate, and valerate were higher ($P < 0.01$) for the supplemented cows during experiment 1 with **NALL**. There were no differences between **ƐNALL** and **diNALL** for rumen escape, duodenal appearance, **VFA** production, or blood **LYS AUC**. Results of the experiment verify significant protection of the N-acetyl **MET** and **LYS** from rumen degradation.

ACKNOWLEDGMENTS

I am incredibly grateful to be here today. I know that there is no way that I could have reached this point without the help of many people. First of all, I would like to thank the many workers in the USU nutrition lab. The many hours that you spent helping me feed cows and take samples is greatly appreciated. I am especially thankful for the help of Dr. Yang, Cody, and Anisa. The time you spent on the farm and in the lab was invaluable to the completion of my project. I would also like to thank the many workers of the Caine Dairy Research Center. The time that all of you spent taking milk samples was essential to the success of my project. I would also like to thank Dr. Rusty Stott. I could not have been successful if it had not been for your time and energy. Thank you for helping to monitor the health of the research cows.

I would like to thank the members of my committee for taking the time necessary to work with me. Dr. Rood, I appreciate that you were willing to support me in my goal to get a master's degree. Thank you for coming to meetings and beings willing to revise my writings. I am grateful to Dr. Eun for helping me get where I am today. It was in your class as an undergraduate that my interest in nutrition was first piqued. I am glad that you had enough faith in me to take me on as a graduate student. I am grateful for the opportunities that I had to work on your research projects as an undergraduate student. Thank you for guiding me as I began my journey in this program.

I would also like to thank Dr. Hall. I know that you do not have much time to spare. I appreciate that you spent so much of it on me. I am grateful that you were willing to answer all of my questions and review all of my papers. I also appreciate the time that

you spent teaching the class on vitamins and minerals. That class helped me to learn a lot that I had not previously known. I also am grateful for how much you have helped me increase my desire to understand new concepts in greater depth. I am amazed at how well you balance knowledge with your ability to teach at the level of your students. Thank you for making time to accept me as your student. I would not have been able to complete my degree without your support.

Finally, I would be remiss if I did not thank my family. Dad, since I was a little girl, you have instilled in me a desire to learn. Thank you for supporting me in my college career. Mom, you have always been my rock. With your help and encouragement, I had the courage to pursue my degree. I cannot thank you enough for the times you have helped me study throughout my years here at Utah State University. Of course, I never would have made it this far without the love and support of my husband, Kevin. Thank you for understanding that my work and my school required so much time. Thank you for always being there when I hit roadblocks. Your encouragement means the world to me.

Sara Menchu

CONTENTS

Page

COPYRIGHT ii

ABSTRACT iii

PUBLIC ABSTRACT v

ACKNOWLEDGMENTS vii

LIST OF TABLES xi

LIST OF ABBREVIATIONS xiii

CHAPTER

I. INTRODUCTION 1

II. REVIEW OF LITERATURE 3

Protein… 3
Crude Protein… 5
Microbial Protein Synthesis 6
Synchronization… 6
Fate of Protein… 7

Liver 7
Mammary Gland 7

Nitrogen Recycling 8
Amino Acids 8
Methionine 11

Coating Technology 11
D,L Isomers 13
HMB 13
HMBi 14
Smartamine 15
NALM 16
Liver Metabolism 16
Utilization 17

DMI 18
Milk 18
Immunity 19

Lysine 19

Coating 20
Forms and Availability 21
Lactation 22
DMI 22

Cobalt as a Marker 23
Conclusion… 23

III. METHIONINE 25

Introduction… 27
Materials and Methods 29

Animals and Diets 29
Sampling 30
Chemical Analysis 32
Calculations and Statistical Analysis 33

Results and Discussion 34

IV. LYSINE 38

Introduction… 40
Materials and Methods 42

Animals and Diets 42
Sampling 43
Chemical Analysis 44
Calculations and Statistical Analysis… 46

Results and Discussion 48

V. CONCLUSION 56

REFERENCES 58

LIST OF TABLES

Table Page

Methionine

1 Ingredients and chemical composition of the experimental diets with varying does of N-acetyl-L-methionine and metasmart supplemented to early lactation Holstein dairy cows.....30

2 Dry matter intake, body weight, and milk parameters of second lactation dairy cows fed rumen protected methionine.....34

3 Ruminal pH, ammonia-N, volatile fatty acid profiles, and blood methionine concentration of second lactation dairy cows fed a diet with rumen-protected methionine.....35

4 Ruminal escape and duodenal appearance **NALM** in second lactation dairy cows fed a diet with rumen-protected methionine.....36

Lysine

1 Ingredients and chemical composition of the experimental diets with varying does of N-acetyl-L-lysine supplemented to early lactation Holstein dairy cows. 43

2 Dry matter intake, body weight, and milk parameters of second lactation dairy cows fed rumen-protected lysine.....48

3 Ruminal pH, ammonia-N, volatile fatty acid profiles, and blood lysine in second lactation dairy cows fed a diet with rumen-protected lysine.....50

4 Ruminal escape and duodenal appearance **NALL** in second lactation dairy cows fed a diet with rumen-protected lysine.....51

5 Dry matter intake, body weith, and milk parameters of second lactation dairy cows fed rumen-protected lysine.....52

6 Ruminal pH, ammonia-N, volatile fatty acid profiles, and blood lysine in second lactation dairy cows fed a diet with rumen-protected lysine 53

7 Ruminal escape and duodenal appearance **NALL** in second lactation dairy cows fed a diet with rumen-protected lysine.....54

LIST OF ABBREVIATIONS

AA = amino acid

ADF = acid detergent fiber

AUC = area under the curve

BCS = body condition score

BHBA = beta-hydroxybutyrate

BUN = blood urea nitrogen

BW = body weight

CP = crude protein

DNA = deoxyribonucleic acid

DIM = days in milk

diNALL = Nα,Ɛ-acetyl-L-Lysine

DM = dry matter

DMI = dry matter intake

EAA = essential amino acid

ECM = energy corrected milk

ƐNALL = ƐN-acetyl-L-Lysine

HMB = DL-2-hydroxy-4-(Methylthio)-butanoic acid

HMBi = isopropyl ester of 2-hydroxy-4-(Methylthio)-butanoic acid

LCFA = long chain fatty acid

LYS = Lysine

MET = Methionine

MPS = microbial protein synthesis

MUN = milk urea nitrogen

N = nitrogen

NALL = N-acetyl-L-Lysine

NALM = N-acetyl-L-Methionine

NDF = neutral detergent fiber

NH_3-N = ammonia nitrogen

NRC = national research council

RNA = ribonucleic acid

RDP = ruminally degradable protein

RPAA = rumen protected amino acid

RUP = ruminally undegradable protein

TMR = total mixed rations

VFA = volatile fatty acids

VLDL = very low-density lipoprotein

CHAPTER 1

INTRODUCTION

Protein Metabolism is a complicated process in ruminants. Rumen microbes degrade much of the protein that is provided in the diet (Giallongo et al., 2016). This means that some protein is not available for systemic use by the animal. Dairy cows require protein to build enzymes and build tissue. Protein is also necessary for synthesis of milk proteins. Inadequate dietary protein makes it impossible for a cow to sustain high milk production (Lee et al., 2015).

Proteins are built using different amino acids (**AA**). There are **AA** that cannot be adequately synthesized by the animal and must therefore be supplied in the diet. These are referred to as essential **AA** (**EAA**) (Schwab et al., 2010). The **EAA** that are not typically supplied in sufficient amounts in the diet are said to be limiting. The two most limiting **AA** in a typical dairy diet are Lysine (**LYS**) and Methionine (**MET**) (Weiss et al., 2002). Simply supplementing diets with free **MET** and **LYS** does not suffice due to the extensive degradation and use of these **AA** by rumen microbes (Chalupa et al., 1976. Onodera et al., 1993).

Many methods have evolved over time to protected **MET** and **LYS** from rumen degradation. Some minerals were bound with **AA** to provide protection. This proved to be ineffective and costly (Graulet et al., 2005). Lipid coatings were fairly effective at ruminally protecting **MET** and **LYS**, but the amount of **AA** that was released in the small intestine was minimal at best (Schwab and Ordway, 2003; Sakkers et al., 2013).

Protecting **LYS** has proven more difficult than for **MET**. Methods are frequently less effective for **LYS** (Rossi et al., 2003). For this reason, **LYS** is still most commonly protected with lipids. Less information is available for **LYS** than for **MET** on the effectiveness of different protection methods (Evans et al., 2015).

When studied by Rossi et al., (2003), ethyl-cellulose coating also released lower amounts of **MET** into the small intestine than had been expected. Various **MET** analogs and precursors have been used as well, but often have low bioavailability (Koenig et al., 1999; Graulet et al., 2005). The protection method that appears to provide the most bioavailability so far appears to be the pH sensitive polymer (Schwab et al., 2010).

N-acetyl-L-Methionine (**NALM**) and N-acetyl-L-Lysine (**NALL**) are both derivatives that are protected in the rumen by attaching an acetyl group to the α-amino group of the **AA**. The acetyl group protects the **MET** or **LYS** from microbial degradation by blocking aminopeptidase from accessing the amino group (Wallace et al., 1993). The acetyl group is then cleaved from the **AA** in the small intestine. The released **AA** should then become available for absorption (Baxter et al., 2001). The current studies were intended to determine the ruminal protection and intestinal availability of **MET** and **LYS** from **NALM** and **NALL**, respectively.

CHAPTER 2

REVIEW OF LITERATURE

In the United States, milk is sold under the multiple component pricing system. This means that milk fat, and milk protein are more valuable to dairy farmers than the liquid portion. Many methods have been tried in order to increase milk protein yield. Supplementation has thus far proven to be the most useful. Protein supplements, however, can be expensive and total protein costs can be up to 80% of feed costs. Therefore, efficient protein utilization is paramount to maintaining the health and high production of the modern dairy cow while also keeping costs as low as possible. Increasing protein utilization in ruminants also decreases the amount of nitrogen that is lost into the environment via urine and manure. This has become more and more important as environmental concerns are receiving increasing attention. Rumen protected amino acids (**RPAA**) have been developed in order to supply the specific amino acids (**AA**) needed by the cow in the amounts necessary. Methionine (**MET**) and Lysine (**LYS**) are generally accepted as the most limiting **AA** when an alfalfa hay and corn silage diet is fed. For this reason, emphasis has been placed on successfully protecting these two **AA** from degradation in the rumen. This literature review will discuss some of the ways that **MET** and **LYS** have been protected, and the ramifications they have on production in dairy cattle.

Protein

Cattle have evolved to utilize diets that were low in digestible protein. The ability to take plant proteins, as well as other protein building blocks, and turn them into meat and milk is an advantageous characteristic of ruminants. Because of this, nitrogen efficiency may decrease as high protein feeds are fed. However, much research is still needed to fully understand the particulars of protein digestion and use in the ruminant digestive tract (Calsamiglia et al., 2010).

Protein utilization is an inefficient process in ruminants. Due to fermentation by rumen microbes, much of the protein that is supplied in the diet is not the protein that is available for the systemic use of the cow. Only 20-35% of dietary crude protein (**CP**) will be used in the production of milk proteins (Giallongo et al., 2016). Many strategies, such as low protein feeds, supplying limited energy, altering rumen degradable (**RDP**) and rumen undegradable protein (**RUP**) ratios, and protecting amino acids from rumen degradation have been employed to improve the efficiency of this process. Although microbial fermentation efficiency has improved, little has been accomplished in overall nitrogen utilization efficiency over the last few decades (Calsamiglia et al., 2010). However, much has been learned about protein use in the dairy cow. After parturition, cows begin producing large quantities of milk, which requires a lot of nutrients. At the same time, dry matter intake (**DMI**) is low in the days following parturition. This results in a negative energy balance for cattle. This is especially true for high producing dairy cows. There is a high demand for high quality protein in the diet during early lactation in order to both maintain milk protein production and to reduce body weight (**BW**) loss. Protein that is consumed by the cow goes to the rumen where microbes break it down into ammonia and peptides. These are used by bacteria and protozoa for their own growth requirements and microbial protein synthesis. These microbes will eventually be washed out of the rumen and into the lower digestive tract. The cow is then able to digest these microbes in the abomasum and utilize their protein. Most protein that is available to the cow post-ruminally comes in the form of microbial protein (NRC, 2001). Generally, protein from bacteria and protozoa contains a good balance of **AA**. However, the proportions are not consistent. The small intestine also uses some **AA** before they can be absorbed into the blood stream (Calsamiglia et al., 2010). This makes it difficult to determine the amount of **AA** that the cow is actually absorbing into the

bloodstream. Knowing the amount of each **AA** that is delivered to the cow is important because dairy cows can function on non-protein nitrogen, but high milk production is not sustainable without the proper balance of **AA** (Lee et al., 2015).

Crude Protein

Traditionally, diets were formulated based on concentration of **CP**. There are two main types of protein within **CP**. Those consist of protein that can be digested by the rumen microbes and that which passes through the rumen and may become available directly to the cow. **RDP** and **RUP** must be balanced in order to maintain rumen health and high production. Adequate **RDP**-about 10% **DM**-will be utilized for microbial growth as long as there is sufficient energy (Weiss et al., 2002). However, feeding an excess of **RDP** results in large amounts of nitrogen that cannot be fully utilized by rumen microbes (Weiss et al., 2002). This excess nitrogen is absorbed into the blood in the form of ammonia. The liver converts the ammonia into urea. The urea can then be excreted in the urine. Excess urea can build up in the blood and can reach toxic concentrations. A high concentration of **BUN** can lead to a decrease in reproductive efficiency (NRC, 2001). In order to decrease the negative effects that feeding excess **CP** has on the body and the environment, many researchers and farmers began putting deficient amounts of **CP** in the diet. This also proved to be problematic in terms of productivity. In one study done by Giallongo et al. (2016), lowering the amount of **CP** in the diet caused **DMI** to decrease. A reduction in **DMI** can be costly for a dairy because it can negatively affect milk production and milk protein yield (Giallongo et al., 2016). This is due to the fact that, as previously discussed, microbes require **RDP** for growth. Furthermore, **RUP** is required to maintain high milk production because this supplies protein for absorption by the cow. In a study by Osorio et al. (2013), it was found that increasing **RUP** during the last trimester of gestation increased milk

production in the following lactation. All of these benefits are lost if **CP** is lowered too far below dietary requirements, or if **RDP** and **RUP** are not provided in proper proportions. Current research shows that much more attention should be paid to the amount of **AA** in the diet rather than overall **CP** (Schwab, 2010).

Microbial Protein Synthesis

Microbial protein synthesis (**MPS**) is important because after the microbes die, they are digested by the cow. Increasing **MPS** increases the amount of microbial protein that flows into the abomasum and is utilized. It simultaneously decreases the amount of excess ammonia-N present in the rumen as microbes incorporate it into their proteins. Small peptides and **AA** supply an important source of nitrogen for microbes. In fact, these take less energy to utilize than complete proteins (Calsamiglia et al., 2010). The efficiency at which these molecules are used can be decreased by protozoa that ingest bacteria. Some of this nitrogen can be recovered by the cow when these microorganisms wash into the abomasum. However, some purines are not bioavailable, and will therefore be lost. The majority of microbial N is present in the form of amino acid-N. These can be broken down and used by the cow. Nucleic acid-N cannot be completely broken down and will be excreted in the urine. (Calsamiglia et al., 2010).

Synchronization

Nitrogen from protein cannot be fully utilized in the rumen unless adequate carbohydrates are also being fed. Carbohydrates supply the energy required by the rumen microbes to break down dietary protein and incorporate it into microbial protein (NRC, 2001). If there are not enough carbohydrates available, the cow must deal with the excess protein. This is costly to the animal. Energy is spent taking excess protein and converting it into urea so that it can excreted from the body via the urine (Evans et al., 2015). When sufficient carbohydrates are present,

microbes in the rumen are able to break dietary protein down to ammonia and peptides. Excess ammonia is then absorbed through the rumen wall into the blood. From there it can be recycled or eliminated from the body (Wu et al., 2012).

Fate of Protein

Liver

The **AA** that are available to the mammary gland are not present in the same amounts that they were absorbed. This is due to the fact that the liver utilizes some **AA**. It catabolizes many proteins and uses many **AA** for the carbon backbones during glucose synthesis (Calsamiglia et al., 2010). Methionine may be removed by the liver in large quantities. The liver may oxidize **MET** or use it to synthesize export proteins (Vyas et al., 2009).

Mammary gland

Nitrogen utilization in the mammary gland is a complex process. The extent to which **AA** are used can vary dramatically. The more **AA** that are available in the bloodstream, the more that can be used for protein synthesis in the mammary gland. When there are less **AA** present, the body will increase overall blood flow (Calsamiglia et al., 2010). This increase in blood flow increases the **AA** that pass through the liver and may increase the **AA** available for use in the mammary gland. This is a process that is most effective during early lactation. The epithelial cells inside the mammary gland also have the ability to increase the percent of **AA** that are absorbed when the supply from the bloodstream is low and there are many proteins present in the mammary gland that 0 g dose the synthesis of proteins once the **AA** have been absorbed (Calsamiglia et al., 2010).

Nitrogen Recycling

Nitrogen recycling is an advantageous characteristic of ruminants. Recycling refers to when nitrogen is brought back from the blood stream into the rumen, where it can be used by microbes. The use of this pathway theoretically increases with the decrease of available dietary nitrogen. Ammonia is absorbed from the rumen and transported to the liver. The ammonia is converted to urea. Urea may be excreted in the urine. The urea that is present in the blood can enter back into the rumen through the saliva. It may also be drawn into the rumen from the blood down the concentration gradient. The urea can then be broken down to ammonia in the rumen (Reynolds, 1992). Rumen microbes require the presence of ammonia for growth and microbial protein synthesis. Maintaining adequate concentrations of ammonia can be energetically expensive for the cow. If little protein is available in the diet, ammonia is more readily absorbed through the rumen wall. Energy must then be used to recycle nitrogen back into the rumen. Although nitrogen recycling has proven useful in maintaining the animal in extreme conditions, this tactic has not been shown to have benefits for high production on dairies. Reducing the nitrogen in the diet does seem to increase the recycling process, but it does not upregulate as quickly as the nitrogen intake is limited (Calsamiglia et al., 2010). Therefore, high milk production cannot be maintained on little protein in a real-world situation (Giallongo et al., 2016).

Amino Acids

Amino acid concentration can be quite different in the diet depending on the ingredients used. A diet that is heavy in corn products will typically be low in **LYS**. Feeding mostly alfalfa generally limits the amount of **MET** that is available in the diet (Weiss et al., 2002). Furthermore, typical plant or animal by-product sources of **AA** are generally more easily broken

down and utilized by rumen microbes. This limits the amount of **AA** that bypass the rumen and become available to the cow (Koenig et al., 2001).

There are many **AA**, however only 20 are used to build proteins. Out of these, 10 are recognized as being necessary in the diet because the cow cannot synthesize them herself. These are known as essential amino acids (**EAA**). These **EAA** are absorbed by the cow from microbial protein and **RUP** as well as the cow utilizing them from endogenous proteins. Every time that a protein is assembled, it requires the same **AA**. The bonding between these **AA**, as well as between side chains, is what gives proteins their shape. These proteins are then utilized in tissue synthesis as well as their role in many other important functions. Many nitrogen-containing compounds are made using **AA**. These include hormones, neurotransmitters, nucleotides for **DNA** and **RNA**, as well as many others (Schwab et al., 2010). Importantly, **AA** are used to synthesize tissue and milk proteins. They are also important for growth, maintaining body weight, and milk production. Amino acids may also be used for gluconeogenesis or fatty acid synthesis (Lobley et al., 1992). The effects of **MET** and **LYS** supplementation are greater in early lactation when demands for **AA** are high and available energy is low (Osorio et al., 2013). Of their many uses, milk protein synthesis uses significant amounts of **AA**. Mammary cells remove free **AA** that are present in the arterial blood and utilize them in the making of milk protein (Patton et al., 2015). However, mammary cells are not always as efficient as is necessary.

In fact, as the supply of **AA** increases, the efficiency at which the body uses these **AA** decreases (Vyas et al., 2009). This happens as the metabolic pathways for **AA** become saturated. As requirements are reached for the mammary gland, **AA** may be oxidized by the liver and used for gluconeogenesis instead of making milk protein. This inefficiency is further compounded by

the use of **AA** by gut epithelial cells and by inefficient reabsorption of endogenous sources (Vyas et al., 2009). In one study by Lee et al. (2015), a negative relationship between supply of **AA** and the efficiency at which the **AA** was used for milk protein synthesis was found to be larger than expected. When **LYS** and **MET** were supplied at 50% of dietary requirement, utilization of these **AA** for milk protein synthesis was at 90%. On the other hand, when 125% of dietary requirement was fed, efficiency of utilization was found to be only 60%. As the percentage of **AA** supplied exceeds the needs of the animal, the metabolic pathways become overwhelmed. The excess **AA** will be excreted and lost. This decreases the overall efficiency of utilization (Lee et al., 2015). Even so, meeting the dietary optimum of **AA** concentration is still important. When insufficient **AA** are supplied, cows can only maintain themselves so long before problems arise. Deficiencies of **AA** in the diet lead to tissue mobilization (Giallongo et al., 2015). After a period of time, production will also be negatively affected by inadequate **AA** in the diet.

Decreased protein or AA can also cause other animal or environmental effects. Decreased immunity may be seen when dietary **AA** are insufficient (Giallongo et al., 2016). Supplying a greater amount of **AA** to the small intestine for absorption increases the amount of protein synthesis in the mammary gland. In a study by Ordway et al. (2009), they found that supplementing **MET** both prepartum and postpartum seemed to prevent protein loss from body tissues. This may help maintain health and longevity of the animal. Furthermore, supplementing **MET** and **LYS** improves the concentration of **EAA** while allowing the overall **RUP** of the diet to be reduced (Ordway et al., 2009). This means that less nitrogen will be excreted into the environment. Thus, the health of the environment can also be maintained. One study by Lee et al (2015) discovered that ammonia emissions from manure were 31% lower when diets were

supplemented with **MET** and diets that were supplemented with **LYS** and **MET** showed an average decrease of 19% in ammonia emissions.

Methionine

Methionine is an **AA** that is important in many different processes. It is utilized in synthesizing proteins, but it is also important as a source of methyl groups. Methionine is used to make cysteine, taurine, creatine, phosphatidylcholine, and carnitine. It also plays an important role in gene expression because **MET** is used to methylate cytosine and histone (Bertoli et al., 2013). According to NRC (2001) recommendations, **MET** should be supplied 2.4% of metabolizable protein (**MP**).

Coating Technology

Free **MET** and **LYS** are rapidly degraded by microbes in the rumen (Chalupa et al., 1976. Onodera et al., 1993). This means that they do not make it to the small intestine where they can be absorbed. Researchers have spent decades testing many different types of protection methods to enhance the amount of **AA** that reaches the small intestine for absorption. Early on, many types of fat and fatty acid coatings were experimentally utilized. These did not prove to be efficient. Lipid coatings that protected **MET** from ruminal degradation did not release much **MET** into the small intestine for absorption (Schwab and Ordway, 2003). Mineral matrices were also researched. Some methods did not prove to be effective or were expensive to produce (Graulet et al., 2005).

In a study by Rossi et al. (2003), hydrogenated fatty acids, calcium soaps, ethyl-cellulose, and pH sensitive polymers were all tested as protective coating methods. The pH polymer showed the most protection in this study. The ethyl-cellulose coating did reduce the nitrogen that was released in the rumen. However, it did not release as much **MET** in the small intestine

as the pH-sensitive polymer. According to research of Koenig et al. (2001), many types of coatings show a negative correlation between increased rumen protection and bioavailability of **MET** in the small intestine. The pH sensitive polymer has thus far had the most positive results as far as providing protection from rumen microbes without decreasing systemic bioavailability. Smartamine (Adisseo, Inc., Antony, France) uses a pH sensitive polymer to protect **MET** (Schwab et al., 2003). Seventy-five percent of Smartamine is a DL-**MET** mixture. It is currently a popular product due to its ability to adequately supply **MET** to the small intestine.

Methionine analogs and precursors have also been studied. These increase the bioavailable **MET** by allowing the analog to pass through the rumen undegraded where it can then be synthesized into **MET** and be utilized by the cow (Koenig et al., 1999; Graulet et al., 2005). 2-hydroxy-4-methylthiobutanoic acid (**HMB**) is a **MET** analog. The α-amino group of the **MET** is replaced with a hydroxyl group. This is designed to protect the **HMB** from rumen microbes. **HMB** can also be esterified to isopropyl ester of the 2-hydroxy-4-(methylthio)-butanoic acid (**HMBi**). Metasmart (Adisseo, Inc., Antony, France) is an **HMBi** product that is relatively popular in the market. This product contains 57% **HMBi**. From the **HMBi** 78% can be converted into **MET** (Graulet et al., 2005). Methionine analogs and precursors have had mixed results and have often had low overall bioavailability (Belasco 1972; Koenig et al., 1999; Graulet et al., 2005).

Attaching chemical tails to protect **MET** from microbial degradation has also been researched in some detail (Wallace et al., 1993; Baxter et al., 2001). This has proven effective but has often been cost prohibitive. It is therefore not currently used for dietary supplementation. N-acetyl-L-Met (**NALM**) has had little research, and little is known about its bioavailability.

The comparison of **NALM** to current commercial products is needed to verify relative effectiveness of various products.

D, L isomers

There are two naturally occurring isomers of **MET**. These are the D and L isomers. Rumen protected **MET** typically contains a mixture of these two isomers. However, D-**MET** must be converted into L-**MET** before it can be utilized. This process is highly inefficient (Baker et al., 1994). The conversion of D-**MET** to L-**MET** means that the D isomer is metabolized more slowly, and so it accumulates in the plasma (Patton et al., 2015). The research is also unclear as to how much of the D isomer is actually converted into the L isomer. Furthermore, some research has shown that increasing the percent of L-**MET** in a supplement improves hepatic lipid metabolism in both calves as well as lactating cows (Osorio et al., 2013).

HMB

2-hydroxy-4-methylthiobutanoic acid (**HMB**) is not actually a rumen protected source of **MET**. It is an analog that must be absorbed and then metabolized into **MET** (Koenig et al., 1999). The body tissues oxidize **HMB** into 2-keto-4-(Methylthio)-butanoic acid. The L-alpha-hydroxy acid oxidase and D-hydroxy acid oxidase enzymes are responsible for this conversion. It is then transaminated into **MET** (Graulet et al., 2005). Methionine synthesis from **HMB** occurs most actively in the liver, kidneys, rumen, and small intestine of growing lambs (Wester et al., 2000).

When compared to free **MET**, **HMB** effectively delivers **MET** to the cow instead of rumen microbes. In studies by Belasco (1972), a batch culture was used to compare the analog with **MET**. After 4 hours, 91% of the analog remained while only 55% of **MET** was still present.

However, the ruminal protection of **HMB** is not as effective as many other forms of rumen protected **MET**. Koenig et al. (1999) found that about 50% of **HMB** bypassed the rumen. Once in the small intestine, it was then absorbed and converted into **MET**. Although 50% bypasses the rumen, there are further losses during the conversion from **HMB** to **MET**. Serum concentrations of **MET** peaked 6 hours post dosing with **HMB** (Koenig et al., 1999).

HMBi

Isopropyl ester of the 2-hydroxy-4-(methylthio)-butanoic acid (**HMBi**) has been shown to be more effective at ruminal protection than **HMB**. It also stimulates rumen microbes, although the mechanism is not yet known (Chen et al., 2011). **HMBi** has also been shown to increase plasma **MET** concentrations more than **HMB** (Ordway et al., 2009). Several studies have been conducted to show the method of ruminal protection and subsequent absorption of **HMBi**. There are many conflicting views. The current consensus is that much of it is absorbed across the rumen wall. This protects it at least in part from rumen degradation. There is also evidence that some **HMBi** makes it to the small intestine where it is absorbed. In a study by Graulet et al. (2005), the products of **HMBi** metabolism were tracked to determine how quickly it was broken down and absorbed. They determined that the **HMBi** was absorbed rapidly across the rumen wall and then hydrolyzed into **HMB** and isopropyl alcohol. **HMB** is then converted into **MET** by tissues of the cow and isopropyl alcohol is converted into acetone by the liver (Wester et al., 2000). In a study by Graulet et al. (2005), isopropyl alcohol increased in the plasma during the first 2 hours after **HMBi** supplementation. It was then slowly but steadily cleared by the liver. There was also a rapid increase in acetone concentrations. Both of these metabolites returned to normal plasma concentrations within 40-46 hours after supplementation. **HMB** was detectable within the blood as early as 10 minutes following supplementation with **HMBi**. It then peaked

an hour later. **HMBi** was never detected in the peripheral blood during this study. This may indicate that it was absorbed across the rumen wall and quickly hydrolyzed into **HMB**. Methionine concentrations in plasma increased during the 3 hours after **HMBi** supplementation. It then decreased back to basal concentrations within 48 hours. When compared with normal **MET** kinetics, it was determined that **HMBi** was absorbed across the rumen wall and then converted to **MET**. The ability for **HMBi** to be absorbed from the rumen is likely due to lipophilic properties. It is not yet known if **HMBi** is hydrolyzed within the digestive tract, in the digestive wall, or in the liver. Ultimately, 50% of **HMBi** was shown to bypass the rumen and therefore avoid microbial degradation (Graulet et al., 2005). In a study by Noftsger et al. (2005), only 2.3% of **HMBi** escaped from the rumen and was detectable in the omasum. But, significant absorption from the rumen would have left a very low concentration for passage. Furthermore, the group treated with **HMBi** in this study had higher milk protein. It was reasoned that the increase in milk protein would have been due to ruminal absorption of **HMBi**.

Smartamine

Because of its size and weight, Smartamine moves with the small, solid particles through the rumen. A study by Schwab and Ordway (2003) showed that as much as 90% of Smartamine bypassed the rumen into the abomasum. When the pH becomes more acidic in the abomasum, the **MET** is released from its capsule. The majority of it is then absorbed from the jejuno-ileal area in the small intestine (Graulet et al., 2005). The amount of Smartamine that is available varies somewhat. The availability of Smartamine is generally around 80%. Robert and Williams (1997) found that it ranged between 75 and 97%.

In a study by Graulet et al. (2005), **HMBi** and Smartamine were compared to see the difference in bioavailability and effects of these two supplementation methods. The **MET**

availability of **HMBi** was estimated to be 48.3 ± 2.05%. The kinetics of absorption seen in this trial suggested that **HMBi** was absorbed across the rumen wall. Methionine concentrations in blood peaked after 4 h and held steady for 4 more h. It then decreased steadily and returned to basal concentrations within 30 h after supplementation. For Smartamine, the **MET** availability was estimated to be 74.4 ± 2.15%. Methionine concentration did not increase in the blood until 10 h after supplementation. It then increased rapidly for 2 h and remained steady for 16 h. The height of the peak concentration did not differ between treatments, but the area under the curve was 1.87 times greater for **HMBi** (Graulet et al., 2005).

NALM

Little research has been done on acetylated **MET**. The acetyl group that is attached to the **MET** prevents deamination by rumen microbes (Wallace, 1992). Once it bypasses the rumen and enters the small intestine, the **AA** and acetyl group are separated by an enzyme called aminoacylase 1 (Baxter et al., 2001). Hydrolysis of the acetylated **MET** allows its absorption by the small intestine.

Liver Metabolism

Much research has been done to determine **AA** metabolism in the liver. Evidence shows that the liver may use significant amounts of **AA** (Patton et al., 2010). It extracts **AA** from the blood once the needs of the mammary gland have been met. High concentrations of **MET** may constrict the blood flow to the mammary gland (Patton et al., 2010). Methionine is used for many different processes in the liver. It is used as a methyl donor for choline synthesis and therefore helps in the formation of very low-density lipoproteins (**VLDL**) (Sharma et al., 1988). In a study by Osorio et al. (2013), **MET** seems to alleviate the stress of fatty liver and ketosis. When in a negative energy balance, dairy cows mobilize adipose tissue to supply energy. The

liver becomes overloaded with these long-chain fatty acids (**LCFA**). The incomplete oxidation of these fatty acids results in the formation of ketone bodies. Supplementation with **MET** decreases the incidence of ketosis by preventing the accumulation of triacylglycerides (Osorio et al., 2013; White 2015).

Isopropyl alcohol increased quickly in plasma after **HMBi** supplementation. It is then cleared by the liver where it is converted it into acetone (Graulet et al., 2005). The body can then use acetone for hepatic gluconeogenesis or it can be utilized in the milk. Acetone can be returned to the rumen via blood capillaries in the rumen wall. It may also be secreted back into the rumen via saliva. The rumen environment allows it to be reduced back into isopropyl alcohol. This can create a metabolic cycle that favors the transfer of reducing hydrogens from the rumen to the liver (Graulet et al., 2005).

Utilization

Methionine is the most limiting **EAA** for milk protein synthesis. Feedstuffs do not provide adequate **MET** in the diet. High producing cows may require as much as 50 to 60 g of supplemental **MET** per day (Koenig et al., 2001). In studies that have researched the effects of rumen protected **MET**, results have been inconsistent. According to Patton et al. (2010) milk protein percentage and milk fat percentage seems to have increased for rumen protected **MET** diets in most studies, but some studies showed no result. Studies have seen an increase of milk fat yield while others have found a decrease in milk fat yield (Patton et al., 2010).

Comparing different supplementation strategies can be difficult because of differences in protection methods. Ration formulations also vary from one study to the next. There have been many inconsistencies in research results for rumen protected **MET**. Many of these could possibly be attributed to study ration variables. It is also important to note that stage of lactation,

breed, and health status may affect **MET** utilization. Therefore, it is important to note that the source of **MET** and its availability is an important factor in affecting production parameters.

DMI

With **MET** supplementation, **DMI** has been shown in some studies to increase and in others to remain the same (Patton et al., 2010). The base diets fed vary so widely, that these discrepancies are often attributed to diet differences. In a study by Chen et al. (2011), Smartamine and **HMBi** were compared. In this study, the **LYS:MET** ratio was 3:1. There were not any **DMI** effects seen for either treatment when compared with a positive 0 g dose diet. There were also not any differences in **BW** gain.

Smartamine and Metasmart –an **HMB** product- were compared in a study by Osorio et al. (2013), and it was found that cows given **MET** supplements had greater **DMI** postpartum and lower **BCS** than cows that were not given supplementation. There were not significant differences between the two products. Mepron is a DL-**MET** that is protected by ethylcellulose and has been shown to decrease **DMI** when compared to Smartamine (Patton et al., 2010).

Milk

In a meta-analysis done by Patton et al. (2010), cows fed alfalfa-based diets while being supplemented with **MET** tended to have a greater increase in milk. Diets that were high in energy resulted in less changes in milk protein percentage between treatments. There are also differences in the protected **MET** products that were used in the studies. The varying bioavailability of these products would explain some of the differences in results. Giallongo et al (2016) also hypothesized that the mixed reports on milk fat content could also be as a result **MET** being used to donate methyl groups and in its use for choline synthesis. Osorio et al.

(2013) discovered that the cows that were fed **MET** had an increase in milk yield, milk protein percentage, energy corrected milk, and milk fat yield despite having a lower **BCS** than 0 g dose cows.

When comparing Metasmart and Smartamine, Ordway et al. (2009) discovered that milk conversion from **DMI** and the ratio of feed nitrogen that became milk nitrogen was lower for Metasmart. It was hypothesized that this was due to the fact that Metasmart must be converted from **HMBi** into **MET** before it can be utilized. However, **HMBi** increased energy-corrected milk and milk protein content. It was hypothesized that this may be due to an increase in microbial protein synthesis. Both **MET** supplemented diets and the positive 0 g dose diet increased milk fat and true protein. Nitrogen efficiency was increased the most in the diet that was supplemented with Smartamine. Cows supplemented with Smartamine had higher plasma **AA** and milk protein. No milk yield differences were observed between **HMBi** and Smartamine when the two **MET** sources were compared by Chen et al. (2011). When compared with Smartamine, Mepron increased milk production according to Patton et al. (2010). Some studies have shown that **HMB** increases milk and milk fat yields, but no increases in milk protein concentration have been observed (Ordway et al., 2009).

Immunity

In a study by Osorio et al. (2013), the immune capacity increased with **MET** supplementation. The lymphocytes seem to have a **MET** requirement for proliferation. This was measured by the amounts of blood neutrophil phagocytosis that occurred. So, it should be understood that **MET** that is used for other processes in the body will not be available for use in milk fat or milk protein synthesis.

Lysine

Lysine, like other **AA** has many uses in the body. Similar to **MET**, it is necessary for growth, and milk protein synthesis. It is also used for repairing and maintaining tissues. Some research shows that it is necessary for gestation. Lysine is also used to make carnitine. Fat is transported into cells by carnitine (Evans et al., 2015). The NRC (2001) recommends that **LYS** be included in the diet of dairy cattle at a 7.2% of **MP**.

LYS Coating

There is limited information available on the **LYS** products and their respective availabilities (Evans et al., 2015). This is partially due to the fact that **LYS** has proven more difficult to coat and protect than **MET**. Lysine is highly water soluble (Watanabe et al., 2003). The positive charge on **LYS** may make it more reactive with coatings made of fat. Even when coated in the same manner as **MET**, it has comparatively higher rates of ruminal degradation (Rossi et al., 2003). Protecting **LYS** from rumen degradation is vital, as little more than 3% passes through into the lower digestive tract (Robinson et al., 2006). Amos and Evans (1978) found that only 5% of L-Lysine-HCl bypassed the rumen. The most common method of **LYS** protection is to coat it with lipid. Several different fatty acid combinations have been used over the years. Current lipid coatings appear to protect **LYS** from ruminal degradation. Lipid coatings that use saturated, long chain fatty acids also raise the melting temperature and increase the shelf life by preventing auto-oxidation (Sakkers et al., 2013). This makes it easier to store and transport. It is hypothesized that this method of protection is also the reason that **LYS** is not sufficiently released into the intestine and, therefore, is not absorbed well (Wu et al., 2012). Some research has suggested that acetylating the **N** terminus of **LYS** would be an effective method of protection. Microbes in the rumen break proteins and amino acids down with the enzyme aminopeptidase (Wallace et al., 1993). This means that blocking access to the amine group restricts the extent of rumen degradation that can occur. Although rumen bypass

appeared to be sufficient in nutrition trials, the high cost of production has prevented acetylated **LYS** from entering the market (Wallace et al., 1993).

Elwakeel et al. (2015) tested hydroxymethyl **LYS** in sheep. Hydroxymethyl **LYS** is created by reacting Lysine-HCl with formaldehyde in a solution of calcium hydroxide. This study showed that hydroxymethyl **LYS** was well protected from ruminal degradation. However, it also inhibited ruminal microbes to the extent that the overall results were ineffective (Elwakeel et al., 2015).

Forms and availability

Lysine is present in fair amounts in many animal by-products, but plants are not a good source. Lysine is typically low in diets that are high in corn-based ingredients. As with other amino acids, **LYS** that is available in the diet is often degraded and then utilized by rumen microorganisms (Evans et al., 2015). For this reason, supplements have been created that ruminally protect **LYS**. This allows farmers to supply cows with adequate amounts of **LYS** (Evans et al., 2015). In a study by Rossi et al. (2003), several types of protection were compared. Lysine that was protected using calcium soaps were degraded more than those that were protected using triglycerides. Another study tested the effects of using different concentrations of oleic acid in the lipid matrix (Wu et al., 2012). The **LYS** was protected with a hydrogenated fat coating that had either 2% or 4% oleic acid. The results of this study indicated that increasing the percentage of oleic acid in the coating decreased ruminal bypass of the product. Increasing the percent of **LYS** present in the product also decreased how much was able to bypass the rumen.

It has proven difficult to protect **LYS** from microbes and yet maintain its bioavailability for the cow. This **AA** has been shown to be sensitive to Maillard reactions. Furthermore, **LYS** does not appear to become highly available in the intestine when it is protected with lipids

(Sakkers et al., 2013). Watanabe et al. (2003) conducted a study using a **LYS** product that was protected with a coat of dehydrogenated tallow. The **AA** was tested in a solution that simulated rumen conditions and then in a solution that simulated abomasal conditions. In this study, 75% of **LYS** remained stable for 24 hours in solution with a pH similar to that of a typical rumen. However, only 2% was released in the solution with a pH similar to that of the abomasum. In the aforementioned study by Wu et al. (2012), the bioavailability of **LYS** protected by hydrogenated fats in the small intestine was also tested. The hypothesis was that polyunsaturated fatty acids would improve the intestinal digestibility and therefore bioavailability of the **LYS**. It was discovered, however, that there was no significant increase of intestinal digestibility when higher concentration of oleic acid was used to protect **LYS**. This, combined with the decreased rumen bypass rate showed that increasing oleic acid was not effective.

Wallace et al. (1993) compared two acetylated products with unmodified peptides in order to see the difference in rumen bypass rate. This study found that acetylated **LYS** (N-ε-methionyl **LYS**) and acetylated **MET** were more stable than the unacetylated peptides. This increased the proportion of amino acid that passed through the rumen into the lower digestive tract.

Lactation

A study by Giallongo et al. (2016) tested a diet with several different amounts of **AA** supplementation. The basal diet was deficient in **MP**. In the cows that were only supplemented with **LYS**, there was an increase in milk protein yield and plasma glucose concentration. When adequate amounts of **MET** and histidine were also supplied with **LYS** supplementation, milk fat yield, **ECM** and feed efficiency were also increased.

DMI

Dry matter intake was decreased for the **LYS** supplemented diet in the study by Giallongo et. al (2016). In another study, however, no milk production or composition effects were seen

when the diet was supplemented with **LYS** (Paz et al., 2013). Effects vary from study to study based on diets, stage of lactation, and many other factors.

Cobalt as a marker

Cobalt leaves the rumen with the liquid portion of the diet. Since **NALL** and **NALM** are quickly dissolved in liquid, they also pass through the rumen with the rest of the liquid. Cobalt can be used effectively to determine the rumen bypass rate of **NALL** and **NALM** because it leaves the rumen at approximately the same rate as the rumen protected **AA** (Sakkers et al., 2013).

Conclusion

Balancing **AA** in the diet of dairy cows is necessary in order to maintain milk production and milk protein synthesis. There have been many methods for protecting **AA** from ruminal degradation over the last few decades. The improved protection allowed for more **AA** to become available for absorption from the small intestine. The **AA** must be released from the coating method before it can be absorbed. Results for overall bioavailability have been variable; especially for **LYS**. It is believed that attaching chemical tails to the α-amino group of **AA** would improve overall bioavailability. Three experiments were designed to test the overall availability of one rumen protected **MET** product and two rumen protected **LYS** products. Rumen bypass and duodenal appearance were measured. Blood **AA** concentration was monitored so that blood **AUC** could be calculated. Milk yield, milk protein, milk fat, and **DMI** were measured. It was hypothesized that **NALM** would protect the **MET** from ruminal degradation while allowing for release in the small intestine. It was also hypothesized that the extra **MET** would increase milk protein synthesis. The first experiment with **NALL** tested two

doses of **ƐNALL**. It was hypothesized that the chemical tail would provide protection in the rumen and be cleaved from the **LYS** in the small intestine. Milk protein synthesis and milk yield was hypothesized to increase in response to **LYS** supplementation. The second experiment with **NALL** compared **ƐNALL** and **diNALL**. It was hypothesized that the two **NALL** products would have similar bioavailability and production effects. These experiments were designed to test the ruminal protection and intestinal digestibility of **NALM** and **NALL**, as well as the effects these products have on milk production.

CHAPTER 3

METHIONINE

Ruminal Protection and Intestinal Availability of Rumen-Protected Methionine in Lactating Dairy Cows

ABSTRACT

Rumen protected Methionine (**MET**) is critical for milk protein synthesis in dairy cows. N-acetyl-L-methionine (**NALM**) is a **MET** derivative that consists of L-**MET** protected with an acetyl group that is attached to the α-amino group. The objective of this study was to quantify the gastrointestinal availability of **NALM** by measuring ruminal escape and intestinal appearance. The experiment was designed as a 3 × 3 Latin square using 3 lactating Holstein cows that had been fitted with cannulas in the rumen and duodenum. Each cow was fed twice daily with a diet that consisted of alfalfa hay, corn silage, steam-flaked corn, cottonseed, canola meal, soybean meal, shredded beet pulp, and a vitamin and mineral mix. The cows were fed this diet for 2 weeks leading up to the experiment so that the rumen microbes had time to adapt. Each period consisted of 10 d of adaptation followed by 2 d of sampling. A dose of 0 g (control), 30, or 60 g of **NALM** was placed under the rumen mat at the time of feeding every day during adaptation and hour 0 of the sampling period. On the first day of sampling, a liquid marker (Co-EDTA) was also administered as a bolus dose at the time of **NALM** administration. Blood, ruminal, and duodenal samples were then taken at hours 0, 1, 3, 6, 9, 12, and 24 after administration of the **NALM** and liquid marker. A nonlinear regression model was used to determine fractional rate constants for the passage of the liquid marker and the disappearance of

NALM from the rumen. There was no difference between treatments for liquid passage rate or ruminal **NALM** passage rate. Ruminal escape as a percentage of the dose was numerically higher for the 30 g dose (69.1%) than for the 60 g dose (46.2%), but no statistical differences were found (P = 0.44). Duodenal **NALM** appearance as a percentage of dose did not differ (P = 0.21) between the 30 g dose (2.16%) and the 60 g dose (3.40%). Dietary treatments did not affect rumen pH or **VFA** production. In conclusion, overall results suggest that **NALM** was effectively protected again microbial degradation in the rumen and was then extensively digested post-ruminally.

INTRODUCTION

Protein is an important nutrient for dairy cows. It is necessary to balance rumen degradable protein (**RDP**) and rumen undegradable protein (**RUP**) such that the microbes are supplied with protein to grow while the cow still receives sufficient for systemic needs (Weiss et al., 2002). Supplying sufficient **CP** is not always adequate to meet production needs. Specific **AA** are necessary in the diet (Schwab et al., 2010). Amino acids are utilized for many different purposes in the body, including tissue growth, milk proteins, and enzymes. Milk protein is reduced if insufficient **AA** are present in the diet (Schwab et al., 2010; Lobley et al.,1992). It is generally accepted that the **AA** that is limited most frequently in regards to milk protein synthesis in dairy cows is **MET** (Schwab et al., 2003; Weiss et al., 2002). In order to ensure that the dietary requirement of this **AA** is achieved, many methods have been developed to protect it from microbial degradation so that it may bypass the rumen and be absorbed from the small intestine of the cow (Graulet et al., 2005; Rossi et al., 2003; Koenig et al, 2001). Along with improving milk components, feeding bypass **AA** also increases the efficiency of nitrogen utilization and therefore decreases the deleterious effects nitrogen can have on the environment (Ordway et al., 2009). The benefits of feeding rumen protected **AA** depends on the efficacy of the individual products to supply **MET** to the cow via absorption from the small intestine. The **NALM** product is a **MET** derivative where the **MET** is protected from rumen degradation by an acetyl group that is attached to the α-amino group of the **AA** (Wallace et al., 1992). The **NALM** product was tested in vitro by Wallace et al. (1992). After 48 h, 67% of the **NALM** was still present in the protected form. The ability of **NALM** to bypass the rumen in vivo needed to be determined. The

present study compared the extent to which **NALM** provided protection from rumen degradation and yet became available for absorption from the small intestine.

MATERIALS AND METHODS

Animals and Diets

Three lactating Holstein dairy cows (second-lactation cows; 631 ± 5.9 kg **BW**) with ruminal (10 cm diameter, Bar Diamond, Parma, ID) and duodenal T-type (Bar Diamond, Parma, ID; placed 10 cm distal to the pylorus) cannulas were used in an experiment of 3 X 3 Latin square design. Cows were adapted to the basal diet for 2 weeks before beginning the experiment. Each of the 3 periods consisted of 10 d adaptation and 48 h sampling.

All care and procedures were approved by the Institutional Animal Care and Use Committee of Utah State University. Cows were housed in individual tie stalls. Each stall had a rubber mat that was bedded daily with straw. Clean water was accessible at all times. Cows were milked twice daily at 0400 and 1530 h. The **DIM** for the three cows averaged 22 days at the beginning of the trial and averaged 98 days by the end.

The **TMR** was mixed daily and was composed of alfalfa hay, corn silage, straw, steam-flaked corn, cottonseed, soybean meal, canola meal and a vitamin and mineral mix (Table 1). Each cow was fed in her individual bunk twice each day at 0600 and 01600 h throughout the experiment. Enough **TMR** was given so that approximately 10% orts were left each morning. Cows were supplemented with 0, 30, or 60g **NALM** each day of the adaptation period and hour 0 of the sampling period. Supplements were placed through the rumen cannula, beneath the rumen mat 15 minutes after the morning feeding.

Table 1. Ingredients and chemical composition of the experimental diets with varying does of N-acetyl-L-methionine supplemented to early lactation Holstein dairy cows.

	Diet		
Item	0 g/d NALM	30 g/d NALM	60 g/d NALM
Ingredient, % of DM			
Alfalfa Hay	29.35	29.31	29.28
Corn Silage	27.08	27.04	27.01
Wheat Straw	3.22	3.22	3.21
Corn, steam-flaked	12.31	12.29	12.28
Cottonseed, whole	5.30	5.30	5.29
Canola meal	6.91	6.90	6.89
Soybean meal	6.91	6.90	6.89
Beet pulp, shredded	5.11	5.11	5.10
Vitamin and mineral mix	2.67	2.67	2.66
Sodium bicarbonate	1.14	1.13	1.13
NALM	0	0.12	0.25
Chemical composition, % of DM			
DM, %	96.7	96.7	96.7
OM	88	88	88
CP	15.8	15.8	15.8
NDF	38.5	38.5	38.5
ADF	25.7	25.7	25.7
Ether Extract	1.5	1.5	1.5
LYS, %MP	6.78	6.74	6.70
MET, %MP	1.92	2.29	2.67
LYS:MET	3.54	2.94	2.51

Sampling

Each period consisted of 10 days of adaptation followed by 48 h of sampling. The amount of **TMR** fed and refusals were recorded every day of the entire trial. Samples of the **TMR** and of individual refusal were taken from day 5 of adaptation through day 2 of sampling. These were dried and ground. Body weights for each cow were taken on days 9 and 10 of each adaptation period. A liquid marker of Co-EDTA was given ruminally at the same time as the **NALM** was administered (0 h of sampling) at the start of the sampling periods. This marker was prepared

according to instructions given in Uden et al. (1980) and was then dissolved in 500 mL of distilled water so that each dose contained 3 g of cobalt.

Rumen, duodenal and blood samples were taken at 0, 1, 3, 6, 9 ,12, and 24 h after the cobalt was given on the first day of sampling. Rumen samples were taken from various parts of the rumen through the cannula. This was then strained to remove solids and microbes. Samples were then acidified with 6 M HCl for ammonia analysis, or HPO_3 for **VFA** analysis and frozen (-40ºC). Duodenal contents (100 ml) were collected and frozen for later analysis. The pH of rumen and duodenal contents was measured at each sampling. At 48 h, the rumen contents of the cows were removed and then weighed. The rumen contents of each cow were then placed into the rumen of the cow that would be the next to receive the corresponding supplementation. This was intended to expedite the adaptation process for the rumen microbes. A sample was taken and dried in a 60º C oven until dry to determine dry matter content. Blood was collected from the coccygeal vein into purple top Vacutainer tubes, centrifuged at 3000 X g for 25 min. Plasma was removed from the cells and then frozen until analyzed. Blood plasma was also collected for blood ketone and glucose analysis 0 and 24 h after Co-EDTA administration.

Milk yield was recorded daily from 14 d before the first adaptation until the end of the experiment. Milk samples were taken a.m. and p.m. of the 2 sampling days and preserved with potassium dichromate and analyzed for fat, protein, lactose, somatic cell count, and **MUN** by Rocky Mountain DHIA lab (Logan, UT).

Chemical Analysis

All **TMR** and refusal samples were dried in a 60◦ C oven for 48 h and weighed to determine **DM**. They were then ground through a 1-mm screen for analysis. Both **NDF** and **ADF** were analyzed according to standard procedures using an Ankom 2000 Fiber Analyzer (Ankom Technology, Macedon, NY). These procedures were based upon the research and fiber analysis work by Van Soest and Robertson (1980). Approximately 0.9-1.0 g from ground sample was sealed in an Ankom XT4 bag and analyzed for fat content using an XT15 ether extract machine (Ankom Technology, Macedon, NY). Each sample was extracted for 40 min at 70°C and then dried at 102°C for 16 hr. Percentage fat was determined as mass lost after extraction and drying.

Rumen and duodenum samples (100 ml) were freeze dried and then ground using a mortar and pestle. These samples were then sent to CJ CheilJedang Bio (Seoul, South Korea) for **MET** and cobalt analyses. Each sample was diluted (X 40 dilution) using deionized water and vortexed until dissolved. These samples were then sonicated for 20 min. The solution was centrifuged for 20 minutes at 4000 X g. The supernatant was filtered through a 0.45 µL syringe filter and then analyzed for **NALM** concentration using UHPLC (Shimadzu Nexera, Seoul, South Korea). An additional 0.1 g of each ground sample was dissolved in 4 mL nitric acid, 4 mL water, and 2 mL hydrogen peroxide before being digested by acid assisted microwave irradiation using an Ethos Easy digestor system (Milestone Srl, Sorisole, Italy) for 30 min at 200° C. These samples were then diluted with 50 mL deionized water and tested for cobalt concentration with plasma mass spectrometry (ICP-MS, Agilent, Santa Clara, CA).

Each rumen sample was analyzed for NH_3-N. First, 2-3 mL of rumen fluid was spun in a microcentrifuge at 12000 X g for 2 min. 100 µL of the supernatant was then diluted with 400 µL

of distilled water and vortexed. Twenty µL of the solution was then placed in a microwell. Each sample was mixed with reagents made of phenol, sodium nitroprusside, sodium hydroxide, sodium hypochlorite, and ammonium sulfate. The preparation of the reagents and the procedure of mixing them with the samples was done according to the protocols established by Weatherburn (1967). After mixing with the reagents, the samples were run in an MRX microplate reader (Dynatech, Alexandria, VA).

Blood **MET** was determined using EZ:faast (Phenomenex, Torrance, CA) reagents and protocol for gas chromatography. Rumen samples were prepared for analysis by centrifugation at 12000 X g for 10 minutes. The supernatant was analyzed for **MET** using a Hewlett-Packard 6890 GC (Agilent, Santa Clara, CA) as explained by Eun and Beauchemin (2007).

Calculations and Statistical Analysis

The fractional rate constants for the cobalt marker and the **MET** were determined by running nonlinear regression analysis in SAS 9.4. The **NALM** is soluble in liquid. It dissolves quickly and is assumed to pass with the liquid portion of the rumen. The fractional rate constant of **NALM** was equated to that of the liquid cobalt marker.

The calculations and statistical analysis for fractional rate constants, ruminal disappearance, duodenal appearance, and duodenal area under the curve (**AUC**) were run according to the protocol set in Koenig et al. (2002).

Data for **DMI**, milk yield, milk protein, milk fat, and blood parameters were run using a Latin square mixed linear model (PROC MIXED; SAS 9.4). Treatment and period were run as fixed effects, and cow was run as a random effect.

RESULTS AND DISCUSSION

No differences were seen in milk yield and composition between treatments (Table 2). This may have been due in part to the fact that the experimental periods were short. Research into **MET** supplementation shows varying results for milk production. In a meta-analysis of **MET** trials, Patton et al. (2010) found that milk fat and milk protein increased in response to **MET** in some trials but decreased in other trials. The varied response of milk fat may be due to the many alternate uses of **MET** in the body (Giallongo et al., 2016). Results for milk yield also varied between trials. Some trials did not see any differences in milk yield (Osorio et al., 2013; Chen et al., 2011; Ordway et al., 2009). No decreases in **DMI** were recorded in response to **NALM** treatment (Table 2). This seems to indicate that no feedback mechanisms were triggered by **NALM** that would decrease appetite - at least not during the length of this trial. Previous research observed differing responses of **DMI** to **MET** supplementation (Osorio et al., 2013; Chen et al., 2011).

Table 2. Dry matter intake, body weight, and milk parameters of second lactation dairy cows fed rumen protected methionine.[1]

	0 g	60 g	30 g	SEM	*P*
DMI (kg/day)	30.0	31.8	31.1	7.36	0.33
Milk Protein	2.8	2.8	2.8	0.07	0.42
Milk Fat	3.8	3.5	3.5	0.20	0.22
Lactose	4.7	4.7	4.7	0.08	0.78
SNF	8.3	8.3	8.3	0.12	0.18
SCC	18.2	17.8	10.8	8.96	0.33
MUN	14.6	14.6	14.4	0.66	0.89
Yield (kg/day)	28.6	28.6	27.3	8.76	0.28
BW (kg)	641.6	645.6	645.9	21.79	0.29

[1] 0 g, 30 g, and 60 g of NALM (CJ).

Rumen pH and **VFA** production did not differ between treatments. There was a trend for rumen pH to be higher for the 0 g dose than for the **NALM** treatments (Table 3; $P = 0.09$). Although it appears that the **NALM** was splitting prior to the duodenal sampling site, there was no significant increase in blood **MET AUC** between treatments (Table 3; $P = 0.43$). The **AUC** 60 g dose was numerically lower than the 30 g dose and the 0 g dose (Table 3; 1610, 2162, and 2361 respectively). This may indicate that the body absorbed and utilized the **MET** that it needed. Previous research on **AA** absorption has indicated that increased supplementation results in decreased efficiency of absorption of the **AA** (Vyas et al., 2009). The 60 g dose may have been high enough that the need for **MET** was exceeded and the absorption pathways down-regulated. This has been seen in previous research on **AA** absorption (Vyas et al., 2009; Lee et al., 2015).

Table 3. Ruminal pH, ammonia-N, VFA profiles and blood methionine AUC of second lactation dairy cows fed a diet with rumen-protected methionine[1]

	0 g	60 g	30 g	SEM	*P*
Rumen pH	6.5	6.3	6.3	0.19	0.09
Duodenum pH	2.9	3.0	2.9	0.08	0.37
NH_3-N, mg/100 mL	7.6	7.6	7.9	0.84	0.93
Individual VFA, mol/100 mol					
Acetate (A)	1.2	1.1	1.1	0.05	0.21
Propionate (P)	0.2	0.2	0.2	0.03	0.80
Butyrate	0.1	0.1	0.1	0.01	0.36
Valerate	0.01	0.01	0.01	0.002	0.37
Isobutyrate	0.01	0.01	0.01	0.001	0.2
Isovalerate	0.01	0.01	0.01	0.001	0.34
A:P	5.0	4.7	4.8	0.22	0.03
Blood methionine Baseline	124.8	117.5	68.8	33.06	0.47
Blood MET AUC, µM/L	2361	1610	2162	441.9	0.43

[1] 0 g, 30 g, and 60 g of NALM (CJ).

Liquid passage rate was determined by the concentration of a liquid cobalt marker as described in Koenig et al. (2002). Treatment did not affect liquid passage rate. Fractional rate constant of **NALM** going into the small intestine was not affected by treatment. Ruminal escape of **NALM** was numerically higher for the 30 g dose (69.1%) than for the 60 g dose (46.2%), but no statistical differences were observed (Table 4; $P = 0.44$). The percentage of **NALM** that escaped the rumen was roughly that noted in vitro by Windschitl et al. (1988). Duodenal appearance was low (2.16%-3.40%) but did not differ significantly between treatments (Table 4; $P = 0.21$). These results are relatively consistent with findings from Koenig et al. (2010). Duodenal appearance of **NALM** may have appeared low due to the fact that the acid environment of the abomasum and aminoacylase 1 in the small intestine split the acetyl group from the **MET** (Baxter et al. 2001). This allows for **MET** to be absorbed in the small intestine. Low duodenal appearance may indicate that the acetyl group of **NALM** was indeed being cleaved from the **MET** prior to the duodenal collection, as intended.

Table 4. Ruminal escape and duodenal appearance of NALM in second lactation dairy cows fed a diet with rumen-protected methionine[1]

	0 g	60 g	30 g	SEM	*P*
Ruminal volume, L	126.9	129.5	127.4	7.43	0.91
Ruminal flow rate, L/h	14.0	13.8	13.7	1.75	0.36
Fractional rate constants, /h					
Liquid	0.113	0.108	0.107	0.016	0.65
NALM	…	0.30	0.13	0.06	0.13
Ruminal escape, % of dose	…	46.2	69.1	18.39	0.45

Duodenal NALM, AUC µg/ml·h	…	145.3	50.9	9.15	<0.0001
Duodenal NALM appearance, % of dose	…	3.4	2.2	0.56	0.21

[1] 0 g, 30 g, and 60 g of NALM (CJ).

The **NALM** product provided reasonable protection of **MET** from ruminal degradation. It appears to have been split prior to the collection site in the duodenum. Future studies are also needed to research the effects of **NALM** on milk production over a longer period of time.

CHAPTER 4

LYSINE

Ruminal Protection and Intestinal Availability of Rumen-Protected Lysine in Lactating Dairy Cows

ABSTRACT

Lysine (**LYS**) is one of the most limiting amino acids for dairy cow production. Rumen protected **LYS** is important for milk protein synthesis. Two different rumen protected **LYS** derivatives were tested in two consecutive trials. Both ƐN-acetyl-L-Lysine (**ƐNALL**) and Nα,Ɛ-acetyl-L-Lysine (**diNALL**) are protected from rumen degradation with an acetyl group attached to the α-amino group. The objective of this study was to quantify the gastrointestinal availability of N-acetyl-L-Lysine (**NALL**) by measuring ruminal escape and intestinal appearance. Two experiments were run as a 3 × 3 Latin square using 3 second lactation Holstein cows that had been fitted with cannulas in the rumen and duodenum. Each period consisted of 10 days of adaptation followed by 2 days of sampling. During the first experiment, a dose of 0 (0 g dose), 60, or 120 g of **ƐNALL** was placed under the rumen mat at the time of feeding every day of adaptation and hour 0 of sampling periods. A dose of 0 (0 g dose), 120 g **ƐNALL**, or 120 g **diNALL** was given similarly during the second experiment. On the first day of sampling, a liquid marker (Co-EDTA) was also administered as a bolus dose at the time of **NALL** administration. Blood, ruminal, and duodenal samples were then taken at hours 0, 1, 3, 6, 9, 12, and 24 of sampling. A nonlinear regression model was used to determine fractional rate constants for the passage of the liquid marker and the disappearance of **NALL** from the rumen.

During experiment 1, there was no difference between treatments for **DMI**, milk production, liquid passage rate and ruminal **NALL** passage rate. Ruminal escape as a percentage of the dose was numerically higher for the 120 g dose (32.7%) than for the 60 g dose (27.2%), but no statistical differences were found ($P = 0.54$). Duodenal **NALL** appearance as a percentage of dose was higher ($P < 0.01$) for the 60 g dose (2.86%) than for the 120 g dose (1.19%). The production of acetate, butyrate, propionate, and valerate (Table 3; $P < 0.01$) was greater for the supplemented cows than for the 0 g dose cows. There was a trend for isobutyrate to be higher for the supplemented cows than for the 0 g dose (Table 3; $P = 0.06$). For experiment 2, no differences were seen in **DMI**, milk yield, and milk composition between treatments. There was a trend ($P = 0.08$) for **Ɛ NALL** to have a higher rumen pH than **di NALL**. There were no differences between treatments for fractional rate constants, ruminal escape, or duodenal appearance. There do not appear to be any significant differences between **Ɛ NALL** and **di NALL**. Both provided significant protection from rumen degradation.

INTRODUCTION

It is generally accepted that dairy cows have a need for specific **AA** rather than a specific amount of **CP**. Lysine is an important **AA** that is used in milk protein synthesis as well as tissue growth. It is also an important player in fat metabolism due to the fact that it is used to make carnitine, which is essential for utilization of mobilized fat stores (Evans et. al, 2015). Lysine is often limited in typical dairy diets, and therefore, must be supplemented (Weiss et al., 2002).

Very little free **LYS** escapes rumen degradation. As little as 3-5% of **LYS** supplemented in the free form or as a salt makes it into the lower digestive tract (Amos and Evans, 1978; Robinson et. al, 2006). Much research has been done to find a way to protect **LYS** from degradation in the rumen (Wallace et al., 1993; Watanabe et al., 2003; Sakkers et al., 2013; Evans et al., 2015). Methods that have previously worked to ruminally protect **MET** have proven inefficient for **LYS** protection (Rossi et al., 2003). The most common method of protecting **LYS** in the rumen is to coat the **AA** with a lipid complex. Lysine is more reactive than **MET** and tends to interact with the lipid matrix and this increased reactivity leads to the **LYS** remaining bound to the lipid coating in the lower digestive tract (Wu et. al, 2012).

Studies found that blocking the amine group of **AA** provides adequate protection in the rumen while allowing for degradation in the abomasum and small intestine (Wallace et al., 1993; Baxter et al., 2001). This happens because attaching a chemical tail to the **N** terminus blocks rumen microbes from breaking down the **AA**. Lower pH in the abomasum weakens the bonds and allows the chemical tail and the **AA** to be cleaved by aminoacylase (Baxter et al., 2001). However, the process of chemical modification to protect **AA** has been cost prohibitive up to this point in time. But, both the **ƐNALL** and the **diNALL** are protected with an acetyl group

attached to the α-amino group using a more efficient process (Wallace et. al, 1993). The present study was intended to compare the rumen protection, bioavailability and production effects of these **NALL** products in dairy cows.

MATERIALS AND METHODS

Animals and Diets

Three lactating Holstein dairy cows (second-lactation cows; 735.7±33.3 kg **BW** for experiment 1 and 783.2±22.3 kg **BW** for experiment 2) with ruminal (10 cm diameter, Bar Diamond, Parma, ID) and duodenal T-type (placed 10 cm distal to the pylorus, Var Diamond, Parma, ID) cannulas were used in 2 experiments of 3 X 3 Latin square design with 3 periods of 10 d adaptation and 48 h sampling.

All care and procedures were approved by the Institutional Animal Care and Use Committee of Utah State University. Cows were housed in individual tie stalls. Each stall was bedded daily with straw. Clean water was accessible at all times. Cows were milked twice daily at 0400 and 1530 h. The average **DIM** was 207-243 days for experiment 1 and 243 -307 days for experiment 2.

Cows were put in tie stalls and adapted to the diets for a 2-week period before the experiment began. The **TMR** was composed of alfalfa hay, alfalfa haylage, corn silage, straw, steam-flaked corn, high moisture corn, cottonseed, soybean/canola meal, beet pulp and a vitamin/mineral mix (Table 1). Each morning of the adaptation, cows were given the supplement under the rumen mat 15 minutes after feeding. Each cow was given the supplement and the cobalt marker hour 0 of sampling. The treatments were 0g (0 g dose), 60g, or 120g **ƐNALL** for experiment 1, and either 0g, 120g **ƐNALL**, or 120g **diNALL** for experiment 2. Each cow was fed in her individual bunk twice each day at 0600 and 01600 h throughout both experiments. Enough **TMR** was given so that approximately 10% orts were left each morning.

Table 1. Ingredients and chemical composition of the experimental diets with varying does of N-acetyl-L-lysine supplemented to early lactation Holstein dairy cows

Item	Diet			
	0 g/d ƐNALL	60 g/d ƐNALL	120 g/d ƐNALL	120 g/d diNALL
Ingredient, % of DM				
Alfalfa Hay	17	17	17	17
Alfalfa Haylage	14.7	14.7	14.7	14.7
Corn Silage	8.6	8.6	8.6	8.6
Wheat Straw	5.9	5.9	5.9	5.9
Corn, steam-flaked	12.5	12.5	12.5	12.5
High Moisture Corn	9.6	9.6	9.6	9.6
Cottonseed, whole	7.3	7.3	7.3	7.3
Canola meal	4.5	4.5	4.5	4.5
Soybean meal	4.2	4.2	4.2	4.2
Beet pulp, shredded	6.7	6.7	6.7	6.7
Vitamin and mineral mix	1.9	1.9	1.9	1.9
Sodium bicarbonate	0.6	0.6	0.6	0.6
NALL	0	0.2	0.4	0.4
Chemical composition, % of DM				
DM, %	94.7	94.7	94.7	94.7
OM, %	90.3	90.3	90.3	90.3
CP	16.4	16.4	16.4	16.4
NDF	39.6	39.6	39.6	39.6
ADF	24.6	24.6	24.6	24.6
Ether Extract	3.58	3.58	3.58	3.58
LYS, % MP	6.54	9.34	12.14	12.14
MET, % MP	1.85	1.82	1.79	1.79
LYS:MET	3.53	5.13	6.78	6.78

Sampling

Each period consisted of 10 d adaptation followed by 48 h of sampling. The amount of **TMR** fed and refusals were recorded every day of the entire trial. Samples of the **TMR** and of individual refusal were taken from day 5 of adaptation through day 2 of sampling. These were

dried and ground. Body weights for each cow were taken on days 9 and 10 of each adaptation period. A liquid marker of Co-EDTA was given ruminally at the same time as the **NALL** supplements (0 h of sampling). This marker was prepared according to instructions given in Uden et al. (1980) and was then dissolved in 500 mL of distilled water so that each dose contained 3 g of cobalt.

Rumen, duodenal and blood samples were taken at 0, 1, 3, 6, 9 ,12, and 24 h after the cobalt and **NALL** were given on the first day of sampling periods. Rumen samples were taken from various parts of the rumen through the cannula. This was then strained to remove solids and microbes. Samples were then acidified with 6 M HCl for ammonia analysis, or HPO_3 for **VFA** analysis and frozen (-40ºC). Duodenal contents (100 mL) were collected and frozen until processed for analyses. The pH of rumen and duodenal contents was measured at each sampling. Blood was collected from the tail vein into purple top Vacutainer tubes, centrifuged at 3000 X g for 25 min. The plasma was then separated from the cells and frozen until analysis. Blood serum was also collected for glucose and ketone analysis in red top Vacutainer tubes at 0 h and 24 h after Co-EDTA administration.

Milk yield was recorded daily from 14 d before the first adaptation until the end of the experimental periods. Milk samples were taken a.m. and p.m. of the 2 sampling days, preserved with potassium dichromate, and analyzed for percent fat, percent protein, lactose, somatic cell count, and **MUN** by Rocky Mountain DHIA (Logan, UT).

Chemical Analysis

All **TMR** and refusal samples were dried in a 60° C oven for 48 hours and weighed to determine **DM**. They were then ground through a 1-mm screen for analysis. **NDF** and **ADF** was analyzed according to standard procedures using an Ankom 2000 Fiber Analyzer (Ankom Technology, Macedon, NY). These procedures were based upon the research and fiber analysis work by Van Soest and Robertson (1980). Approximately 0.9-1.0 g from ground sample was sealed in an Ankom XT4 bag and analyzed for fat content using an XT15 ether extract machine (Ankom Technology, Macedon, NY). Each sample was extracted for 40 min at 70°C and then dried at 102°C for 16 hr. Percentage fat was determined as mass lost after extraction and drying.

Rumen and duodenum samples (100 mL) were freeze dried and then ground using a mortar and pestle. These samples were then sent to CJ CheilJedang Bio (Seoul, South Korea) for **LYS** and cobalt analysis. Each sample was diluted (X 40 dilution) using deionized water and vortexed until dissolved. These samples were then sonicated for 20 minutes. The solution was centrifuged for 20 minutes at 4000 X g. The supernatant was filtered through a 0.45 µL syringe filter and then analyzed for **NALL** concentration using UHPLC (Shimadzu Nexera, Seoul, South Korea). An additional 0.1 g of each ground sample was dissolved in 4 mL nitric acid, 4 mL water, and 2 mL hydrogen peroxide before being digested by acid assisted microwave irradiation using an Ethos Easy digestor system (Milestone Srl, Sorisole, Italy) for 30 minutes at 200° C. These samples were then diluted with 50 mL deionized water and tested for cobalt concentration with plasma mass spectrometry (ICP-MS, Agilent, Santa Clara, CA).

Each rumen sample was analyzed for NH_3-N. First, 2-3 mL of rumen fluid was spun in a microcentrifuge at 12000 X g for 2 minutes. 100 µl of the supernatant was then diluted with 400

µL of distilled water and vortexed. 20 µL of the solution was then placed in a microwell. Each sample was mixed with reagents made of phenol, sodium nitroprusside, sodium hydroxide, sodium hypochlorite, and ammonium sulfate. The preparation of the reagents and the procedure of mixing them with the samples was done according to the protocols established by Weatherburn (1967). After mixing with the reagents, the samples were run in an MRX microplate reader (Dynatech, Alexandria, VA).

Blood **LYS** was determined using EZ:faast (Phenomenex, Torrance, CA) reagents and protocol for gas chromatography. Rumen samples were prepared for analysis by centrifugation at 12000 X g for 10 minutes. The supernatant was analyzed for **LYS** using a Hewlett-Packard 6890 gas chromatograph (Agilent, Santa Clara, CA) as explained by Eun and Beauchemin (2007).

Calculations and Statistical Analysis

The fractional rate constants for the cobalt marker and for the **NALL** were determined by running nonlinear regression analysis in SAS 9.4. The **NALL** products dissolve quickly in the rumen fluid. Therefore, the rate of passage from the rumen was assumed to be equal to that of the liquid marker.

The calculations and statistical analysis for fractional rate constants, ruminal disappearance, duodenal appearance, and duodenal area under the curve (**AUC**) were run according to the protocol set in Koenig et al. (2002).

Data for **DMI**, milk yield, milk protein, milk fat, and blood parameters were run using a Latin square mixed linear model (PROC MIXED; SAS 9.4). Treatment and period were run as fixed effects, and cow was run as a random effect.

RESULTS AND DISCUSSION

There were no significant differences in **DMI** and milk yield for experiment 1 (Table 2). Milk production parameters such as protein and fat were also not altered significantly during experiment 1 (Table2). Some of this may have been due to the short length of the test period. The lack of effect on milk protein could also be attributed to the stage of lactation. Dairy cows are more sensitive to **AA** supplementation during early lactation when their needs are typically greater than their intake (Calsamiglia et al., 2010; Lee et al., 2015).

Table 2. Dry matter intake, body weight, and milk parameters of second lactation dairy cows fed rumen protected lysine[1]

	0 g	120 g	60 g	SEM	*P*
DMI (kg/day)	36.5	36.7	37.0	2.22	0.89
Milk Protein	3.2	3.3	3.2	0.08	0.60
Milk Fat	3.7	3.8	3.9	0.32	0.60
Lactose	4.2	4.3	4.2	0.16	0.47
SNF	8.2	8.4	8.3	0.21	0.48
SCC	201.5	187.3	175.1	55.55	0.74
MUN	13.6	15.0	14.5	0.64	0.12
Yield (kg/day)	27.7	26.8	26.5	6.99	0.43
BW (kg)	740.2	727.5	738.6	15.96	0.09

[1]0 g, 60 g, and 120 g of ƐNALL (CJ Bio).

During experiment 1, we observed a numerically greater percent of the 120 g dose of **NALL** escaped the rumen (Table 4; 32.7%) than the 60 g dose (Table 4; 27.2%). This could be explained by the greater concentration of **NALL** in the rumen and a saturation of breakdown capacity. A greater percent would be expected to escape the rumen microbes. This difference was not statistically significant.

Blood **LYS AUC** was greater for the 0 g dose than the supplemented groups during experiment 1. We observed greater blood **LYS AUC** for the 120 g dose than for the 60 g dose (Table 3, $P = 0.02$). The higher blood **LYS AUC** for the 0 g dose may indicate that the chemical tail was not cleaved properly. The dosage of **LYS** may have been high enough to cause a downregulation of **LYS** absorptive pathways, which has been seen in previous research (Vyas et al., 2009). Acetate, propionate and valerate production was higher (Table 3; $P < 0.01$) for the supplemented groups than for the group that was given 0 g **NALL**. Butyrate production was significantly higher (Table 3; $P < 0.01$) for the 120 g dose of **ƐNALL** than for the 0 g dose. We noted a trend (Table 3; $P = 0.06$) for isobutyrate production to be higher in the group supplemented with 120 g of **ƐNALL** than for the 0 g dose. The 0 g dose group had a higher acetate to propionate ratio (Table 3; $P < 0.01$) than the group supplemented with the 120 g dose. An increase in **VFA** production seems to indicate that the **ƐNALL** that was not protected was being used by the rumen microbes. Increased **VFA** production leads to increased energy available for use by the cow. This increase in **VFA** production in response to supplementation may be responsible for the increase in milk production and milk fat seen in other research (Giallongo et al., 2016). We observed a higher acetate to propionate ratio for the 0 g dose than for the 60 g or 120 g doses (Table 3; $P < 0.01$). The lower acetate to propionate ratio coupled

with the overall increase in VFA production for the cows supplemented with LYS means that there is more propionate available for gluconeogenesis.

Table 3. Ruminal pH, ammonia-N, VFA profiles, and blood lysine concentration in second lactation dairy cows fed a diet with rumen-protected lysine[1]

	0 g	120 g	60 g	SEM	*P*
Rumen pH	6.28	6.05	6.20	0.150	0.23
Duodenum pH	2.86	2.88	3.02	0.147	0.71
NH_3-N, mg/100 mL	5.60	5.61	5.48	0.608	0.97
Individual VFA, mol/100 mol					
Acetate (A)	0.60	0.70	0.67	0.043	<0.01
Propionate (P)	0.19	0.25	0.23	0.027	<0.01
Butyrate	0.13	0.16	0.14	0.015	<0.01
Valerate	0.018	0.022	0.021	0.0017	<0.01
Isobutyrate	0.011	0.012	0.012	0.001	0.06
Isovalerate	0.02	0.02	0.02	0.002	0.21
A:P	3.13	2.85	2.99	0.1	<0.01
Blood Lysine					
Baseline	157.3	123.3	134.6	60.78	0.57
Blood LYS AUC, μM/L	2624	2222	2108	314.3	<0.01

[1]0 g, 60 g, and 120 g of ƐNALL (CJ Bio).

We observed a trend (Table 4; $P = 0.07$) for the 60 g dose to have a higher duodenal **AUC** of **NALL** than the 120 g dose. A higher percentage of **NALL** appeared in the duodenum for the 60

g dose than for the 120 g dose (Table 4; $P < 0.05$). Rumen flow was numerically lower for the 120 g dose (Table 4; 16.3) than for the 60 g dose (Table 4; 18.9). This slower escape from the rumen may have allowed enzymes to more completely break down the **NALL** before it reached the duodenum. This would result in a lower **AUC** and duodenal appearance of **NALL** for the 120 g dose than for the 60 g dose.

Table 4. Ruminal escape and duodenal appearance NALL in second lactation dairy cows fed a diet with rumen-protected lysine[1]

	0 g	120 g	60 g	SEM	*P*
Ruminal flow rate, L/h	18.0	16.3	18.9	2.78	0.12
Fractional rate constants, /h					
Liquid	0.14	0.13	0.14	0.022	0.91
Ruminal NALL	…	0.45	0.52	0.066	0.51
Ruminal escape, % of dose	…	32.7	27.2	6.9	0.54
Duodenal NALL, AUC g/kg●h	…	82.8	88.9	14.6	0.07
Duodenal NALL appearance, % of dose	…	1.2	2.9	0.6	0.01

[1]0 g, 60 g, and 120 g of ƐNALL (CJ Bio).

For experiment 2, we did not see any differences for **DMI**, milk yield, milk protein, or milk fat (Table 5). There was a trend (Table 6; $P = 0.08$) for cows treated with **ƐNALL** to have a higher rumen pH than cows treated with **diNALL**. The acetate to propionate ratio was greater (Table 6; $p < 0.01$) for **ƐNALL** than for the 0 g dose or **diNALL**. There were no differences seen in the blood **LYS AUC** (Table 6; $P = 0.41$). The **diNALL** product had blood **LYS AUC** that was numerically lower than the 0 g dose and the **ƐNALL.** This may indicate that the **LYS** was not being absorbed as well as had been anticipated. We also did not observe any significant differences between treatments for the percentage of **LYS** that escaped the rumen, duodenal **AUC** or duodenal appearance of **NALL** between the two forms (Table 7).

Table 5. Dry matter intake, body weight, and milk parameters of second lactation dairy cows fed rumen protected lysine[1]

	0 g	ƐNALL	diNALL	SEM	*P*
DMI (kg/day)	37.7	38.9	37.1	3.28	0.61
Milk Protein	3.21	3.21	3.26	0.091	0.51
Milk Fat	3.75	3.73	3.68	0.252	0.74
Lactose	4.15	4.07	4.13	0.208	0.35
SNF	8.17	8.08	8.21	0.303	0.50
SCC	240.1	267.8	266.1	51.51	0.57
MUN	13.1	13.1	13.3	0.651	0.89
Yield (kg/day)	15.45	15.27	16.01	0.868	0.65
BW (kg)	786.7	786.5	586.5	113.41	0.46

[1]0 g, and 120 g ƐNALL (CJ Bio); 120 g diNALL (CJ Bio).

Table 6. Ruminal pH, ammonia-N, VFA profiles, and blood lysine AUC in second lactation dairy cows fed a diet with rumen-protected lysine[1]

	0 g	ƐNALL	diNALL	SEM	*P*
Rumen pH	6.23	6.31	6.18	0.120	0.08
Duodenum pH	3.12	3.14	3.20	0.047	0.57
NH_3-N, mg/100 mL	5.89	4.69	5.45	0.604	0.28
Individual VFA, mol/100 mol					
Acetate (A)	0.63	0.61	0.63	0.052	0.83
Propionate (P)	0.20	0.19	0.21	0.027	0.29
Butyrate	0.13	0.13	0.13	0.014	0.38
Valerate	0.02	0.02	0.02	0.001	0.12
Isobutyrate	0.01	0.01	0.01	0.001	0.51
Isovalerate	0.02	0.02	0.02	0.002	0.19
A:P	3.11	3.31	3.16	0.202	0.03
Blood lysine					
Baseline	171.8	56.6	122.2	50.42	0.30
Blood LYS, AUC, µM/L	3430	3335	2897	51.23	0.41

[1]0 g, and 120 g ƐNALL (CJ Bio); 120 g diNALL (CJ Bio).

During experiment 1, the rumen escape for **ƐNALL** was 32.7% and 27.2 % for the 120 g and 60 g doses respectively (Table 4). Rumen escape was 44.2% for **ƐNALL** and 42.8% for **diNALL** during experiment 2 (Table 7). This is much greater than the expected 3% of free dietary **LYS** that reaches the small intestine (Robinson et al., 2006). A study by Amos and

Evans (1978) found that only 5% of L-Lysine-HCl bypassed the rumen. Watanabe et al. (2003) found that some **LYS** products provide as much as 75% rumen bypass, but the true amount that becomes available for absorption in the small intestine varies, and is often quite low. Further research is needed to determine the overall bioavailability of **NALL**.

Table 7. Ruminal escape and duodenal appearance NALL in second lactation dairy cows fed a diet with rumen-protected lysine[1]

	0 g	ƐNALL	diNALL	SEM	*P*
Ruminal volume, L	140.47	139.64	140.70	0.456	0.25
Ruminal flow rate, L/h	13.24	20.06	18.82	3.543	0.36
Fractional rate constants, /h					
Liquid	0.14	0.09	0.13	0.025	0.32
Ruminal NALL	…	0.43	0.42	0.115	0.51
Ruminal escape, % of dose	…	44.2	42.8	20.8	0.82
Duodenal NALL, AUC g/kg●h	…	153.6	175.0	25.0	0.36
Duodenal NALL appearance, % of dose	…	2.4	2.1	0.7	0.78

[1]0 g, and 120 g ƐNALL (CJ Bio); 120 g diNALL (CJ Bio).

The percentage of **NALL** that appeared in the duodenum was low for all of the treatments for experiment 1 (Table 4) and for experiment 2 (Table 7). According to Baxter et. al (2001), the low pH of the abomasum allows enzymes to split the acetyl group from the **NALL**. The low percentage of appearance may indicate that the acetyl group is being efficiently cleaved from the amino acid prior to duodenal collection. Further research could test this by testing for the presence of different end products in the small intestine. Highly degradable **NALL** would supply more absorbable **LYS** in the small intestine.

The increased overall **VFA** production for **NALL** seen in experiment 1 would mean better feed efficiency. However, these same effects were not seen in experiment 2. This may be due to increased **DIM**. Previous research by Stockdale et al., (1987) saw decreased **VFA** response to treatments as lactation progressed. A decrease in **DMI** may largely be responsible for the decreased response to treatments (Friggens et al, 1998). Further research would need to be undertaken to see if the effects on **VFA** could be recreated and utilized. Overall, **NALL** shows potential to provide rumen protection while allowing degradation into absorbable **LYS** in the small intestine. Furthermore, increased **VFA** production could increase efficiency in dairy cows.

CHAPTER 5

CONCLUSIONS

The main focus of this study was to observe the ability of the **NALM** and **NALL** products to effectively protect **MET** and **LYS** in the rumen and still allow these **AA** to be released for absorption in the small intestine. The **NALM** and **NALL** were fed along with a typical dairy diet to measure rumen protection and see effects of supplementation on milk production and **DMI**. It was expected that milk protein and milk yield would be affected by the rumen-protected **MET** and **LYS**. It was found that the acetylated **MET** and **LYS** were protected from rumen degradation but appeared to be changed post-ruminally. No negative effects on **DMI** were observed for either the **NALM** or the **NALL**. Adequate rumen protection of **MET** was observed for both treatments of the **NALM**. The 30 g had a higher percent escape than the 60 g dose. This indicates that the optimum dosage is closer to the 30 g than the 60 g dosage. Future studies are needed to determine where the optimum dosage is so that the benefits of supplementation can be maximized. The **NALL** product also protected **LYS** from degradation in the rumen. Neither **NALL** product appeared to be better than the other. Testing this dosage against other dosages to determine the optimum is also necessary for the **NALL** products. The **NALM** and both **NALL** products had low duodenal appearance. It needs to be determined if this low appearance is due to the effective cleaving of the acetyl group from the **AA**. Future studies should test for the products of this cleavage to see if they are present in the duodenum. Although low duodenal appearance seems to indicate release of the **AA**, neither study observed significantly increased blood concentration of **MET** or **LYS**. However, neither of these studies were designed to specifically evaluate the absorption into the blood. The **NALM** product had numerically higher blood concentration of the **AA** for the lower dosages than the 0 g dose. The absorption of the

AA may have been inhibited if the dosages were too high. A high dose during the adaptation period may have caused a down-regulation of absorption pathways. During the first experiment with **NALL**, significantly increased **VFA** production was observed. Adequate rumen protection and intestinal availability combined with increased **VFA** production would be beneficial for dairy herds. Having more **VFA** availability increases the energy available for use by the cow. Overall, these studies indicate that **NALM** and **NALL** have potential to provide rumen protection. The effectiveness of releasing the **AA** in the small intestine requires further research. Determining the optimum dosages will help to increase the benefit of these products. There also appears to be potential to increase rumen fermentation products, which is important for maintaining high milk production in dairy cows.

REFERENCES

Amos, H. H., and J. J. Evans. 1978. Abomasal levels of lysine and methionine in wethers fed polymerized L-lysine-HCl and polymerized L-methionine. J. Anim. Sci. 46:778-786.

Baker, D. H. 1994. Utilization of precursors for L-amino acids. Amino Acids in Farm Animal Nutrition Cab International ed. 37-47.

Baxter J., Y. Kim, and M. Snowden. 2001. Hydrolysis of N-acetyl-L-glutamine by acylase I. J. Food Sci. 66:1428-1433.

Belasco, I. J. 1972. Stability of methionine hydroxyl analog in rumen fluid and its conversion in vitro to methionine by calf liver and kidney. J. Dairy Sci. 55:353-357.

Bertoli, R. F., and L. E. McBreairty. 2013. The nutritional burden of methylation reactions. Curr. Opin. Clin. Nutr. Metab. Care. 16:102-108.

Calsamiglia, S., A. Ferret, C. K. Reynolds, N. B. Kristensen, and A. M. van Vuuren. 2010. Strategies for optimizing nitrogen use by ruminants. Animal. 4:1184-1196.

Chalupa, W. 1976. Degradation of amino acids by mixed rumen microbial population. J. Anim. Sci. 43:829-834

Chen, Z. H., G. A. Broderick, N. D. Luchini, B. K. Sloan, and E. Devillard. 2011. Effect of feeding different sources of rumen-protected methionine on milk production and N-utilization in lactating dairy cows. J. Dairy Sci. 94:1978-1988.

Elwakeel, E. A., E. C. Titgemeyer, B. R. Faris, D. W. Brake, A. M. Nour, and M. E. A. Nasser. 2015. Hydroxymethyl lysine is a source of bioavailable lysine for ruminants. J. Anim. Sci. 90:3898-3904.

Eun, J. S., and K. A. Beauchemin. 2007. Enhancing in vitro degradation of alfalfa hay and corn silage using feed enzymes. J. Dairy Sci. 90:2839-2851.

Evans, E., J. Lamont, and H. Leclerc. 2015. Methods to evaluate rumen protected lysine for dairy cows. Open J. of An. Sci. 5:495-499.

Friggens, N. C, G. C. Emmans, I. Kyriazakis, J. D. Oldham, and M. Lewis. 1998. Feed intake relative to stage of lactation for dairy cows consuming total mixed diets with a high or low ratio of concentrate to forage. J. Dairy Sci. 81:2228-2239.

Giallongo, F., M. T. Harper, J. Oh, J. C. Lopes, H. Lapierre, R. A. Patton, C. Parys, I. Shinzato, and A. N. Hristov. 2016. Effects of rumen-protected methionine, lysine, and histidine on lactation performance of dairy cows. J. Dairy Sci. 99:4437-4452.

Giallongo, F., A. N. Histrov, J. Oh, T. Frederick, H. Weeks, J. Werner, H. Lapierre, R. A. Patton, A. Gehman, and C. Parys. 2015. Effects of slow-release urea and rumen-protected methionine and histidine on performance of dairy cows. J. Dairy Sci. 98:3292-3308.

Graulet, B., C. Richard, and J. C. Robert. 2005. Methionine availability in plasma of dairy cows supplemented with methionine hydroxy analog isopropyl ester. J. Dairy Sci. 88:3640-3649.

Koenig, K. M., and L. M. Rode. 2001. Ruminal degradability, intestinal disappearance, and plasma methionine response of rumen-protected methionine in dairy cows. J. Dairy Sci. 84:1480-1487.

Koenig, K. M., L. M. Rode, C. D. Knight, and P. R. McCullough. 1999. Ruminal escape, gastrointestinal absorption, and response of serum methionine to supplementation of liquid methionine hydroxyl analog in dairy cows. J. Dairy Sci. 82:355-361.

Lee, C., F. Giallongo, A. N. Hristov, H. Lapierre, T. W. Cassidy, K. S. Heyler, G. A. Varga, and C. Parys. 2015. Effect of dietary protein level and rumen-protected amino acid

supplementation on amino acid utilization for milk protein in lactating dairy cows. J. Dairy Sci. 98:1885-1902.

Lobley, G. E. 2014. 0 g dose of the metabolic fate of amino acids in ruminants: a review. J. Anim. Sci. 70:3264-3275.

Lobley, G. E. 1992. 0 g dose of the metabolic fate of amino acids in ruminants: a review. J. Anim. Sci. 70:3264– 3275.

Martinov, M. V., V. M. Vitvitsky, R. Banerjee, and F. I. Ataullakhanov. 2010. The logic of the hepatic methionine metabolic cycle. Biochimica et Biophysica Acta. 1804:89-96.

National Research Council. 2001. Nutrient requirements of dairy cattle. 7th rev. ed. National Academy Press, Washington, DC.

Onodera, R. 1993. Methionine and lysine metabolism in the rumen and the possible effects of their metabolites on the nutrition and physiology of ruminants. Amino Acids. 5:217-232.

Ordway, R. S., S. E. Boucher, N. L. Whitehouse, C. G. Schwab, and B. K. Sloan. 2009. Effects of providing two forms of supplemental methionine to periparturient Holstein dairy cows on feed intake and lactational performance. J. Dairy Sci. 92:5154-5166.

Osorio, J. S., P. Ji, J. K. Drackley, D. Luchini, and J. J. Loor. 2013. Supplemental Smartamine M or MetaSmart during the transition period benefits postpartal cow performance and blood neutrophil function. J. Dairy Sci. 96:6248-6263.

Patton, R. A. 2010. Effect of rumen-protected methionine on feed intake, milk production, true milk protein concentration, and true milk protein yield, and the factors that influence these effects: A meta-analysis. J. Dairy Sci. 93:2105-2118.

Patton, R. A., A. N. Hristov, C. Parys, and H. Lapierre. 2015. Relationships between circulating plasma concentrations and duodenal flows of essential amino acids in lactating dairy cows. J. Dairy Sci. 98:4707-4734.

Paz, H. A., M. J. de Veth, r. S. Ordway, and P. J. Kononoff. 2013. Evaluation of rumen-protected lysine supplementation to lactating dairy cows consuming increasing amounts of distillers dried grains with solubles. J. Dairy Sci. 96:7210-7222.

Reynolds, C. K. 1992. Metabolism of nitrogenous compounds by ruminant liver. J. Nutrition. 122: 850-854.

Robinson, P. H., E. J. DePeters. I. Shinzato, and H. Sato. 2006. Rumen lysine escape, rumen fermentation, and productivity of early lactation dairy cows fed free lysine. Anim. Feed. Sci. Technol. 128: 31-41.

Rossi, F., M. Maurizio, M. Fracesco, C. Giovanni, and P. Gianfranco. 2003. Rumen degradation and intestinal digestibility of rumen protected amino acids; comparison between in situ and in vitro data. An. Feed Sci. and Tech. 108:223-229.

Sakkers, M., L. J. Erasmus, P. H. Robinson, R. Meeske, and J. E. Garrett. 2013. Determining in vivo ruminal stability of three ruminally protected nutrients in lactating jersey cows. An. Feed Sci. and Tech. 185:133-139.

Schwab, C. G. 2010. Balancing diets for amino acids: nutritional, environmental and financial implications. Tri-State Dairy Nutrition Conference.

Schwab, C. G., R. S. Ordway, and N. L. Whitehouse. 2003. Amino acid balancing in the context of MP and RUP requirements. in: Proc. Four-State Applied Nutrition and Management Conference. ;25-34.

Stockdale, C. R., A. Callaghan, T. E. Trigg. 1987. Feeding high energy supplements to pasture-fed dairy cows. Effect of stage of lactation and level of supplement. Australian J. of Agr. Research. 38:927-940.

Van Soest, P. J., and J. B. Robertson. 1980. Systems for analysis of fibrous feeds. p. 49-60. *In* W. G. Pigden et al. (ed.) Standardization of analytical methodology for feeds. Publ. IDRC-134e. Int. Res. Dev. Ctr. Ottawa, ON.

Vyas, D., and R. A. Erdman. 2009.meta-analysis of milk protein yield responses to lysine and methionine supplementation. J. Dairy Sci. 92:5011-5018.

Wallace, R. J. 1992. Acetylation of peptides inhibits their degradation by rumen micro-organisms. British J. Nutr. 68:365-372.

Wallace, R. J., P. P. Frumholtz, and N. D. Walker. 1993. Breakdown of N-terminally modified peptides and an isopeptide by rumen microorganisms. Applied and Environmental Microbiology. 59:3147-3149.

Watanabe, K., H. Sato, T. Kobayashi, K. Katoh, and Y. Obara. 2003. Determination of intestinal disappearance of lysine and methionine derived from ruminally protected lysine and methionine in Holstein heifers. Asian-Aust. J. Anim. Sci. 16:549-554.

Weatherburn, M. W. 1967. Phenol-hypochlorite reaction for determination of ammonia. Analytical Chemistry. 39:971-974.

Weiss, W.P. 2002. Protein and carbohydrate utilization by lactating dairy cows. North Carolina Dairy Nutrition Management Conference. P. 44.

Wester, T. J., M. Vazquez-Anon, D. Parker, J. Dibner, A. g. Calder, and G. E. Lobley. 2000. metabolism of 2-hydroxy-4-methylthiobutanoic acid (HMB) in growing lambs. J. Dairy Sci. 79:397-400.

White, H. M. 2015. The role of TCA cycle anaplerosis in ketosis and fatty liver in periparturient dairy cows. Animal. 5:793-802.

Wiltbank, M. C., A. Garcia-Guerra, P. D. Carvalho, K. S. Hackbart, R. W. Bender, A. H. Souza, M. Z. Toledo, G. M. Baez, and R. S. Surjus, R. Sartori. 2014. Effects of energy and protein nutrition in the dam on embryonic development. Anim. Reprod. 11:168-182.

Wu, Z., J. K. Bernard, R. B. Eggleston, and T. C. Jenkins. 2012. Ruminal escape and intestinal digestibility of ruminally protected lysine supplements differing in oleic acid and lysine concentrations. J. Dairy Sci. 95:2680-2684.

Windschitl, P. M., and M. D. Stern. 1988. Influence of methionine derivatives on effluent flow of methionine from continuous culture of ruminal bacteria. J. Anim. Sci. 66:2937-2947.