Effects of reduced dietary protein and supplemented rumen protected amino acids on the nitrogen efficiency of dairy cows

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Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

Master of Science

In

Dairy Science

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September 30th, 2011

Blacksburg, Virginia

Keywords: nitrogen efficiency, rumen protected amino acids, milk production, protein synthesis

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ABSTRACT

Dairy cows are extremely inefficient at converting dietary nitrogen (N) to productive N. Approximately 25-30% of dietary N is used for milk protein while the remaining N is lost to the environment. According to National Research Council (NRC, 2001) recommendations, dairy cow rations are formulated in terms of metabolizable protein (MP) which often causes many amino acids (AA) to be fed in excess. A better understanding of protein and AA requirements could help to improve the nitrogen efficiency of dairy cows. The objective of this work was to examine the effects of feeding a low protein diet supplemented with rumen protected (RP) AA on production and N efficiency of dairy cows. Twenty-four Holstein and 24 Holstein x Jersey crossbred cows were used in a Youden square design consisting of 3 periods. Cows were randomly assigned to one of 8 treatments: 1) a standard diet containing 17% crude protein (+Con), 2) a 15% crude protein diet (-Con), 3) –Con plus RP methionine (+M, 16g/d), 4) –Con plus RP lysine (+K, 47g/d), 5) –Con plus RP leucine (+L, 181g/d), 6) –Con plus RP methionine and lysine (+MK), 7) –Con plus RP methionine and leucine (+ML), and 8) –Con plus RP methionine, lysine, and leucine (+MKL). Cows fed the –Con as well as the +MKL diet experienced a reduction in milk production and milk protein yield ($P < 0.05$). Dry matter intake decreased only for those animals on the +ML diet ($P < 0.05$). Milk urea N (MUN) decreased for all diets when compared to the +Con treatment ($P < 0.05$). In accordance with the decrease in MUN, N efficiency was numerically increased in the diets supplemented with RP AA, but this improvement was not significant. Phosphorylation of signaling proteins important for protein synthesis were also examined. Animals fed the +MK treatment increased phosphorylated and

total forms of eukaryotic elongation factor 2 (eEF2) when compared to the +Con and –Con ($P < 0.05$), but this increase in abundance did not affect the ratio of phosphorylated to total abundance. Feeding dairy cows a low protein diet supplemented with RP AA has the ability to alleviate the loss in milk production associated with feeding a low protein diet as well as to increase nitrogen efficiency.

ACKNOWLEDGEMENTS

I would like to thank my committee members for their constant guidance during my research here at Virginia Tech. To my advisor, Dr. Hanigan, thank you for being so willing to take me on as your first Masters' student. I am grateful for your patience, constructive criticism, ability to always make time for me as well as for your other students, and contagious excitement for research. Thank you for everything. To Dr. Escobar, thank you for convincing me to go to graduate school. Without your persuasiveness, I would not have had the opportunity to enjoy this amazing experience. Dr. McGilliard, thank you for all of your assistance on the statistics for my research. I greatly appreciate all of the late night e-mails you would send to help me fix my SAS code. Dr. Akers, thank you for your enthusiastic guidance, which has always kept me interested in my research.

I also want to thank all of the members of the Dairy Science department faculty and staff for all of their contributions during my time at Virginia Tech. I especially want to thank Dr. Gwazdauskas. Your passion for teaching and for your students is such an inspiration. I have enjoyed every minute of being your teaching assistant for the past two years.

To Shane, Woody, Curtis and the rest of the VT dairy farm crew, thank you for all of the fun times you have given me while out at the dairy. I will greatly appreciate your willingness to always lend a helping hand whether it was to help me take blood samples, set up the sample bottles before the 3:00 AM milkings, or fix the Data Ranger every time it broke down.

To the Hanigan Lab, without all of you, none of this would have been possible. Tara, thank you for generously running all of my GC-MS analyses and for your constant help throughout my research. To Ranga, Joby, and Agustin, thank you for all of the excitement and knowledge you have given me when it comes to research. To Sebastian, Deepthi, Sandip, Michelle, and Tyler, thank you for all of your help whether on my study, helping me out in the lab, or just answering my simple questions. I wish all of you the best of luck in your future projects. To the undergrads: Katie, Emma, Carenda, Holley, Niki, and Derek, you were the true backbone to my experiment. Without your constant willingness to help during my trial, I would never have gotten everything accomplished.

To Callie, thank you for being on this crazy ride with me. Through the good times and bad, you have always been right by my side through it all. I greatly appreciate your friendship over the past two years, and will cherish all the memories we have shared together. Hayden, thank you for being a wonderful officemate. Your laid-back nature and up-beat personality are characteristics I have always admired about you. Emily, Brittany, Kayla, Jamie, Andrea, Jen, Christina, Liying, Partha, Manisha, Becky, and to all the recent graduates, thanks for all of the laughs, smiles, and words of encouragement I have received from each of you during my time here. I will miss each and every one of you.

To Dave McKee, Will Petersen, Tony Marinello, and all of the Marching Virginians, thank you for being my home away from home. Being a part of such a wonderful organization has blessed me with a multitude of memories that I will never forget.

Finally, I would like to thank my family whose endless love and support has given me the determination to overcome any obstacle that gets in my way as well as the drive to always do my best. Mom, thank you for always being a phone call away. Your constant guidance and encouraging words were always heard and appreciated. I greatly value our relationship, and know that without you I would not be the person I am today. Dad, thank you for always encouraging me to follow my dreams. I greatly appreciate all of the care packages you sent on holidays or just when you knew I was feeling overwhelmed. Aaron, I could never repay you for all of the support and confidence you have given me over the past year. I hope I have made all of you proud.

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CHAPTER 1

INTRODUCTION

Ammonia emissions from concentrated animal feeding operations (CAFO) are a significant source of N pollution to the environment. Excretion of N contaminates ground and surface water and enters the atmosphere leading to acid rain, climate change, and human health concerns (Tamminga, 1992; Pope et al., 2002). Dairy cows make large contributions to this pollution due to poor N utilization. Approximately 30% of dietary N consumed by dairy cows is converted to milk protein while the remaining 70% is excreted in the urine and feces (Chase, 1994; Tamminga, 1992). This inefficiency in N utilization by dairy cows is partially caused by feeding protein above the amount required by the animal. Overfeeding of protein and the loss of excess N to the environment is a concern to producers as protein is typically an expensive nutrient in dairy cow rations (Brown and Arscott, 1960; Clark et al., 1987). An improvement in N efficiency would reduce dairy contributions to pollution and may decrease feed costs for farmers.

The National Research Council (NRC; 2001) recommendations for protein requirements are defined in terms of metabolizable protein (MP). Feeding according to NRC requirements of MP may overestimate the need for many AA to ensure maximum levels of milk production are achieved. Unlike swine and poultry, there are no defined AA requirements for dairy cows. Determining AA requirements for dairy cows is challenging because of the variety of feedstuffs used in rations and the remodeling of nutrients by rumen microbes (Lapierre et al., 2006). Obtaining accurate AA estimates for dairy cows would allow the feeding of lower crude protein (CP) diets supplemented with

specific AA to support milk production and milk protein synthesis, which is the strategy used in the swine and poultry industries.

In grass silage diets, histidine appears to be a limiting AA for milk production; however, corn-based diets, used predominately in the United States, are most limiting in methionine (Met) and lysine (Lys). Numerous production experiments have been conducted to determine the effects of adding Met and Lys on milk yield and milk protein production. Fisher (1972) observed an increase in protein yield with jugular infusion of Met compared to saline infusions ($P < 0.05$). Jugular infusion of the combination of Met and Lys increased protein yield and content compared to a saline infusion ($P < 0.05$) (Appuhamy et al., 2011). In addition, supplementing a 16.1% CP diet with 10 g/d of rumen-protected (RP) Met caused an increase in milk production and improved N efficiency compared to animals on a 18.6% CP diet with no RP Met supplementation ($P < 0.05$) (Broderick et al., 2008). A combination of RP Met and Lys also numerically increased milk protein and milk fat when compared to a diet without supplementation (Broderick et al., 2008).

The addition of leucine (Leu) has also been shown to improve production characteristics. Duodenal infusions of 40 g/d of Leu increased milk protein content and yield when corn-based diets were sufficient in Lys or Met ($P < 0.05$) (Rulquin and Pisulewski, 2006). Determination of limiting AA would help in the creation of an AA profile that could be used to supplement low protein diets with specific AA for milk production.

In addition to AA being used as the building blocks for proteins, AA have been shown to activate protein synthesis through the mammalian target of rapamycin (mTOR)

pathway. Amino acid supply has been shown to activate mTOR along with hormones, growth factors, and energy supply (Wang and Proud, 2006). Activation of mTOR leads to the subsequent phosphorylation of p70 S6 kinase (S6K1), ribosomal protein S6 (rpS6), eukaryotic initiation factor 4E binding protein 1 (4EBP1), and the dephosphorylation of eukaryotic elongation factor 2 (eEF2).

Leucine specifically had significant effects on the stimulation of mTOR when added to AA deficient media (Kimball, 2001). Leucine has also been shown to activate mTOR signaling in skeletal muscle cells of neonatal pigs (Escobar et al., 2006). Therefore, addition of Leu increases protein synthesis rates through the activation of the mTOR pathway. Based on the above findings, one would expect that the addition of AA and specifically Leu to dairy cow diets would increase both protein synthesis rates and milk protein yield.

The objective of this experiment was 1) to determine whether supplementation of RP Met, Lys, Leu, or combinations of these AA could alleviate the loss in milk production associated with feeding a low protein diet, and 2) to examine the effects of a low protein diet and RP AA on cell signaling proteins which regulate protein synthesis. The overarching goal of this work is to improve the nitrogen efficiency of dairy cows.

CHAPTER 2

REVIEW OF LITERATURE

Impact of Nitrogen on the Environment

Nitrogen is a significant source of pollution to the atmosphere as well as waterways. Nitrate (NO^-_3) can leech into the soil and contaminate groundwater and pollute surface waters through runoff (NRC, 2001). Ammonia (NH_3) released into the atmosphere can result in acid rain and eutrophication. In addition, volatilized NH_3 in the atmosphere contributes to the formation of fine particulate matter. These particles can decrease visibility and air quality, and increase human health problems (Pope et al., 2002). Graff et al. (2009) determined that fine particulate matter induces pulmonary inflammation in healthy young adults. There has also been an increased incidence of wheezing and asthma diagnoses in children attending schools near farms with high levels of NH_3 (Mirabelli et al., 2006; Sigurdarson and Kline, 2006). Radon et al. (2001) also concluded that farmers exposed to high NH_3 production experienced a higher rate of chronic respiratory diseases such as chronic bronchitis. Animal health and production can also be affected by high NH_3 levels. Beker et al. (2004) observed that poultry exposed to NH_3 exhibit lower performance and increased disease susceptibility. Therefore, decreasing the amount of nitrogen pollution from NO^-_3 and NH_3 would improve waterways and air quality as well as animal and human health.

The United States Environmental Protection Agency (EPA) has implicated concentrated animal feeding operations (CAFO) as a leading contributor of NH_3 emissions. Battye (1994) concluded that livestock contribute approximately 80% of the

NH_3 emissions in the United States, of which cattle are the biggest contributor. Specifically, dairy cows are responsible annually for 19.9% and 23.1% of the global and national NH_3 emissions, respectively (Ritz et al., 2004). Kebreab et al. (2001) determined on average dairy cows release approximately 150 g of N in urine per day. In addition, 40-50% of dairy cow manure N is made up of urea and NH_3 (NRC, 2001). Van Horn et al. (1994) concluded that 50-75% of the N lost to the environment is released as NH_3 before NO^-_3 can be produced. Improving the N retention of dairy cows is one potential strategy to control NH_3 emissions to the environment.

Improving Nitrogen Efficiency of Dairy Cows

Dairy cows are particularly inefficient at converting dietary N to productive N. Approximately 30% of N intake is converted to milk N, while the remaining is lost to the environment in urine and feces (Tamminga et al., 1992; Chase, 1994). Nutritional strategies should be used to improve the N efficiency of dairy cows.

Decreasing dietary protein has been repeatedly shown to improve N efficiencies for dairy cows. Ipharraguerre and Clark (2005) reported that increasing dietary crude protein (CP) from 14.8% to 18.7% decreased N efficiency from 33% to 25%. Additionally, reducing dietary N from 11% to 9.6% caused a decline in NH_3 emissions from the manure of Holstein heifers (James et al., 1999). Rius et al. (2008) concluded that reducing CP from 18.7% to 15.5% while maintaining a high energy level of 1.55 Mcal increased N efficiency from 37.1% to 43.0% with only a tendency for a reduction in milk production.

While feeding reduced dietary protein increases N efficiency, it often negatively impacts milk production. Kalscheur et al. (2006) observed an increase in N efficiency from 28.2% to 36.5% associated with a decrease in dietary CP from 17.1% to 12.3%; however, the N reduction also caused a decrease in production. Klusmeyer (1990) also reported a loss in milk production when CP was lowered from 14.5% to 11%. A better understanding of the protein requirements of dairy cows would help reduce harmful N pollution to the environment while still maintaining a high level of production.

Protein Requirements of Dairy Cattle

Dairy cows have requirements for 10 of the 21 amino acids (AA). These 10 AA are arginine (Arg), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine, threonine, tryptophan, and valine (Val) (NRC, 2001). These AA are classified as essential because they cannot be produced in body at a rate adequate to support needs for maintenance, growth, and production. While dairy cows require specific AA, no defined AA requirements have been established. Lapierre et al. (2006) indicated the usage of a variety of feed products and the remodeling of nutrients in the rumen by microorganisms as a challenge for determining AA requirements for ruminants. The current NRC (2001) protein requirements for dairy cows are therefore defined in terms of metabolizable protein (MP). When cows are fed acceptable levels of MP for production, many AA are fed in excess. Overfeeding AA is important because protein has been identified as one of the most expensive nutrients in dairy cow diets (Brown and Arscott, 1960; and Clark et al., 1987). Feeding excessive protein results in

increased production input costs as well as decreases in N efficiency compared to other production animal species.

Monogastric species, such as swine and poultry, have greater N efficiencies compared to dairy cows. Rotz (2004) reported that N efficiency of pigs and poultry is approximately 35% or higher. Bequette et al. (2003) calculated that N efficiency of growing pigs was about 33%, while growing broilers and egg-laying hens were 45% and 34% efficient, respectively. This improvement in N conversion is associated with precise AA requirements for monogastric species allowing for more accurate diet formulation. Swine and poultry producers are able to supply low protein diets supplemented with the specific AA needed to maintain production (Baker, 1997). Determination of dairy cow AA requirements would allow for the application of this kind of approach in dairy nutrition and ration formulation.

Amino Acid Supplementation to Dairy Cattle Diets

Supplementation of individual AA necessary for milk production to low protein diets would increase N efficiency and reduce N excretions to the environment. Instead of feeding high protein diets, adding individual AA allows for the feeding of a minimal amount of N in dairy cow diets. Noftsger and St-Pierre (2003) concluded that providing absorbed AA similar to the required AA for milk protein synthesis can improve N utilization.

In corn based diets, typically utilized in the United States, Met and Lys seem to be the most limiting for milk protein synthesis, while His appears to be limiting in grass silage diets. Clark (1987) and Schwab et al. (1976) observed the most deficient

intestinally available AA to be Met and Lys. Lactating dairy cows infused postruminally with Met showed linear increases in milk protein yield and content when Met concentrations increased from 6 g/d to 12 g/d (Pisulewski et al., 1996). Leonardi et al. (2003) observed that dairy cow diets supplemented with ruminally protected (RP) Met increased milk protein concentration. Furthermore, the addition of RP Met increased milk fat and protein content (Misciattelli et al., 2003). A meta-analysis of 129 RP Met studies determined that supplementation increased milk protein content and yield and slightly increased milk production (Patton, 2010).

In addition to Met supplementation alone, the addition of Lys in combination with Met also increases lactational performance in dairy cows. Abomasal infusion of a combination of Met and Lys improved milk protein synthesis in lactating dairy cows (Seymour et al., 1990; Weekes et al., 2006). Weekes et al. (2006) also saw that infusion of Met and Lys caused a 35% reduction in dry matter intake due to an imbalance in AA supply. The supplementation of Met and Lys increased milk production, protein yield, milk fat content, and N efficiency in Chinese Holstein cattle above that of the control, Met, and Lys treatments (Wang, et al., 2010). Also, lactating dairy cows supplemented with RP Met plus Lys showed increased milk protein and fat production during lactation (Robinson et al., 1998; Robinson et al., 1995); however, supplementation of Lys alone resulted in equivalent production to that of the unsupplemented treatment (Robinson et al., 1998). Socha et al. (2005) observed an increase in milk, milk fat, and milk protein yields with the supplementation of RP Met and Lys compared to the basal and Met treatments. The combination of Met and Lys to dairy cattle rations appears to have additive effects on milk production characteristics.

Dairy cattle rations also may be deficient in branched chain AA (BCAA) and in particular Leu. Rulquin and Pisulewski (2006) observed that duodenal infusion of 40 g/d of Leu increased milk protein content and yield. However, reports have been inconsistent with the findings of Rulquin and Pisulewski (2006). Korhonen et al. (2002) concluded that abomasal infusion of His and BCAA to dairy cows feed grass silage diets did not increase milk protein yield. Additionally, Appuhamy et al. (2011) saw no differences in milk protein yield or content between animals infused with Met plus Lys and animals infused with the combination of Met, Lys, and BCAA. When animals were supplemented with RP Leu in addition to Met and Lys, there were no differences in DMI, milk yield, milk protein, milk fat, lactose, and urea (Křižova et al., 2008). Each of these studies implied that BCAA or Leu provided no additional benefits when supplemented to lactating dairy cow rations; however, Leu has been shown to increase protein synthesis rates through cellular signaling activation in cultured cells and in other species (Avruch et al., 2009).

The mTOR Pathway and Effects of Amino Acids on Regulation

The mammalian target of rapamycin (mTOR) is a multidomain protein with kinase activity specific to phosphorylation of proteins at serine and threonine residues (Wang and Proud, 2006). Raptor and rictor bind with mTOR to form the complexes mTORC1, which is sensitive to the effects of rapamycin, and mTORC2, which is rapamycin insensitive, respectively (Sarbassov, 2005; Yang et al., 2006). Protein synthesis is regulated through the mTORC1 signaling cascade. Activation of the mTOR

pathway through mTORC1 is caused by a variety of controls including hormones, growth factors, energy status, and AA supply (Wang and Proud, 2006).

Activation of mTOR causes the phosphorylation of direct downstream substrates, p70 S6 kinase (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4EBP1) (Avruch et al., 2009). Phosphorylation of S6K1 and hyperphosphorylation of 4EBP1 increase rates of protein synthesis. Activation of S6K1 causes the phosphorylation of ribosomal protein S6 which enhances mRNA translation and increases ribosomal biogenesis, while 4EBP1 phosphorylation allows for the release of eukaryotic initiation factor (eIF) 4E to form the active eIF4F complex with eIF4G to facilitate the binding of mRNA to the 40S ribosomal subunit (Bolster et al., 2004; Kimball, 2001). Additionally, mTOR appears to regulate the phosphorylation of eukaryotic elongation factor 2 (eEF2) through eEF2 kinase. Phosphorylation of eEF2 kinase by S6K1 causes dephosphorylation and activation of eEF2 which enhances translation elongation (Proud, 2004). Increased phosphorylation of eIF2α by general non-repressible kinase 2 (GCN2) appears to be independent of the mTOR pathway, and causes inactivation of eIF2β which decreases mRNA translation (Kimball, 2001).

Amino acids positively regulate mTOR signaling. Orellana et al. (2007) determined that increased AA supply to neonatal pigs increased skeletal muscle protein synthesis and increased phosphorylation of mTOR, 4EBP1, and S6K1. However, abomasal infusion of casein had no effect on the phosphorylation of rpS6 or mTOR in bovine mammary tissue (Rius et al., 2010). Hara et al. (1998) observed rapid deactivation of S6K1 and decreased phosphorylation of 4EBP1 when AA were withdrawn from CHO-IR cells. In MAC-T cells and mammary tissue slices, addition of

essential AA affected translation initiation and elongation through the activation of mTOR, S6K1, and 4EBP1, and the dephosphorylation of eEF2 and eIF2α (Appuhamy et al., 2011).

Supplementations of individual AA can also regulate protein synthesis rates. Avruch et al. (2009) found that withdrawal of Leu or Arg from media deactivates mTOR as effectively as the removal of all AA. Escobar et al. (2006) observed stimulation of skeletal and cardiac muscle protein synthesis due to increases in Leu concentrations. However, Moshel et al. (2006) indicated that phosphorylation of 4EBP1 and S6K1 were more affected by complete AA withdrawal than by withdrawal of Leu alone. While Leu has been shown to increase protein synthesis in skeletal muscle, little work has been done with mammary tissue of lactating dairy cows fed diets with specific AA contents. Toerien et al. (2010) infused dairy cows with various AA and collected mammary biopsies. They observed a 71% and 61% increase in phosphorylation of S6K1 with the infusion of Leu and Met plus Lys, respectively. Additionally, Leu showed a 101% increase in rpS6 activation and Met plus Lys showed a 95% increase. In our experiment we looked at these same AA effects in mammary biopsies, but with the feeding of RP AA rather than intravenous infusion.

Hypothesis

We hypothesized that lactational performance could be maintained following a reduction in dietary CP if supplemented with individual or combinations of RP Lys, Met, and Leu. We also hypothesized that diets supplemented with RP AA would also increase dairy cow N efficiency compared with cows fed a high protein diet. In addition, we

hypothesized that supplementation of RP AA would increase the cellular signals necessary for protein synthesis in mammary epithelial cells.

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CHAPTER 3

Effects of reduced dietary protein and supplemented rumen protected amino acids on the nitrogen efficiency of dairy cows

ABSTRACT

When fed to meet National Research Council (2001) protein recommendations, dairy cows consume an excess of many amino acids (AA) resulting in approximately 70% of dietary nitrogen (N) being lost to the environment as urine and feces. Reductions in environmental N release could be attained through an improvement of N efficiency. The objective of this study was to determine if the typical reduction in milk yield associated with feeding a low protein diet to lactating dairy cows could be avoided by dietary supplementation with one or more ruminally protected (RP) AA. Fourteen multiparous and 10 primiparous Holstein cows and 24 multiparous Holstein x Jersey crossbred cows were used in a Youden square design consisting of 8 treatments and 3 periods. The 8 dietary treatments were 1) a standard diet containing 17% crude protein (+Con), 2) a 14% crude protein diet (-Con), 3) –Con plus RP methionine (+M, 16g/d), 4) –Con plus RP lysine (+K, 47g/d), 5) –Con plus RP leucine (+L, 181g/d), 6) –Con plus RP methionine and lysine (+MK), 7) –Con plus RP methionine and leucine (+ML), and 8) –Con plus RP methionine, lysine, and leucine (+MKL). Cows given the –Con and +MKL diets had lower milk production and milk protein yield than the +Con cows ($P < 0.05$). The yield of milk and milk protein for all other AA treatments were not different from either control diets. Milk fat yield was unchanged across all treatments. Dry matter intake was unchanged except for a decrease on the +ML diet compared to the +Con. Milk urea N was less for all diets compared to the +Con suggesting that greater N

efficiency can be attained by feeding a low protein diet ($P < 0.05$). The addition of one or more RP AA did not positively or negatively affect milk production.

Keywords: nitrogen efficiency, rumen protected amino acids, milk production, dairy

INTRODUCTION

Dairy cows have requirements for specific amino acids (AA) during lactation. The current National Research Council (NRC; 2001) recommendations for protein requirements are expressed in terms of metabolizable protein (MP) rather than individual AA. By feeding in terms of MP, animals are often overfed many AA to ensure high levels of milk production. Excess nutrients which are not efficiently used for milk production will be excreted and pollute the environment (Chandler, 1996). Approximately 30% of the nitrogen (N) consumed by dairy cows is converted into productive N, while the remaining N is excreted in urine and feces (Chase, 1994; Tamminga, 1992). Feeding animals low protein diets is one way to improve N efficiency (Kalscheur et al., 2006), but the practice is typically associated with decreased production which is economically disadvantageous to producers. Improving N efficiency through diet manipulation will increase N retention in dairy cows and reduce N pollution to the environment, but it is important to maintain production.

Delineating AA requirements for dairy cows is especially difficult compared to pigs and poultry because of the use of a variety of feed products and the remodeling of nutrients in the rumen by microorganisms (Lapierre et al., 2006). A better understanding of AA requirements could allow the feeding of less concentrated crude protein (CP) diets supplemented with AA needed specifically for production, as seen with swine and

poultry. Implementation of this type of feeding strategy in dairy cattle would allow for greater N utilization by tissues allowing supplemented amino acids to be used for milk production. Lysine (Lys) and methionine (Met) have been generally accepted to be the main limiting AA for milk protein synthesis on corn-based diets (NRC, 2001; Clark, 1975; Schwab et al., 1976). Extensive research has been performed utilizing rumen protected (RP) Lys and Met supplementation. Cows fed both RP Met and Lys experienced increased milk protein and milk fat yield (Robinson et al., 1998).

In addition to Met and Lys, dairy cow rations may also be limiting in leucine (Leu). Duodenal infusions of 40 g/d of Leu to corn-based diets improved both milk protein content and yield when diets were not limiting in Lys or Met (Rulquin and Pisulewski, 2006). Additionally, Leu has been shown to increase protein synthesis in skeletal muscle of growing pigs (Escobar et al., 2006). Amino acids, in particular Leu, increase phosphorylation of mammalian target of rapamycin (mTOR) which controls protein synthesis rates (Wang and Proud, 2006). Therefore, including RP Leu to dairy cow rations may result in an increase in milk protein synthesis through the activation of the mTOR pathway.

The objectives of this study were: 1) to determine whether the reduction in milk production associated with feeding a 15% CP (DM basis) diet could be alleviated by supplementation of RP Met, Lys, Leu, or combinations of the RP AA, 2) to determine the effects of feeding RP AA on cell signaling proteins associated with the mTOR pathway, and 3) to improve N efficiency of dairy cows.

MATERIALS AND METHODS

Animals

All experimental procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee. Fourteen multiparous and 10 primiparous Holstein cows and 24 multiparous Holstein x Jersey cross-bred cows were utilized in a Youden square design consisting of 8 treatments and 3 periods. Cows were randomly assigned to treatment, and treatment sequences were balanced for crossover effects. Experimental periods lasted 15 d and consisted of 10 d of diet adaptation and 5 d of sample collection. Cows were housed in a free stall barn with Calan Broadbent individual animal feeders (American Calan Inc., Northwood, NH) and free access to water. Four cows were removed from the study (1 low milk production, 1 health issues, 2 theft of feed from other doors).

Treatments

The eight dietary treatments were **1)** a standard diet containing 17% CP (+Con), **2)** a 15% CP diet (-Con), **3)** –Con diet plus RP Met (+M), **4)** –Con diet plus RP Lys (+K), **5)** –Con diet plus RP Leu (+L), **6)** –Con diet plus RP Met and Lys (+MK), **7)** –Con diet plus RP Met and Leu (+ML), and **8)** –Con diet plus RP Met, Lys, and Leu (+MKL). Diets were formulated to meet NRC (2001) recommendations for a lactating cow weighing 612 kg, consuming 22 kg of DM, and producing 35 kg/d of milk with 3.0% CP and 3.5% fat. Final diets contained 45.1% forage and 54.9% concentrate, and three fourths of the diets contained corn-based ingredients (Table 3-1). Three concentrate mixes were utilized in the experiment. A moderate fat grain with high protein was used

for the +Con, a moderate fat grain with low protein was used for the –Con, and a low fat grain with low protein was used in conjunction with the moderate fat, low protein mix to construct the diets supplemented with AA. The moderate fat –Con and the low fat –Con grains were mixed in varying proportions to construct the +AA diets so that dietary fat was equalized across treatments. Diets were mixed as a total mixed ration (TMR), provided in quantities to maintain between 5 and 10% refusals, and delivered to the Calan gates once daily by 1400 h. Sixteen grams of rumen protected DL-Met (Balchem Corporation, New Hampton, NY), 47g rumen protected L-Lys (AminoShure™-L; Balchem Corporation, New Hampton, NY), and 181g rumen protected L-Leu (Ajinomoto Co,. Inc., Japan; Balchem Corporation, New Hampton, NY) were supplied in diets containing those AA. These amounts were adequate to achieve predicted absorbed AA supplies that were equivalent to the +Con diet.

Sample Collection and Analysis

Feed offered and orts were recorded daily to determine dry matter intake. Dry matter content was determined weekly on the major components of the TMR (grain mixes, alfalfa haylage, and corn silage) and used to adjust the ration to maintain the targeted DM inclusion rates. Samples of the TMR, grains, and forage mix were collected on d 11, 13, and 15 of each period. Feed samples were stored at -20°C for later analysis. Individual feed samples were thawed and then dried at 55°C in a forced air oven for 48 h to determine dry matter concentrations. Dried samples were ground on a Wiley mill through a 1-mm screen (Arthur H. Thomas, Philadelphia, PA), and subsequently

composited by diet and period. Composited samples were submitted to Dairyland Laboratories, Inc. (Arcadia, WI) for nutrient analyses.

Milk weights were recorded at each milking and BW were recorded as cows exited the milking parlor following milking. Milk samples were taken on d 11, 12, and 13 of each period at the morning and afternoon milkings. Milk samples were analyzed following each sampling by the Virginia Tech DHIA lab (Blacksburg, VA) for true protein, fat, non-fat milk solids (SNF), lactose, somatic cell count (SCC), and milk urea nitrogen (MUN).

Blood samples were taken from the coccygeal vessels following the afternoon milking on d 13, 14, and 15. Blood samples were collected into 5 mL sodium heparin vacuutainer tubes, placed on ice, and centrifuged at 3,000 × g for 5 min at 4°C to collect plasma, which was stored at -20°C until analysis. Plasma samples were pooled by period for each cow and assessed for AA concentration using gas chromatography-mass spectrometry as described by El-Kadi et al. (2006).

Mammary biopsies were taken once from a rear quarter of each cow on the +Con, -Con, and +MK diets after the afternoon milking on either d 16 or 17 of the third period. Prior to biopsies, cows were restrained in a standing position in a headlock. Cows were administered an epidural block by injection of 2% lidocaine-HCl at the sacrococcygeal site. The entry point of the rear quarter was washed sequentially with alcohol, iodine, and alcohol. Approximately 100 mg of tissue was collected by biopsy from differing locations through a common entry point using a Magnum® Core Biopsy System (Bard, Covington, GA) equipped with a 12 gauge needle. A subsample of the tissue (~20 mg) was immediately homogenized in the presence of protease and phosphatase inhibitors,

spun, and the supernatant was boiled in Laemmli SDS sample buffer as described by Escobar et al. (2006). The remaining tissue was snap frozen in liquid N. Following collection and processing, samples were transported to the laboratory and stored at -80°C for later analysis.

Cell Signaling Analysis

An aliquot (20µL) of the biopsy supernate was separated by gel electrophoresis (Laemmli, 1970) and transferred to a PVDF membrane (Bio-Rad, Hercules, CA). Membranes were blocked for 1 h with StartingBlock™ (TBS) Blocking Buffer (Thermo Scientific, Rockford, IL), and probed with primary antibodies against the phosphorylated and total forms of the signaling proteins of interest. Proteins of interest included α-tubulin, ribosomal protein S6 (rpS6; Ser235/236), p70 S6 kinase (S6K1; Thr389), eukaryotic initiation factor 4E binding protein 1 (4EBP1; Thr37/46), eukaryotic initiation factor 2 (eIF2α; Ser51), and eukaryotic elongation factor 2 (eEF2; Thr56). Primary antibodies were obtained from Cell Signaling Technology (Danvers, MA) and consisted of monoclonal rabbit, polycolonal rabbit, or monoclonal mouse antibodies. For rpS6, S6K1, and eIF2α, primary antibodies for the total and phosphorylated forms were from different species allowing simultaneous probing for both protein forms using secondary antibodies (goat anti-mouse and goat anti-rabbit; LI-COR® Biosciences, Lincoln, NE) with different fluorescing compounds. Proteins were visualized using an Odyssey Infrared Imaging System (LI-COR® Biosciences, Lincoln, NE). α-Tubulin was used as a covariate for loading differences. For 4EBP1 and eEF2, the phosphorylated form of the protein was quantified first followed by stripping for 1 h using Gentle ReView™

Stripping Buffer (Amersco, Solon, OH), and re-probed to determine the total form. Bands were quantified using Odyssey Software (Ver. 3).

Statistics

Data were analyzed using SAS 9.2 (Cary, NC; 2001). The effects of dietary treatment were assessed using the Mixed models procedure and the statistical model:

$$Y_{ijk} = \mu + T_i + P_j + e_{ijk},$$

where Y_{ijk} = the dependent variable, μ = the grand mean of Y, T_i = the effect of treatment, P_j = the effect of period, and e_{ijk} = the residual error. All values were reported as least squares means and comparisons between treatments were achieved using a Tukey-Kramer test.

Statistical significance was declared at $P < 0.05$ and a statistical trend was declared at $P \leq 0.10$.

RESULTS & DISCUSSION

Ingredient Composition of Experimental Diets

Chemical composition of feed ingredients in the experimental diets is presented in Table 2, and chemical composition of the experimental diets is presented in Table 3. Acid detergent fiber (ADF), NDF, and CP were lower and NFC was higher for all treatments than the NRC formulated values. This resulted in NFC and starch concentrations that were greater than intended which likely contributed to milk fat content below normal concentrations (Table 4). However, Orskov (1986) indicated that high levels of corn in diets supply little benefit to the animal because of limited starch

digestion in the rumen. Therefore, corn-based diets with low rumen degradability are less likely to cause the negative side effects normally associated with high rumen degradability starch sources. Had it not been for the large amount of corn in the treatment diets, experimental animals in this study could have experienced acidosis or other metabolic conditions. Diet composition was also considerably affected by dietary treatment. When compared to +Con, all diets had lower CP, NDF, ADF, ash, ADICP, NDICP, lignin, and sugar while fat, TDN, NFC, and starch were higher.

Predicted AA supply

Duodenal AA flows for the experimental diets as predicted by the NRC model (2001) are presented in Table 5. Estimates for duodenal flow of Leu, Lys, and Met for the +Con treatment were 8.9, 6.1, and 1.8% of MP, respectively, and estimates for the –Con were 9.3, 6.2, and 2.0% of MP, respectively. Estimates for both Lys and Met appeared to be limiting for the +Con and –Con as values were less than the suggested values to obtain maximal milk protein synthesis for Lys (7.2%) and Met (2.5%) (NRC, 2001). Leu did not appear to be limiting in either treatment as the level of Leu required for optimal milk protein synthesis is 8.9% of MP (Rulquin and Pisulewski, 2006). Supplementation of RP Leu, Lys, and Met increased digestible AA flow from 214, 144, and 45 g/d, respectively, to 274, 154, and 51 g/d, respectively, which equates to 11.8, 6.6, and 2.2% of MP flow, respectively. Thus supplementation of Lys and Met increased duodenal supply to values that were closer to target values established by the NRC (2001).

DM and nutrient intake

Least squares means of dry matter (DMI), CP, N, ADF, and NDF intakes were all affected by treatment (Table 4). Intake of CP, N, ADF, and NDF were significantly reduced for all treatments when compared to the +Con. Cows on the +ML treatment experienced significantly reduced DMI compared to cows on the +Con diet. Met supplementation has been shown to cause depressed DMI in dairy cows (Robinson et al., 2000; Patton, 2010; Satter et al., 1975; Socha et al., 2005); however, because of the lack of effect on DMI in the other treatment diets supplemented with RP Met, it appears that RP Leu may have contributed to the decrease as it was in excess in the diets and may have caused an AA imbalance. Multiple experiments in rats and cockerels have shown that AA imbalances cause reductions in feed intake (Peng et al., 1972; Sanahuja and Harper, 1963b; Tobin and Boorman, 1979). Rogers et al. (1967) and Peng et al. (1973) observed a decrease in feed intake when male rats were offered a diet containing excess Leu. In contrast, reductions in DMI are not common with Leu supplementation in dairy cows whether infused (Korhonen et al., 2002; Rulquin and Pisulewski, 2006) or in rumen protected form (Křižova et al., 2008). According to Table 6, it appears that the decrease in DMI may have been caused by supplementation of Leu as plasma concentrations of Leu on the +ML diet were significantly elevated, likely causing an AA imbalance which resulted in changes in AA concentrations in the brain. An AA imbalance or deficiency is recognized in the prepyriform cortex of the brain and amygdala and results in an aversion to the imbalanced diet (Gietzen, 1993). In particular, Leu supply appears to control feed intake by acting on the central nervous system. Avruch et al. (2009) discussed that

increased Leu concentrations in the hypothalamus of rats stimulated hypothalamic mTOR signaling and caused anorexia.

Milk yield and composition

Cows on the –Con and +MKL treatments had significantly lower milk yield when compared to +Con (Table 4). All other treatments did not differ from the +Con or –Con. The decrease in milk associated with the –Con was predicted by the NRC model and is consistent with the literature (Gardner and Park, 1973; Kalscheur et al., 2006; Klusmeyer, 1990; Rius et al., 2010). The significant reduction in the +MKL treatment indicates that the addition of all 3 RP AA had no effect on improving milk yield of the –Con diet; however, previous observations in the literature often report no significant effect on milk yield associated with AA supplementation (Hopkins et al., 1994; Korhonen et al., 2002; Robinson et al., 1998, Socha et al., 2005). Appuhamy et al. (2011) observed no effect on milk yield when high producing dairy cows were infused with Met and Lys or Met, Lys, and branched-chain AA (BCAA).

Milk protein yields were also significantly reduced on the –Con and +MKL compared to the +Con. All other treatments were not significantly different from either the +Con or –Con indicating that the decrease in milk yield could be attributed to the decrease in milk protein yield. Socha et al. (2005) and Weekes et al. (2006) both indicated an increase in milk protein yield when lactating dairy cows were fed corn-based diets supplemented with Met or Lys.

The effect of Leu or BCAA supplementation on milk protein synthesis is inconsistent. No effect of supplementation has often been reported in the literature

(Hopkins et al., 1994; Korhonen et al., 2002; Weekes et al., 2006); however, Rulquin and Pisulewski (2006) and Appuhamy et al. (2011) both observed a significant increase in milk protein yield when cows fed corn-based diets were infused with Leu or BCAA, respectively. However, in both instances, animals were also supplemented with Met and Lys to ensure that those AA were not limiting for milk protein synthesis which may explain the significant increase.

Milk lactose production was also significantly reduced with the +MKL treatment compared to the +Con, but was not different from –Con, again indicating that a combination of all 3 RP AA cannot make up for the decrease in lactational performance associated with the feeding of a low protein diet. Robinson et al. (1995) observed a decrease in milk lactose composition when lactating dairy cows were fed corn-based diets supplemented with RP Met and Lys. In contrast, much of the literature indicates that supplementation of Met, Lys, or BCAA does not significantly impact lactose content (Appuhamy et al., 2011; Broderick et al., 2008; Korhonen et al., 2002; Robinson et al., 1998).

Milk fat content was not significantly affected by treatment compared to +Con, but it was significantly elevated for +MKL as compared to the +K treatment. This infers that Leu or Met supplementation was responsible for increases in milk fat composition although the effects were not evident when either AA was supplemented alone or when they were provided in combination in the absence of Lys. Robinson et al. (1995) observed a significant increase in milk fat composition in cows supplemented with RP Met and Lys. Socha et al. (2005) also saw a significant increase in milk fat composition when RP Met was supplemented to an 18.5% CP diet compared to a basal diet and RP

Met and Lys treatments. Appuhamy et al. (2011) and Korhonen et al. (2002) both saw no effect of BCAA or Leu supplementation on milk fat yield or composition, respectively. However, there was no significant increase in milk fat content or yield for either the +M or +L treatments in this study.

MUN and Nitrogen Efficiency

Milk urea nitrogen and nitrogen efficiency results are presented in Table 4. Gooden et al. (2001) indicated that MUN concentrations were useful in determining nitrogen efficiency of dairy cows. MUN was significantly reduced on all treatments compared to the +Con. In accordance with Broderick et al. (2008), these results suggest animals fed the –Con or any of the RP AA supplemented diets exhibited increased nitrogen efficiency. Nitrogen efficiency values were not different among treatments; however, there was a numerical increase for all of the RP AA supplemented diets. Nitrogen efficiency increased from 29.7% on the +Con treatment to an average of 32.7% for the treatments supplemented with RPAA. There tended to be a significant difference between the two control diets and those diets with supplemented AA ($P = 0.07$). This indicates that the addition of RPAA tended to alleviate the depression in milk protein yield. Kalscheur et al. (2006) observed a significant increase in N efficiency of dairy cows from 28.2% to 36.5% when dietary CP was decreased from 17.1% to 12.3%. Ipharraguerre and Clark (2005) also concluded that a reduction in CP from 18.7% to 14.8% increased N efficiency from 25% to 33%. The increases in nitrogen efficiency will reduce N losses to the environment and decrease the amount of N pollution to the atmosphere and waterways.

Plasma Concentrations of AA

Plasma concentrations of nonessential AA were not affected by treatment (Table 6). Plasma concentrations of isoleucine and valine were significantly reduced on the –Con diet indicating that isoleucine and valine may have been limiting in the –Con diet. Levels of plasma Leu on the +ML treatment were significantly elevated compared to the –Con and were numerically higher than the +Con. The increased concentration suggests that the +ML diet was not limited by Leu. Rulquin and Pisulewski (2006) also observed a rise in plasma Leu of 55% when cows were duodenally infused with 40 g/d of Leu. This rise in plasma Leu also substantiates the decrease in DMI associated with the +ML diet due to an excess in Leu rather than Met.

Cell Signaling Proteins

Effects of dietary treatments on signaling proteins from mammary biopsy samples are given in Table 7. Representative Western immunoblotting images for the phosphorylated (P) forms of signaling proteins are shown in Figure 3-1, and representative total (T) images are shown in Figure 3-2. The relative abundance of P-S6K1 and rpS6 were significantly increased with the +MK compared to the –Con, and the abundance of the T forms of these proteins were not affected by treatment. Phosphorylated 4EBP1 values were significantly reduced with the +MK compared to the +Con, but were not different from the -Con. These results suggest that +MK was unable to make up for the decreased phosphorylation of 4EBP1 associated with the –Con treatment. Also, T and P-eEF2 abundances were both significantly higher in the +MK

compared with the +Con and –Con. An increase in eEF2 phosphorylation reduces its ability to initiate elongation. The relative abundance of P-eIF2α was significantly increased compared to the +Con, but was not different from the –Con.

Phosphorylated to total relative abundance ratios for each of the signaling proteins was unaffected by treatment. Relative abundance ratios represent the phosphorylation status of the signaling proteins. 4EBP1 and S6K1 ratios of +MK were equivalent to the ratio of the +Con. This indicates that supplementation of Met and Lys to low protein diets can activate the mTOR pathway to the same extent as the high protein diet. Hara et al. (1998) observed that withdraw of AA from CHO-IR cells in-vitro rapidly deactivates S6K1 and 4EBP1. The phosphorylation status of rpS6 and eEF2 were relatively similar across treatments. Activation of downstream proteins was not as strong as the direct substrates of mTOR. Appuhamy et al. (2011) concluded that the addition of essential AA to MAC-T cells and mammary tissue slices from dairy cows significantly increased phosphorylation of mTOR, S6K1, 4EBP1, and decreased phosphorylation of eEF2. Independent of the mTOR pathway, the abundance ratio of eIF2α was decreased with the +MK treatment compared to the –Con, but not to the extent of the +Con. Avruch et al. (2009) discussed that activation of GCN2 increases as AA concentrations decline, and as a result decreases the rate of translation initiation. Increasing Met and Lys supply to low protein diets will increase translational initiation; however, the signal will not be as strong as that seen with a high protein diet.

The effect of signaling proteins on milk protein production is presented in Table 8. The variation of the phosphorylated proteins represented approximately one-

seventh of the total variation and approximately one-tenth of the total variation for the total forms of the signaling proteins.

CONCLUSIONS

Results from this study show that supplementation of individual RPAA or a combination of 2 RPAA were able to prevent the significant loss in milk production and protein yield associated with feeding low dietary protein, but production and protein yield were numerically lower than a high protein diet. The combination of all 3 RPAA, however, was not able to alleviate this reduction. All of the supplemented diets also showed a significant reduction in MUN and numerical increases in nitrogen efficiency suggesting that feeding a 15% CP (% DM basis) diet supplemented with RPAA would be able to help in eliminating nitrogen pollution to the environment and decrease feeding costs while maintaining a high level of production.

ACKNOWLEDGEMENTS

Funding for this study was provided by the Virginia Agricultural Council. The authors also acknowledge the support of Balchem Corporation for the donation of rumen-protected lysine and methionine, as well as protecting the leucine product.

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Table 1. Dietary formulas and nutrient compositions, and predicted nutrient requirements as determined from NRC (2001).

Item	+Con	-Con	-Con Low Fat
	---------- % of DM ----------		
Forage			
Corn silage	35.37	35.37	35.37
Mix grass + legume silage	9.73	9.73	9.73
Concentrate			
Corn grain, ground, dry	24.11	33.38	34.29
Corn Dry Distiller Grain+sol	8.18	8.18	8.40
Soybean meal, solvent (48% CP)	11.58	3.58	3.68
Protected soybean meal	1.77	0.00	0.00
Soybean hulls	6.41	4.96	5.09
Urea	0.00	0.88	0.90
Tallow	1.77	2.65	0.00
Calcium carbonate	0.49	0.50	0.51
Calcium phosphate (Di-)	0.00	0.18	0.18
Sodium bicarbonate	0.20	0.20	0.20
Salt	0.33	0.33	0.34
0.06% selenium premix	0.03	0.03	0.03
Trace mineral mix	0.01	0.01	0.01
Trace vitamin premix	0.01	0.01	0.01
Vitamin E (60000)	0.01	0.01	0.01
CP, % of DM	17.5	15.5	15.9
NDF, % of DM	30.3	29.0	29.7
ADF, % of DM	18.3	17.3	17.7
RDP, % of DM	10.3	10.3	10.6
RUP, % of DM	7.3	5.1	5.2
NFC, % of DM	44.0	46.2	47.4
Ether Extract, % of DM	5.4	6.5	3.9
NE_L Mcal/kg	1.55	1.55	1.55
MP supplied, kg/d	2.75	2.31	2.25
MP allowable milk, kg/d	43.7	33.9	32.8
NEl allowable milk, kg/d	39.9	39.7	36.4

Table 2. Observed chemical composition of major feed ingredients used in experimental diets.

Item	Corn silage	Haylage	Conc. A[1]	Conc. B[2]	Conc. C[3]	SEM
DM, %	31.49	54.03	89.98	90.36	89.80	1.45
	---------- % of DM ----------					
NDF	36.08	43.02	18.49	16.28	17.37	0.25
ADF	20.37	35.41	9.86	7.84	8.51	0.14
CP	7.90	18.78	21.26	19.71	18.75	0.08
Fat	3.52	2.92	6.91	4.95	9.19	0.08
NFC	49.68	25.39	49.16	55.13	51.32	0.23
Lignin	2.92	8.94	1.21	1.19	0.96	0.05
Starch	36.79	1.14	33.74	45.38	45.11	0.31
Sugar	2.13	4.22	6.56	3.67	3.06	0.09
Minerals	---------- % of DM ----------					
Calcium	0.19	1.18	0.64	0.65	0.51	0.01
Phosphorous	0.23	0.32	0.49	0.47	0.48	0.002
Magnesium	0.16	0.32	0.23	0.20	0.19	0.001
Potassium	1.16	2.98	1.10	0.81	0.76	0.01
Sulfur	0.10	0.24	0.27	0.22	0.20	0.002

[1]Conc. A = High Protein grain mix;
[2]Conc. B = Low Protein-Low Fat grain mix;
[3]Conc. C = Low Protein grain mix;

Table 3. Observed chemical composition of experimental diets (% DM basis).

	Experimental Diets[1]								
Item	+Con	-Con	+M	+K	+L	+MK	+ML	+MKL	SEM
DM, %[2]	52.49[a]	51.62[b]	52.61[a]	50.98[c]	52.89[ad]	51.58[b]	53.15[d]	51.08[c]	0.15
	---------- % of DM ----------								
CP	16.85[a]	15.03[b]	15.24[c]	15.15[d]	15.40[e]	15.05[b]	15.49[f]	15.44[ef]	0.02
NDF	27.67[a]	26.85[b]	21.84[c]	24.69[d]	25.91[e]	24.08[f]	23.20[g]	25.20[h]	0.05
ADF	17.63[a]	16.20[b]	13.96[c]	14.70[d]	16.19[b]	15.54[e]	15.08[f]	15.67[g]	0.03
Fat	5.36[a]	6.15[b]	7.10[c]	6.64[d]	6.71[d]	6.85[e]	6.94[f]	6.56[g]	0.02
Ash	5.15[a]	4.74[b]	4.45[c]	4.51[d]	4.70[e]	4.63[f]	4.57[g]	4.63[f]	0.006
TDN	77.62[a]	79.86[b]	83.73[c]	81.73[d]	80.97[e]	82.78[f]	83.31[g]	82.18[h]	0.05
ADICP	1.81[a]	1.69[b]	1.62[b]	2.43[c]	2.43[c]	1.66[b]	1.50[d]	0.85[e]	0.02
NDICP	3.11[a]	2.88[a]	5.34[b]	5.01[c]	4.47[d]	3.70[e]	4.29[d]	3.10[a]	0.05
NFC	45.81[a]	47.97[b]	52.14[c]	49.76[d]	48.04[b]	50.13[e]	50.57[f]	48.93[g]	0.05
Lignin	3.25[a]	2.75[b]	2.71[b]	2.73[b]	2.81[c]	2.16[d]	2.26[e]	2.21[e]	0.01
Starch	28.42[a]	33.67[b]	37.73[c]	36.16[d]	34.15[e]	36.08[d]	35.77[f]	35.45[g]	0.06
Sugar	3.92[a]	2.98[b]	3.17[c]	3.30[d]	2.67[e]	3.16[c]	2.86[f]	3.30[d]	0.02
Minerals	---------- % of DM ----------								
Calcium	0.586[ac]	0.582[a]	0.564[b]	0.513[d]	0.553[e]	0.589[ac]	0.593[c]	0.558[be]	0.002
Phosphorous	0.380[a]	0.377[b]	0.374[c]	0.370[d]	0.364[e]	0.366[f]	0.366[f]	0.362[e]	0.0005
Magnesium	0.227[a]	0.207[b]	0.193[c]	0.197[d]	0.194[c]	0.190[e]	0.187[f]	0.197[d]	0.0003
Potassium	1.33[a]	1.12[b]	1.06[c]	1.07[d]	1.16[e]	1.14[f]	1.11[g]	1.18[h]	0.01
Sulfur	0.213[a]	0.180[b]	0.194[c]	0.180[b]	0.177[d]	0.197[e]	0.193[c]	0.186[f]	0.0004

[1]+Con= high CP diet; -Con= low CP diet; +M= -Con diet + RP methionine; +K= -Con diet + RP lysine;
+L= -Con diet + RP leucine; +MK= -Con diet + RP methionine and lysine;
+ML= -Con diet + RP methionine and leucine; +MKL = -Con diet + RP methionine, lysine and leucine

[2] Different letters within a row indicate significantly different LSM ($P<0.05$)

Table 4. Least squares means of intakes, milk production and composition, body weight, and nitrogen efficiency for lactating dairy cows fed experimental diets.

	Experimental Diets[1]								
Variables	+Con	-Con	+M	+K	+L	+MK	+ML	+MKL	SEM
Intake, kg/d									
DM[2]	22.64^{a}	21.17^{ab}	21.99^{a}	21.86^{a}	20.87^{ab}	20.93^{ab}	19.65^{b}	21.05^{ab}	0.75
CP	3.77^{a}	3.15^{b}	3.31^{bc}	3.34^{bd}	3.50^{acd}	3.04^{b}	3.02^{b}	3.13^{b}	0.08
N	0.60^{a}	0.50^{b}	0.53^{bc}	0.53^{bd}	0.56^{acd}	0.49^{b}	0.48^{b}	0.50^{b}	0.01
NDF	6.18^{a}	5.64^{b}	4.75^{ce}	5.44^{bd}	5.88^{ab}	4.87^{c}	4.54^{c}	5.07^{de}	0.13
ADF	3.95^{a}	3.40^{be}	3.03^{cd}	3.25^{bd}	3.70^{ae}	3.14^{bd}	2.95^{cd}	3.18^{bc}	0.08
Milk Production									
Milk yield, kg/d	34.34^{a}	31.06^{b}	32.17^{ab}	31.35^{ab}	31.86^{ab}	32.13^{ab}	31.75^{ab}	29.95^{b}	1.48
True Protein, kg/d	1.09^{a}	0.98^{b}	1.01^{ab}	0.99^{ab}	1.01^{ab}	1.01^{ab}	1.00^{ab}	0.95^{b}	0.04
True Protein, %	3.21	3.23	3.18	3.28	3.20	3.23	3.21	3.28	0.07
Fat, kg/d	1.03	0.98	0.99	0.91	0.95	0.99	0.98	0.97	0.05
Fat, %	3.03^{ab}	3.25^{ab}	3.08^{ab}	2.90^{a}	2.99^{ab}	3.13^{ab}	3.12^{ab}	3.31^{b}	0.12
Lactose, kg/d	1.63^{a}	1.46^{ab}	1.51^{ab}	1.48^{ab}	1.50^{ab}	1.51^{ab}	1.50^{ab}	1.41^{b}	0.07
Lactose, %	4.74	4.68	4.69	4.70	4.70	4.71	4.72	4.70	0.04
SCC, 1000 cells/mL	491	606	361	143	150	200	158	365	230
BW, kg	579	581	578	575	582	578	574	583	8.80
MUN, mg/dL	11.88^{a}	9.78^{b}	10.08^{b}	10.20^{b}	10.02^{b}	9.60^{b}	10.58^{b}	10.56^{b}	0.33
N efficiency[3], %	29.7	29.2	31.3	33.8	33.4	32.2	33.1	32.3	2.20

[1]+Con= high CP diet; -Con= low CP diet; +M= -Con diet + RP methionine; +K= -Con diet + RP lysine;
+L= -Con diet + RP leucine; +MK= -Con diet + RP methionine and lysine;
+ML= -Con diet + RP methionine and leucine; +MKL = -Con diet + RP methionine, lysine and leucine

[2] Different letters within a row indicate significantly different LSM ($P<0.05$)

[3] N efficiency = N yield / N intake x 100

Table 5. Duodenal flows of digestible essential amino acids as predicted by NRC 2001/CPM Dairy 5.0 from experimental diets.

	Experimental Diets[1]																					
	+Con		-Con		+M			+K			+L			+MK			+ML			+MKL		
EAA	AA Flow (g/d)	%MP	AA Flow (g/d)	%MP	RPAA Supp.[2] (g/d)	AA Flow (g/d)	%MP	RPAA Supp.[3] (g/d)	AA Flow (g/d)	%MP	RPAA Supp.[4] (g/d)	AA Flow (g/d)	%MP	RPAA Supp. (g/d)	AA Flow (g/d)	%MP	RPAA Supp. (g/d)	AA Flow (g/d)	%MP	RPAA Supp. (g/d)	AA Flow (g/d)	%MP
Arg	129	4.7	105	4.5		105	4.5		105	4.5		105	4.5		105	4.5		105	4.5		105	4.5
His	61	2.2	52	2.3		52	2.3		52	2.3		52	2.3		52	2.3		52	2.3		52	2.3
Ile	130	4.7	112	4.8		112	4.8		112	4.8		112	4.8		112	4.8		112	4.8		112	4.8
Leu	244	8.9	214	9.3		214	9.3		214	9.3	181	274	11.8		214	9.3	181	274	11.8	181	274	11.8
Lys	168	6.1	144	6.2		144	6.2	47	154	6.6		144	6.2	47	154	6.6		144	6.2	47	154	6.6
Met	49	1.8	45	2.0	16	51	2.2		45	2.0		45	2.0	16	51	2.2	16	51	2.2	16	51	2.2
Phe	137	5.0	115	5.0		115	5.0		115	5.0		115	5.0		115	5.0		115	5.0		115	5.0
Thr	128	4.7	112	4.8		112	4.8		112	4.8		112	4.8		112	4.8		112	4.8		112	4.8
Val	146	5.3	126	5.4		126	5.4		126	5.4		126	5.4		126	5.4		126	5.4		126	5.4
Total	1192		1024			1031			1034			1085			1040			1220			1230	

[1]+Con= high CP diet; -Con= low CP diet; +M= -Con diet + RP methionine; +K= -Con diet + RP lysine; +L= -Con diet + RP leucine;
+MK= -Con diet + RP methionine and lysine; +ML= -Con diet + RP methionine and leucine; +MKL = -Con diet + RP methionine, lysine and leucine
[2]Supplementation based on 60% bioavailability and 67% Met in product
[3]Supplementation based on 60% bioavailability and 37% Lys in product
[4]Supplementation based on 60% bioavailability and 55% Leu in product

Table 6. Concentrations of free AA in plasma of cows fed experimental diets

	Experimental Diets[1]								
	+Con	-Con	+M	+K	+L	+MK	+ML	+MKL	SEM
Essential AA, *μM*									
Arg	140.4	123.4	131.0	142.0	137.3	135.0	132.8	136.3	8.78
His	51.8	48.0	50.1	52.6	50.8	48.9	50.4	48.6	1.58
Ile[2]	118.0[a]	94.2[b]	99.2[ab]	110.2[ab]	101.4[ab]	106.4[ab]	108.4[ab]	108.3[ab]	5.14
Leu	180.6[ab]	160.5[a]	180.2[ab]	188.9[ab]	181.0[ab]	176.5[ab]	193.6[b]	183.3[ab]	7.80
Lys	90.3	81.3	83.5	94.0	87.4	88.2	94.6	88.4	4.54
Met	25.0	26.4	27.4	28.3	27.2	27.9	28.1	27.1	1.05
Phe	49.6	44.3	46.4	48.8	46.5	48.5	49.6	48.4	1.43
Thr	117.3	92.9	91.2	102.6	102.2	99.2	102.2	95.0	9.22
Val	271.3[a]	222.7[b]	233.3[ab]	259.7[ab]	247.1[ab]	246.8[ab]	262.9[ab]	252.8[ab]	11.38
Nonessential AA, *μM*									
Ala	261.2	258.1	277.2	280.7	268.9	286.6	280.5	258.9	11.02
Asp	94.5	95.6	93.8	95.9	95.2	99.0	94.4	95.0	1.82
Glu	100.8	105.8	104.9	112.3	105.5	109.8	109.6	106.7	3.55
Gly	380.9	473.4	441.9	450.3	466.6	431.4	415.9	406.2	26.08
Pro	109.6	106.9	116.2	121.2	117.0	111.8	111.3	108.7	4.11
Ser	98.9	105.5	106.1	111.8	114.4	108.4	106.4	101.4	5.21
Tyr	52.9	45.8	49.3	51.4	48.4	48.3	53.3	46.9	2.23

[1]+Con= high CP diet; -Con= low CP diet; +M= -Con diet + RP methionine; +K= -Con diet + RP lysine;
+L= -Con diet + RP leucine; +MK= -Con diet + RP methionine and lysine;
+ML= -Con diet + RP methionine and leucine; +MKL = -Con diet + RP methionine, lysine and leucine

[2] Different letters within a row indicate significantly different LSM ($P<0.05$)

Table 7. Effects of experimental diets on the relative abundance of signaling proteins in mammary biopsies.

Signaling Protein[1]	Experimental Diets[2]			
	+Con	-Con	+MK	SEM
T-S6K1[3]	1.00	0.97	1.30	0.18
P-S6K1	1.00^{ab}	0.61^{a}	1.43^{b}	0.22
S6K1 ratio	1.00	0.57	1.00	0.23
T-rpS6	1.00	1.35	1.81	0.50
P-rpS6	1.00^{ab}	0.52^{a}	1.44^{b}	0.30
rpS6 ratio	1.00	1.05	0.93	0.39
T-4EBP1	1.00^{x}	0.38^{y}	0.47^{xy}	0.20
P-4EBP1	1.00^{x}	0.40^{xy}	0.36^{y}	0.23
4EBP1 ratio	1.00	1.12	1.00	0.11
T-eEF2	1.00^{a}	0.89^{a}	1.67^{b}	0.15
P-eEF2	1.00^{a}	0.89^{a}	1.74^{b}	0.14
eEF2 ratio	1.00	0.92	0.95	0.19
T-eIF2α	1.00	1.08	1.45	0.25
P-eIF2α	1.00^{a}	2.10^{ab}	2.31^{b}	0.39
eIF2α ratio	1.00	1.72	1.30	0.31

[1]Total (T) and Phospho (P) abundances of signaling proteins were expressed as ratios of abundance/abundance of tubulin. Ratios of signaling proteins were expressed as phospho abundance/total abundance. All values were standardized to the +Con treatment.

[2]+Con= high CP diet; -Con= low CP diet; +MK = -Con diet + RP methionine and lysine

[3] Different letters (a, b) within a row indicate significantly different LSM ($P<0.05$); (x, y) within a row indicate statistical trend ($P<0.10$)

Table 8. Effect of signaling proteins on milk protein production in dairy cows.

Signaling Protein	Estimate of coefficient	P-value	R^2
Phosphorylated			0.15
4EBP1	0.00035	0.06	
eEF2	-0.00042	0.04	
Total			0.11
rpS6	-0.09067	0.10	
4EBP1	0.00203	0.11	

Figure 1. Representative Western blot images of the phosphorylated forms (P) of analyzed signaling proteins from mammary biopsies.

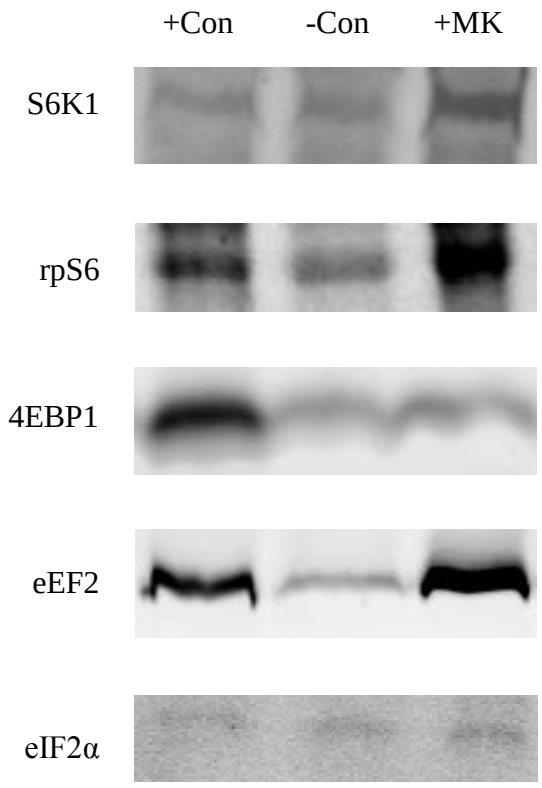
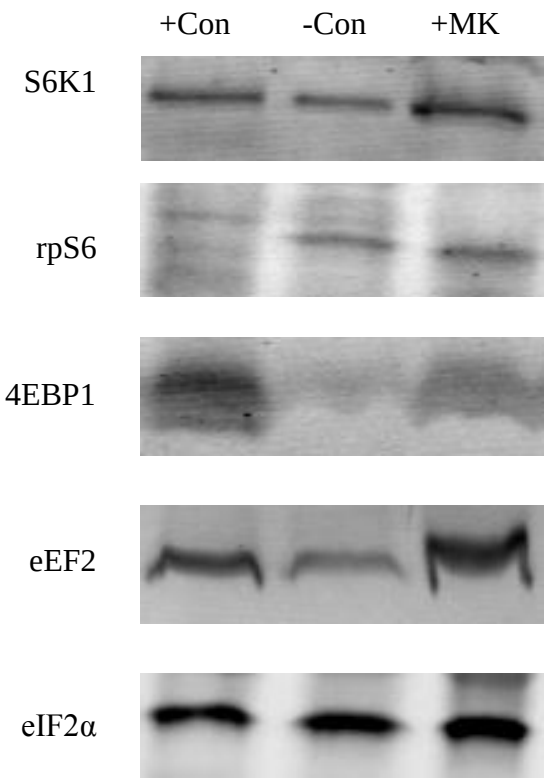


Figure 2. Representative Western blot images of the total forms (T) of analyzed signaling proteins from mammary biopsies.



CHAPTER 4

CONCLUSIONS AND IMPLICATIONS

Decreases in ammonia emissions from dairy cattle have been shown to occur when dietary protein levels are lowered; however, this results in a reduction in milk production at the expense of the dairy farmer. Supplementation of rumen protected amino acids, such as Met and Lys, have shown to improve lactational performance of dairy cows fed lower protein diets. The objective of this study was to improve the N efficiency of dairy cows through the feeding of a low protein diet while alleviating the reduction in milk yield with the supplementation of RP Met, Lys, Leu either individually or in combination. Milk production was significantly decreased on the –Con and +MKL treatments when compared to the +Con, and all other treatment diets did not significantly differ from either the –Con or +Con. These results suggest that the supplementation of RP AA except the combination of all 3 AA can prevent the loss in milk production and milk protein associated with a low protein diet. All of the diets supplemented with RP AA had significantly lower MUN and improved N efficiency from 29.7% to 32.7% on average indicating these animals were able to increase N utilization and reduce N excretions to the environment. Phosphorylation status of cellular signaling proteins was not significantly affected by treatment. The addition of Met plus Lys was able to numerically increase the phosphorylation status of 4EBP1 and S6K1 to that of the +Con implying that increases in protein synthesis rates could be achieved when RP Met and Lys are applied to a low protein diet.

APPENDIX

Anova Table for Statistical Model

Effect	Type	df[1]	ddfm[2]
Treatment	Fixed	7	38
Period	Fixed	2	38
Residual	Random	38	
Total		47	

[1] df = degrees of freedom
[2] ddfm = denominator degrees of freedom