Investigation of Methionine and Lysine Derivatives as a Source of Rumen-Protected Amino Acids for Lactating Dairy Cows

Mark Avila Fagundes
Utah State University

Follow this and additional works at: https://digitalcommons.usu.edu/etd

Part of the Dairy Science Commons

Recommended Citation

This Dissertation is brought to you for free and open access by the Graduate Studies at DigitalCommons@USU. It has been accepted for inclusion in All Graduate Theses and Dissertations by an authorized administrator of DigitalCommons@USU. For more information, please contact digitalcommons@usu.edu.
INVESTIGATION OF METHIONINE AND LYSINE DERIVATIVES AS A SOURCE OF RUMEN-PROTECTED AMINO ACIDS FOR LACTATING DAIRY COWS

by

Mark Avila Fagundes

A dissertation submitted in partial fulfillment of requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Animal, Dairy, and Veterinary Sciences

Approved:

______________________  ______________________
Jeffery O. Hall, D.V.M., Ph.D.  Jong-Su Eun, Ph.D.
Major Professor  Committee Member

______________________  ______________________
Kerry A. Rood, D.V.M., M.P.H., M.S.  Allen J. Young, Ph.D.
Committee Member  Committee Member

______________________  ______________________
Juan J. Villalba, Ph.D.  Dirk K. Vanderwall, D.V.M., Ph.D.
Committee Member  Department Head

______________________
D. Richard Cutler, Ph.D.
Interim Vice Provost of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

2021
ABSTRACT

Investigation of Methionine and Lysine Derivatives as a Source of Rumen-Protected Amino Acids for Lactating Dairy Cows

by

Mark Avila Fagundes, Doctor of Philosophy

Utah State University, 2021

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

The N-acetyl amino acids, such as N-acetyl-L-methionine (NALM), ƐN-acetyl-L-lysine, and Nα, ɛ-acetyl-L-Lysine are amino acid (AA) derivatives and developmental forms of rumen-protected (RP) AAs for dairy cows (CJ CheiJedang). The AA derivates are produced via N-acetylation of the L-Met α-amino, L-Lys α-amino or ɛ-amino group, respectively. Three independent studies were conducted to investigate the use of Met and Lys acetylation derivatives as sources of RPAAs for lactating dairy cows. The initial study investigated production responses and ruminal fermentation characteristics of lactating dairy cows when supplemented with NALM as a source of RPMet in metabolizable protein (MP) -deficient or MP-agdequate diets. Supplementation of NALM increased milk fat concentration and yield and 3.5% fat-corrected milk (FCM) yield and tended to increase energy-corrected milk (ECM) yield regardless of MP difference.
Dietary treatments had similar effects on ruminal fermentation characteristics and microbial protein yield. Results from the first study suggest that NALM exerted minor influence on ruminal metabolism, but increased milk fat concentration, resulting in increases in milk fat yield and feed efficiency.

The subsequent study investigated lactational performance of dairy cows when supplemented with Nε-acetyl-L-Lysine (εNALL), Nα, ε-acetyl-L-Lysine (diNALL), or AjiPro®-L (AP) as a source of RPLys to a control diet. Feeding the diNALL and AP diets decreased dry matter intake. Stage of lactation may have altered DMI in cows fed the diNALL and AP diets because energy requirements decrease during mid-lactation and control of feed intake is regulated by hepatic oxidation. Milk yield was not altered by RPLys supplementation. Findings from the study indicate that supplementing Nα, ε-acetyl-L-Lysine in mid to late lactation dairy cows decreases DMI and maintained milk yield leading to improved milk production efficiency (milk yield/DMI).

The final study evaluated the bioavailability (BA) and lactational performance of lactating dairy cows supplemented with NALM at two different supplementation rates. Overall, production parameters were not influenced by NALM supplementation. Bioavailability, as assessed by plasma appearance of Met was higher for the 30g NALM dose compared with 60g NALM and control, suggesting that optimal supplementation rate for NALM is 30 g/cow/d. Residue of NALM was not detected in plasma, milk, liver or muscle samples. Overall, findings suggest that NALM is deacetylated before reaching central circulation, increases plasma Met concentrations, therefore supplying lactating dairy cows with a BA source of RPMet.
PUBLIC ABSTRACT

Investigation of Methionine and Lysine Derivatives as a Source of Rumen-Protected Amino Acids for Lactating Dairy Cows

Mark Avila Fagundes

Cows have a protein requirement for growth, maintenance, and lactation. In order to meet those protein requirements, dairy farmers can supplement or feed cattle with specific amino acids, the building blocks that make-up protein. However, in order for the amino acid product to be effective it must avoid degradation in the rumen and be delivered in the small intestine for absorption. Lysine and methionine have traditionally been recognized as the most limiting amino acids for lactating dairy cows. Therefore, nutrition companies have focused on finding ways to encapsulate or protect lysine and methionine from rumen microbes. The N-acetyl-L-methionine and N-acetyl-L-lysine are amino acid derivatives and developmental forms of amino acids for dairy cows. Three separate nutrition studies were conducted to investigate the use of lysine and methionine acetylated derivatives as sources of amino acids for lactating dairy cows. The initial study investigated milk production responses of lactating dairy cows when supplemented with N-acetyl-L-methionine. Supplementation of N-acetyl-L-methionine increased milk fat
concentration and yield and 3.5% fat-corrected milk yield. Results from the first study suggest that N-acetyl-L-methionine did not impact rumen metabolism, but increased milk fat concentration by providing methyl donors to the mammary gland, resulting in increases in milk fat yield. The second study investigated lactational performance of dairy cows when supplemented with Ne-acetyl-L-lysine, Nα, e-acetyl-L-lysine, or AjiPro®-L as a source of rumen-protected lysine. Feeding the Nα, e-acetyl-L-lysine and AjiPro®-L diets decreased dry matter intake. Milk yield was not altered by rumen-protected lysine supplementation. Findings from the study suggest that supplementing Nα, e-acetyl-L-lysine to dairy cows decreases dry matter intake, but maintained milk yield leading to improved milk production efficiency. The final study evaluated the bioavailability and lactational performance of dairy cows supplemented with N-acetyl-L-methionine. Overall, milk production parameters were not affected by N-acetyl-L-methionine supplementation. Bioavailability was higher for diets with N-acetyl-L-methionine compared with the control diet. Residue of N-acetyl-L-methionine was not detected in plasma, milk, liver, or muscle samples. Overall, findings suggest that N-acetyl-L-methionine is deacetylated and absorbed as methionine into the bloodstream of cows, supplying lactating dairy cows with a bioavailable source of rumen-protected methionine.
ACKNOWLEDGMENTS

First, I would like to thank all the employees of the Caine Dairy Research Center, veterinary staff of the USU Clinical Veterinary Services and students in the ruminant nutrition lab for aiding in sample collections, maintaining animal health, and feeding of experimental animals. A special thanks is given to both the USU School of Veterinary Medicine and CJ Bio for financial support. Upmost gratitude is expressed to all committee members for accepting the task to direct me through graduate school at USU. I appreciate the time given by Dr. Rood, Young, and Villalba attending committee meetings and mentoring me in your areas of expertise. Dr. Hall, I am grateful for your willingness to take me on as your graduate student, partway through my program, which I know has not always been the most convenient with your busy schedule. To this day your mineral course is one of the best I have taken in my academic career and I have enjoyed our discussions of veterinary medicine. Dr. Eun, I am especially appreciative that you accepted me as your graduate student. Over the last few years, you have not only provided mentorship, but you have become a great friend. Although, you are over 5,000 miles away that doesn’t prevent you from having an active role in my graduate program. This willingness speaks to your passion, thoughtfulness, and leadership in advising students to help them succeed. Lastly, I would like to give a special thanks to my wife, friends, and family members who have supported me throughout this process.

Mark Avila Fagundes
# CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>PUBLIC ABSTRACT</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xiv</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>REVIEW OF LITERATURE</td>
<td>3</td>
</tr>
<tr>
<td>AA</td>
<td>3</td>
</tr>
<tr>
<td>AA Nutrition in Dairy Cows</td>
<td>3</td>
</tr>
<tr>
<td>Structural Configuration and Biological Functions of Met and Lys</td>
<td>7</td>
</tr>
<tr>
<td>RPMET and RPLYS</td>
<td>9</td>
</tr>
<tr>
<td>RPMet Supplements</td>
<td>9</td>
</tr>
<tr>
<td>RPLys Supplements</td>
<td>14</td>
</tr>
<tr>
<td>Effects on Intake</td>
<td>17</td>
</tr>
<tr>
<td>Effects on Milk Yield and Composition</td>
<td>20</td>
</tr>
<tr>
<td>Effects on Immunity</td>
<td>26</td>
</tr>
<tr>
<td>INTESTINAL ABSORPTION OF AA</td>
<td>30</td>
</tr>
<tr>
<td>MET METABOLISM</td>
<td>32</td>
</tr>
<tr>
<td>MAMMARY GLAND AA METABOLISM</td>
<td>34</td>
</tr>
<tr>
<td>Lys</td>
<td>34</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1.</td>
<td>Ingredient and chemical composition of the experimental diets without or with N-acetyl-L-Met (NALM) at 2 different levels of MP fed to lactating Holstein dairy cows (n = 4).</td>
</tr>
<tr>
<td>1-2.</td>
<td>Intake, milk production, feed efficiency, and net energy utilization of lactating Holstein dairy cows supplemented without or with N-acetyl-L-Met (NALM) at 2 different levels of MP.</td>
</tr>
<tr>
<td>1-3.</td>
<td>Plasma AA concentrations (µmol/L) of lactating Holstein dairy cows supplemented without or with N-acetyl-L-Met (NALM) at 2 different levels of MP.</td>
</tr>
<tr>
<td>1-4.</td>
<td>Ruminal fermentation characteristics of lactating Holstein dairy cows supplemented without or with N-acetyl-L-Met (NALM) at 2 different levels of MP.</td>
</tr>
<tr>
<td>2-1.</td>
<td>Ingredient and chemical composition of the experimental diets supplemented with rumen-protected lysine fed to lactating Holstein dairy cows (n = 40).</td>
</tr>
<tr>
<td>2-2.</td>
<td>Dry matter intake, productive performance, body weight (BW) change, and net energy utilization of mid to late lactation Holstein dairy cows supplemented with rumen-protected lysine.</td>
</tr>
<tr>
<td>2-3.</td>
<td>Plasma AA concentrations (µmol/L) of mid to late lactation Holstein dairy cows supplemented with rumen-protected lysine.</td>
</tr>
<tr>
<td>2-4.</td>
<td>Utilization of N by mid to late lactation Holstein dairy cows supplemented with rumen-protected lysine.</td>
</tr>
<tr>
<td>2-5.</td>
<td>Ruminal fermentation characteristics of mid to late lactation Holstein dairy cows supplemented with rumen-protected lysine.</td>
</tr>
<tr>
<td>3-1.</td>
<td>Ingredients and chemical composition of the basal diet.</td>
</tr>
<tr>
<td>3-2.</td>
<td>Energy and protein supply and AA balance of the basal diet supplemented with N-acetyl-L-Met (NALM) at 2 different doses.</td>
</tr>
</tbody>
</table>
3-3. Intake, milk production, and feed efficiency of lactating Holstein dairy cows supplemented with N-acetyl-L-Met (NALM) at 2 different doses........ 174

3-4. Plasma AA concentrations (µmol/L) of lactating Holstein dairy cows supplemented with N-acetyl-L-Met (NALM) at 2 different doses................. 176

3-5. Determination of bioavailability and kinetic parameters in lactating Holstein dairy cows supplemented with N-acetyl-L-Met (NALM) at 2 different doses................................................................. 178

3-6. Residue potential in lactating Holstein dairy cows supplemented with N-acetyl-L Met (NALM) at 2 different doses........................................ 180
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Biological functions of Met and hepatic Met metabolism. BHMT = Betaine-homocysteine methyltransferase; CBS = cystathionine-β-synthase; CSE = cystathionase; DMG = dimethylglycine; GSH = glutathione; GNMT = glycine N-methyltransferase; HCY = homocysteine; MAT1A = Met adenosyltransferase 1A; MTR = Met reductase; MT = methyltransferase; 5-MTHF = 5-methyltetrahydrofolate; SAH = S-adenosylhomocysteine; SAHH = SAH hydrolase; SAM = S-adenosyl-Met and THF = tetrahydrofolate. Adapted from Zhang and White, (2017)</td>
<td>64</td>
</tr>
<tr>
<td>1-1.</td>
<td>Proposed mechanism of N-acetyl-L-Met (NALM) on its potential effects on milk fat and milk production. SAM = S-adenosyl-Met; VLDL = very low-density lipoprotein (Nelson and Cox, 2013)</td>
<td>103</td>
</tr>
<tr>
<td>3-1.</td>
<td>Plasma Met concentration (µM) versus time plot for lactating Holstein dairy cows abomasally infused with N-acetyl-L-Met (NALM) at 2 different supplementation doses</td>
<td>181</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

AA = amino acid

ADF = acid detergent fiber

AP = AjiPro®-L

AUC = area under the curve

BA = bioavailability

BCAA = branched chain amino acids

BW = body weight

CL = control

CNCPS = cornell net carbohydrate and protein system

CP = crude protein

DIM = days in milk

diNALL = Nα, ε-acetyl-L-lysine

dLys = digestible lysine

DM = dry matter

dMet = digestible methionine

DMI = dry matter intake

EAA = essential amino acid

ECM = energy corrected milk

εNALL = εN-acetyl-L-lysine

FCM = fat corrected milk
His = histidine
HMB = DL-2-hydroxy-4-(methylthio)-butanoic acid
HMTBa = DL-2-hydroxy-4-(methylthio)-butanoic acid
HMBi = isopropyl ester of 2-hydroxy-4-(methylthio)-butanoic acid
Ile = isoleucine
KMB = 2-keto-4-(methylthio) butanoic acid
Leu = leucine
LFI = liver functionality index
LPS = lipopolysaccharide
Lys = lysine
MCP = microbial crude protein
ME = metabolizable energy
Met = methionine
MHA = methionine hydroxy analogue
MP = metabolizable protein
MPA = metabolizable protein adequate
MPD = metabolizable protein deficient
mTOR = mammalian target of rapamycin
MUN = milk urea nitrogen
N = nitrogen
NALL = N-acetyl-L-lysine
NALM = N-acetyl-L-methionine
NAM = N-acetyl-DL-methionine
NDF = neutral detergent fiber
NEAA = non-essential amino acids
NEFA = non-esterified fatty acid
NE_{L} = net energy for lactation
NFC = non fiber carbohydrates
NH_{3}-N = ammonia nitrogen
NRC = national research council
OM = organic matter
PD = purine derivatives
PMNL = polymorphonuclear leukocytes
RDP = rumen degradable protein
RP = rumen-protected
RPAA = rumen-protected amino acid
RPLys = rumen-protected lysine
RPMet = rumen-protected methionine
RUP = rumen undegradable protein
SAM = S-adenosyl-methionine
TMR = total mixed rations
Val = valine
VFA = volatile fatty acids
VLDL = very low-density lipoproteins
INTRODUCTION

Lysine (Lys) and methionine (Met) have traditionally been recognized as the most limiting amino acids (AA) for lactating dairy cows fed corn-based diets (Schwab et al., 1992; NRC, 2001). Protein nutrition of dairy cows in recent years has shifted from the use of dietary crude protein (CP) towards meeting the ammonia and AA needs for ruminal fermentation in order to maximize microbial protein (MCP) synthesis (Schwab and Broderick, 2017). Another nutritional shift seen recently is the balancing of individual AA in metabolizable protein (MP) as a method to deliver bioavailable AAs to the small intestine. However, in order for an AA product to reach the small intestine for absorption, it must be protected from rumen microbial degradation.

Protection of free AAs from ruminal degradation dates to the 1960s (Schwab and Broderick, 2017) and numerous rumen-protected AA (RPAA) products, have been developed using a variety of technologies. In terms of Met, encapsulation of a Met molecule with coating materials, such as carbohydrates or polymer allows RPAA products to avoid ruminal degradation (Schwab and Ordway, 2003). Altering the structural configuration of AAs to avoid microbial degradation is another method to escape rumen degradation. Methionine analogues utilize a hydroxyl group to protect them from ruminal degradation and are converted to useable Met by enzymatic reactions (Schwab and Ordway, 2003). Rumen-protected Lys (RPLys) products are often coated by a series of lipid or fatty acid calcium-based salts (Ji et al., 2016). Encapsulated rumen-protected Met (RPMet) and Met analogues can impact milk yield, but their effects tend to be more prominent in altering milk fat and protein composition (Patton, 2010; Zanton et al., 2014). Encapsulated RPLys supplementation has minimal impact on production parameters, unless RPLys is supplemented with a RPMet product (Robinson, 2010). Lactational responses to both
supplemental RPMet and RPLys in the literature are inconsistent and require further research. Inconsistent findings may be related to AA protection methods, varying supplementation rates, or metabolizable protein concentrations in the diets.

The N-acetyl-L-Met (NALM), εN-acetyl-L-Lys (εNALL), and Nα, ε-acetyl-L-Lys (diNALL) are AA derivatives and developmental forms of RPAAAs for lactating dairy cows. Based on prior research, it is thought that the acetyl group acts as a barrier to block the hydrolysis of the AA N-terminal (Wallace, 1992). However, to our knowledge there is limited production and bioavailability data available on the supplementation of acetylated AA derivatives to lactating dairy cows (Amaro et al., 2019; Digenis et al., 1974; Liang et al., 2019; Windschitl and Stern 1988).

Therefore, a series of lactation studies were performed to investigate the use of acetylated Met and Lys derivatives as a source of RPAAAs for lactating dairy cows. Three independent studies were conducted, and it was hypothesized that; for study 1) increased Met from NALM would increase milk protein synthesis and milk yield, but the degree of the response would be dependent upon the MP supply in the diets; for study 2) increased post-ruminal Lys from developmental NALL products would increase milk protein synthesis and milk yield compared with the control diet and for study 3) Met from abomasal infusion of NALM would increase plasma Met concentration, and the bioavailability of NALM would be greater for each dose compared with the control leading to a dose response, while lactational performance would be similar across all treatments due to the short study length.
REVIEW OF LITERATURE

Methionine and Lys are recognized as the 2 most limiting AAs for milk protein synthesis and milk production in lactating dairy cows. Individual or combined supplementation of RPAAs in ruminant diets is not a new concept, but in recent years has become more popular due to the beneficial effects of RPLys and RPMet on lactational performance (Robinson, 2010; Zanton et al., 2014). Supplementation of RPMet can improve milk fat (Overton et al., 1998; Samuelson et al., 2001) and protein yield (Rulquin and Delaby, 1997; Patton, 2010), while RPLys tends to exert its beneficial effects on milk protein yields (Robinson, 2010). As the dairy industry continues to find solutions to reduce environmental impact, nitrogen (N) efficiency can be improved by reducing protein concentrations in ruminant diets with strategic supplementation of RPAAs. For these reasons, feeding RPAAs has gained popularity in the dairy industry. This literature review will cover the common RPLys and RPMet products available for use in ruminants, as well as their effects on feed intake, milk and milk component production, immunity, metabolism, intestinal absorption of AA, mammary gland AA metabolism, and bioavailability. Main focus will be placed on encapsulated RPAAs, Met analogues, and AA derivatives, specifically NALM and N-acetyl-L-Lys (NALL).

AA

AA Nutrition in Dairy Cows
In recent decades, significant progress has been made in understanding the AA nutrition of dairy cows. Ruminal protein metabolism and the discovery of bacterial protein synthesis have been comprehensively researched (Wegner et al., 1940). Dairy cows can utilize nonprotein N (e.g., urea) and free ammonia from ruminal AA degradation for MCP synthesis. Ruminal MCP is the major form of MP supplied to (50-80%) and absorbed from the small intestine of dairy cows (Storm and Ørskov, 1983). Microbial protein is considered a high-quality protein for dairy cows because of its high digestibility and favorable AA composition (Schwab and Broderick, 2017). However, MCP synthesis requires large amounts of energy with the efficiency of energetic transformations for protein synthesis from AA being less than 75% (Wu, 2013). Therefore, much attention has been placed on the development of RPAAs to escape rumen degradation and supply high-quality, systemically available AAs in ruminant diets.

Historically, AA dietary formulations for dairy cows have been based on the single-limiting AA theory (Mitchell and Block, 1946), which was adapted from the law of the minimum proposed by von Lieberg (1863; Liu et al., 2017). Recently, the single-limiting AA theory has been refuted (Appuhamy et al., 2012; Liu et al., 2017). A revised conceptual framework with the NRC (2001) model that represents the individual and additive effects of all 10 essential AA (EAA) is believed to improve model accuracy compared with the single-limiting AA model in ruminants (Liu et al., 2017). Thus, when a ration is formulated based on the single-limiting AA theory, the additive effects of EAA for lactational AA requirements are ignored, and production responses may be altered. Balancing rations for EAA requirements of dairy cows and for multiple-limiting AAs may help to reduce overfeeding of protein, improve post-ruminal AA absorptive efficiency, reduce N excretion, and improve lactational performance (Liu et al., 2017).
Of the 20 primary AAs that occur in proteins, 10 are classified as being “essential” (or indispensable) (NRC, 2001). Essential AAs cannot be synthesized by animal tissues or if they can (arginine and histidine), not at rates sufficient to meet requirements at early stages of growth or for high levels of production (NRC, 2001). Lysine and Met have been identified as the 2 most-limiting EAAs for lactating dairy cows, with current evidence suggesting that histidine (His) may be limiting in grass silage-based diets (Vanhatalo et al., 1999; Giallongo et al., 2016). Since His concentrations are lower in ruminal MCP compared with milk protein and common feedstuffs (NRC, 2001), His may be a limiting EAA for lactating cows fed MP-deficient diets. This is of most significance when MCP provides or encompasses most of the MP supplied to ruminants (Patton et al., 2014; Giallongo et al., 2016).

Branched chain amino acids (BCAA) are used by the mammary gland for cellular, milk protein synthesis and comprise 50% of the EAA in milk proteins (Mackle et al., 1999). It has been suggested that BCAAs may be as limiting as Met and Lys in high producing dairy cows (Appuhamy et al., 2011), because of obligate use of BCAAs (isoleucine (Ile), leucine (Leu), and valine (Val)) by intestinal tissue as energy sources. A meta-analysis was performed (Lean et al., 2018) to predict the effects of metabolizable AAs on dairy cattle performance. The researchers found a positive association of Cornell Net Carbohydrate and Protein System estimated metabolizable Leu with milk protein and milk yield responses, suggesting that Leu should be given greater consideration as a potential co-limiting AA (Lean et al., 2018). However, the effects of BCAA supplementation on dairy cow production is varying and further evidence is needed to determine if BCAAs should be considered as limiting AAs in dairy diets for incorporation into a multiple-limiting AA model.
Methionine and Lys are the most researched AAs in regards to dairy cattle nutrition. They are considered the most limiting EAA for the synthesis of milk and milk protein in the high-producing dairy cow (Schwab et al., 1992; Rulquin et al., 1993). Methionine is one of 4 sulfur-containing AAs and along with cysteine is proteogenic (Brosnan and Bronson, 2006). In ruminants, Met is not only used for protein synthesis, but serves multiple biological roles (Figure 1). Milk protein synthesis by lactating dairy cattle can be altered if Met is in the shortest dietary supply of available dietary AAs requirements based on the single-limiting AA theory (Mitchell and Block, 1946). Methionine functions as a methyl donor and precursor to antioxidant and lipotropic compounds including carnitine, choline, creatine, cysteine, glutathione, metallothionein, taurine and S-adenosyl methionine (SAM) (Lehninger, 1977; Seymour, 2016; Figure 1).

Previously, research has focused on the role of Met as a co-limiting AA for milk protein and fat synthesis, and maintaining metabolic balance (Polan et al., 1991; Seymour, 2016). Recently, the metabolic functions of Met such as its role in supporting liver function, oxidative balance, and immunity have been assessed (Osorio et al., 2013). Methionine deficiency in non-ruminants produces fatty liver disease, a reduction in cellular antioxidants, reduced methyl donors, and generalized hepatic inflammation and fibrosis (Schugar and Crawford, 2012). Unlike the variability of Met’s biological functions, the predominant biological function of Lys is protein synthesis (milk protein, growth, pregnancy, and maintenance) (NRC, 2001). It is evident that Met and Lys are essential for milk and milk component production and serve many biological roles. However, the chemical form of AA fed to dairy cattle can alter the absorption, utilization efficiency, and subsequent production response to AA supplementation.
Structural Configuration and Biological Functions of Met and Lys

Methionine and all protein AAs, except glycine, can have both D- and L-isomers. L-AAs are physiological isomers in organisms and account for greater than 99.98% of the total AAs in the body (Wu, 2013). In dairy cows, the D-isomer is not bioavailable (Lapierre et al., 2012). The D-isomer must be converted to the L-isomer before it can be incorporated into mammalian proteins. Supplementation of the L-isomer of Met can be utilized directly for protein synthesis without conversion to any other isoforms. Since chemical synthesis of AAs results in a racemic mixture of the D- and L-enantiomers of Met, the bioavailability (BA) of the D-isomer of Met in dairy cows depends on the rate of transformation into the L-isomer (Lapierre et al., 2012).

For ruminants, BA data of D-Met are limited and somewhat unclear. In small ruminants, lower utilization of D-Met than L-Met has been described (Doyle, 1981), while the utilization of D-Met and L-Met appear to be equally effective for supporting wool growth (Doyle, 1981; Reis et al., 1989). In growing steers, D- and L-Met produced similar increases in N retention (Campbell et al., 1996), although this tended to be lower with DL-Met infusion compared with an equimolar dose of L-Met (Titgemeyer and Merchen, 1990). In both bovine studies, plasma total Met concentrations (D plus L) were greater when either D- or DL-Met was provided compared with similar supplementation of the L-enantiomer (Lapierre et al., 2012). Lobley et al., (2001) found that feeding RPMet with the 2 enantiomers (D- and L-) to dairy cows results in accumulation of plasma D-Met, which could be an indication of poorer systemic utilization of D-Met. From this research, it seems that both enantiomers increase D-Met plasma concentration, but is D-Met utilized by the dairy cow?
To date, the research by Lapierre et al., (2012) is the most thorough to determine the BA of D-Met in dairy cows. The researchers sought to quantify the conversion of D-Met to the L-isomer, proportion of D-Met plasma removal, and L-Met conversion, and determine if the conversion of D- to L-Met occurs in the mammary gland of dairy cows (Lapierre et al., 2012). The findings from this study indicate that the D-enantiomer of Met, constituting half of the Met in rumen-protected products, is transformed to the L-enantiomer in dairy cows. The whole body rate of disappearance of D-Met is, however, much slower than that of L-Met, with the half-life of D-Met being 6 to 7 times longer than that of L-Met (Lapierre et al., 2012). The non-utilization of D-Met by the mammary gland and a lower hepatic extraction of D-Met compared with L-Met would contribute to the slower rate of disappearance (Lapierre et al., 2012). The longer half-life of D-Met could be useful as it offers the opportunity to delay the clearance of the absorbed Met or act as a reservoir for L-Met synthesis (Lapierre et al., 2012).

The diversity of AAs is influenced by structural configuration (e.g., side chains), which determines a specific AAs chemical property, such as reactivity, solubility, stability, and taste (Wu, 2013). The R-group of Met contains a methyl group covalently bonded to a sulfur atom. Methionine is among the most hydrophobic of the AAs and its electrophilic qualities are attributed to the terminal methyl group and sulfur atom (Brosnan et al., 2007). This means that most of the Met residues in globular proteins are found in the interior hydrophobic core, in membrane-spanning protein domains, and Met is often found to interact with the lipid bilayer (Brosnan and Brosnan, 2006). Methionine must bind to initiator tRNA in order for the pre-initiation complex to form during the beginning of protein synthesis in eukaryotes (N-formyl-Met in bacteria) and it is believed that the hydrophobic nature of Met is essential for protein synthesis (Drabkin et al., 1998).
Lysine is a basic AA characterized by the presence of an amino group at the end of a 4-carbon aliphatic side chain and bears a positive charge at physiological pH (Tomé and Bos, 2007). This structure makes Lys a reactive component in different chemical reactions including carbonyl-amine interactions. As a consequence, a segment of Lys can be degraded, and another part can become unavailable in different food systems because of its involvement in different interactions with AA side chains or with other components including carbohydrates or lipids (Tomé and Bos, 2007). As a result, its ε-amino group side chain easily forms covalent bonds with other AAs and reducing sugars known as the Maillard reaction (Ordway and Aines, 2010). Even when Lys is incorporated into peptides or proteins, its ε-amino group remains reactive and can still form covalent bonds (Nursten, 2005). Although all AA, peptides, and proteins can participate in the Maillard reaction, Lys is the most common and prevalent AA that participates (Ordway and Aines, 2010).

**RPMet and RPLys**

**RPMet Supplements**

Protection of free AAs from ruminal degradation dates back to the 1960s when researchers began to understand the importance of balancing AA in order to meet dairy cows AA requirements (Schwab and Broderick, 2017). The first RPMet supplement was developed by Delmar Chemicals (LaSalle, QC, Canada). This product consisted of a core of 20% DL-Met, colloidal kaolin, and tristearin (Schwab and Broderick, 2017). The encapsulated DL-Met product when fed to lactating cows at 5, 15 and 45 g/d had no effect on milk production or milk
component yields (Broderick et al., 1970). However, plasma Met concentrations were increased at the higher supplementation rates (Broderick et al., 1970). Abomasal infusion of the encapsulated DL-Met product to lactating cows increased both milk protein yield and concentration. Plasma AA concentrations were altered when casein plus the encapsulated DL-Met were abomasally infused. These results suggest that the Delmar product was a successfully developed commercial RPMet product (Broderick et al., 1970); however, it was later found to have poor intestinal digestibility (Grass and Unangst, 1972). These findings highlighted the reality that developing encapsulated products with both high rumen protection and intestinal release was not an easy task.

Since this initial RPMet research, many approaches have been investigated to increase ruminal protection of Met products. In the 1980s and 1990s, Met analogues and derivatives were screened to find alternatives to encapsulation. Methionine analogues and derivatives provide the precursors for enzymatic conversion to Met. However, the screening was unsuccessful, and attention was again focused on improving encapsulation technologies (Schwab and Broderick, 2017). Today RPAA products are marketed as being either; physically encapsulated or protected AA, AA analogues, or AA derivatives. The most popular RPMet products currently fed to dairy cows are physically protected and Met analogues. A brief review will be given on the 2 popular physically protected RPMet products, and special attention will be given to Met analogues in this review as they are structurally and functionally similar to Met derivatives.

Physical protection approaches have been utilized other than lipid coating to protect Met with a surface coated carbohydrate (Mepron M85®, Evonik Inc., Kennesaw, GA). The Mepron® pellets consist of an 85% DL-Met core and are coated with several thin layers of ethylcellulose and stearic acid. Rumen enzymatic digestion of ethyl cellulose is negligible; therefore,
degradation of the products occurs through physical action and abrasion. This results in minimal rumen degradation and slow release of DL-Met in the small intestine (Schwab and Ordway, 2003). Smartamine® M (Adisseo Inc., Alpharetta, GA) is a lipid/pH-sensitive polymer coated product. The core contains 75% DL-Met and is covered with ethylcellulose, stearic acid, and a small droplet of 2-vinylpyridine-co-styrene. This copolymer arrangement allows the product to escape ruminal degradation and become solubilized at the low abomasal pH of 2, thus allowing free DL-Met to be released in the abomasum (Schwab and Ordway, 2003).

The Met analogues currently used in the dairy industry are Met hydroxy analogue (MHA or HMTBa) (ALIMET®, Novus International Inc., St. Louis, MO), calcium bis MHA (MFP®, Novus International Inc.), the isopropyl ester of 2-hydroxy-4-methylthiobutanoic acid (HMBi) (MetaSmart® Liquid, Adisseo Inc.) and butanoic acid, 2-hydroxy-4-(methylthio)-1-methylethyl ester (MetaSmart® Dry, Adisseo Inc.). The ALIMET® is a liquid 2-hydroxy-4-methylthiobutanoic acid (HMB) product with the substitution of the α-amino group of Met with a hydroxyl group. The manufacturers’ data sheet does not state a minimum L-Met or DL-Met hydroxy analogue percentage (Novus International Inc. 2015). The minimum Met activity as a percent of DM in beef and dairy is 100%. The minimum activity is 88% in all other species. The product has a chemical formula of C₅H₁₀O₃S. The ALIMET® is a light brown to brown liquid with a characteristic sulfur odor. The pH of the product is less than 1. Boiling point is observed at 121°C and the flash point using Pensky-Martens Closed Cup is greater than 116°C. The vapor pressure is 16 mm Hg at 25°C. The material density is heavier than water, but is completely soluble. Shelf life is a minimum of 2 years when stored as directed (Novus International Inc. 2015).
The MFP® is the dry-pelleted HMB calcium with substitution of the α-amino group of Met with a hydroxyl group (Novus International Inc. 2015). The product has a minimum DL-Met hydroxy analogue calcium content of 95%. The minimum Met activity is 84%. The product has a chemical formula of C₅H₁₀O₃S . ½ Ca. The MFP® is a light tan to tan granular powder with a cherry odor/flavor and a pH of 11 at 5% solution. The MFP® has a solubility of 74 g/kg at 25°C, and the material density is 800-850 kg/m³ (loose). The MFP® is not oxidative and has dust explosion class ST1 (weak explosion). The shelf life is a minimum of 5 years when stored as directed (Novus International Inc. 2015).

The MetaSmart® Liquid (Adisseo Inc. 2018) is a liquid HMBi (95%) product with the substitution of the α-amino group of Met with an isopropyl ester group. The product has a chemical formula of C₈H₁₆O₃S. It has a colorless to brown liquid appearance and a pH of 3.6. It has a melting/freezing point of -35.2°/-30.4°C. The product degrades prior to boiling. The flash point using Pensky-Martens Closed Cup is 115°C. The vapor pressure (Pa) is 0.6% at 25°C. The specific gravity is 1.07 at 20°C and has a solubility in water of 25.1 g/L at 30°C (Adisseo Inc. 2018).

The MetaSmart® Dry is a dry HMB product with the substitution of the α-amino group of Met with an ester group (Adisseo Inc. 2018). The product has a chemical formula of C₆H₁₂O₃S. It is free-flowing, cream to beige-colored powder, consisting of butanoic acid, 2-hydroxy-4-(methylthio)-1-methylethyl ester (50-75% by weight) adsorbed onto a silicon dioxide carrier (25-50% by weight). The product has a flash point of > 93°C. The specific gravity is 0.75 g/cm³ (loose), 0.80 g/cm³ (packed), and the product is insoluble in water (Adisseo Inc. 2018).

The first Met analogue extensively studied was MHA (DL-α-hydroxy-γ-mercaptobutyrate) or more appropriately called HMB (Schwab and Ordway, 2003). The HMB is more resistant to
ruminal degradation than free Met and its production is less expensive than that of DL-Met protected supplements such as Smartamine M (Wilson et al., 2008). Estimated bypass rates of HMB range from 99 (Jones et al., 1988) to 6% (Noftsger et al., 2005). Supplementation of HMB has a moderate effect on increasing plasma Met concentrations in dairy cows (Koenig et al., 1999; Koenig et al., 2002). In order for the body to utilize HMB as useable Met, HMB must be converted to L-Met in a two-step process. In the first step, HMB is enzymatically converted to 2-keto-4-(methylthio) butanoic acid (KMB). In the second step, transamination of KMB yields useable body Met (L-Met) (Dibner and Knight, 1984).

Several esters of HMB have been developed in an attempt to increase its rumen protection. Comparing microbial degradation of several HMB esters, HMBi was estimated to have 60% protection, but methyl, ethyl, butyl, and cyclohexyl esters were more readily hydrolyzed to HMB in the rumen (Robert et al., 2001). The ability of HMBi to avoid hydrolysis suggests that isopropyl esters present a steric hindrance of ruminal bacterial esterases. In order for HMBi to be converted to useable body Met, HMBi must be hydrolyzed to HMB and isopropanol. Then, HMB is converted to KMB and L-Met (Dibner and Knight, 1984).

Methionine derivatives are the least studied form of RPMet. A Met derivative is a free Met molecule with a chemical blocking group added to the α-amino group or a Met molecule in which the acyl group has been modified (Schwab and Ordway, 2003). Methionine derivatives add a chemical tail to the α-amino group, while Met analogues change the structural configuration of the α-amino group. There are 2 enzymes capable of converting AA derivatives to their corresponding AAs, to be utilized as body AAs. The enzyme aminoacylase I is present in multiple mammalian organs (small intestine, liver, and kidney) (Giardina et al., 1997). The bovine liver contains the enzyme, α-N-acylamino acid hydrolase which is capable of hydrolyzing
α-N-acylated AAs to yield acetate and the corresponding AAs (Met or Lys) (Gade and Brown, 1981).

Digenis et al. (1974) screened 31 nitrogenous compounds with possible Met activity. The compounds were screened in vitro with rumen microbes and compounds not supporting cellulose digestion were included in studies to confirm resistance to rumen microbial degradation. N-acetyl-DL-Met (NAM) and DL-homocysteine thiolactone hydrochloride were the most stable toward microbial degradation (Digenis et al., 1974).

Windschitl and Stern (1988) reported that Met flowing out of continuous cultures increased with NAM supplementation without a change in bacterial Met flow, which was attributed to greater escape (67%) of the NAM from bacterial degradation. Daabees et al., (1984) supplemented NALM to young pigs and revealed that plasma Met concentration increased, but no NALM was present in either portal or vena caval plasma at any sampling time, thus indicating NALM deacetylation to Met in the intestinal lumen or mucosal cells with release of free Met to the plasma. Additionally, Amos et al. (1974) abomasally infused sheep with NAM and found that plasma Met concentration increased 1 h post infusion compared with the control treatment. Based on the prior data, it seems that NAM can bypass the rumen and that both NAM and NALM can increase plasma Met concentrations. However, research for NALM has not reported the BA of NALM fed to lactating dairy cows. These in vitro and in vivo findings revealed the potential use of Met derivatives as a RPMet source to lactating dairy cows; however, it was not until the past few years that interest in these products has grown.

**RPLys Supplements**
Extensive research has focused on RPMet products. However, there are limited publications on RPLys products. This is primarily due to difficulties with development and production of rumen stable and efficacious RPLys products. As previously discussed, the Lys side chain is highly reactive and can react with encapsulation materials, such as polymer. Encapsulated Lys has a lack of resistance to TMR mixing and ruminant mastication (Swanepoel et al., 2010).

Product stability of RPLys in wet TMR has been overlooked in recent years. A study conducted by Ji et al. (2016) assessed whether mechanical mixing of TMR or TMR with low or high moisture content alters Lys release from 6 RPLys products. The authors concluded that mechanical feed mixing, dietary moisture content, and exposure of RPLys to other feed ingredients in the TMR over time may damage the protective coating and reduce efficacy of RPLys products (Ji et al., 2016). Although differences in Lys release among RPLys products were found, inferences about RPLys product BA is cautioned, as altered protection of Lys does not determine free Lys delivery to the plasma and systemic availability to the cow (Block and Jenkins, 1994; Ji et al., 2016).

Inconsistent post-ruminal polymer degradation and high production costs have also limited the development of RPLys products (Swanepoel et al., 2010). However, there has been success since the early 1990s in developing rumen stable AA matrixes and a number of RPLys products have become available for commercial use (Robinson, 2009b). Available commercial RPLys products use encapsulation, matrix technology, or a combination of both to protect a Lys core from ruminal degradation (Ji et al., 2016). These RPLys products are often coated by a series of lipid or fatty acid calcium-based salts (Ji et al., 2016). Wu and Papas (1997) found that RPLys coating protection efficacy in the rumen depends on coating composition, pellet surface smoothness, inner core pellet strength, pellet solubility, and pellet size (Wu and Papas, 1997).
Because the majority of RPLys products are lipid-coated, questions have arisen about the rumen environment (i.e., water content and acidity) and stability of lipid coated RPLys products. Acid can catalyze the hydrolysis of triglycerides to fatty acids and glycerol (Bender et al., 1961; Carey, 2003), and acid-catalyzed hydrolysis occurs in environments with high water concentrations, such as in the rumen (Bender et al., 1961; Carey, 2003). Thus, Reiners et al. (2018) sought to determine the effects of acidity and silage source on Lys retained by 2 RPLys products and to estimate ruminal degradation of Lys retained by RPLys after mechanical mixing with alfalfa or corn silage with different amounts of acidity (pH). The authors concluded that physical and chemical dietary characteristics can affect Lys provided to ruminants from RPLys products (Reiners et al., 2018). They state that Lys concentration was reduced from the two RPLys products when mixed in silage-based diets before cows consume the diet (Reiners et al., 2018). Because chemical and physical dietary factors along with on-farm feeding practices (mixing times) can impact RPLys efficacy, additional research is needed to find ways to minimize Lys loss. The most popular RPLys products currently fed to dairy cows are physically protected. A brief review will be given on 2 popular physically protected lipid RPLys products.

AjiPro-L® (Ajinomoto Co. Inc., Tokyo, Japan) is a small white oval bead composed of L-Lys monohydrochloride (50% minimum) core, vegetable oil (49%) and lecithin (1%). The hydrogenated vegetable oil matrix in AjiPro-L resists physical damage while its hydrophobic layer prevents the microbial breakdown of Lys (Reiners et al., 2018). USA Lysine® (Kemin Industries, Des Moines, IA) is a beige to light brown particle composed of a L-Lys monohydrochloride (65% minimum) core. It is manufactured by extrusion of L-Lys-HCl and lipid (vegetable oil) into small particles and subsequently the Lys lipid particles are coated with multiple layers of a lipid matrix (Reiners et al., 2018).
Lysine derivatives, like Met derivatives are the least studied form of RPLys. A Lys derivative is a free Lys molecule that has a chemical blocking group added to either the α-amino or ε-amino group. The focus of this dissertation is on AA derivatives, specifically NALM and NALL. For now, the review will focus on production responses of lactating dairy cows to encapsulated AA supplementation, Met analogue supplementation, and briefly cover NALM supplementation. To our knowledge, NALL supplementation to lactating dairy cows has not been described in the literature and therefore only encapsulated RPLys production responses will be discussed.

**Effects on Intake**

Dry matter intake (DMI) is the amount of feed an animal consumes that does not include moisture content. Dry matter intake is significant in nutrition, because it determines the amount of nutrients available to an animal for health, production, and other physiologic processes (NRC, 2001). Actual or estimated DMI is important for the formulation of diets to prevent underfeeding or overfeeding of nutrients and to promote efficient nutrient use (NRC, 2001). A variety of factors can influence DMI including forage quality, TMR nutrient balance, feeding delivery, feeding method, TMR palatability, ration dry matter (DM), environmental conditions, housing facilities, and managerial practices.

Amino acids, especially His have been shown to increase DMI in lactating dairy cows (Lee et al., 2012b; Giallongo et al., 2016). The exact mechanism of increased DMI is unknown, but it is speculated that His and other EAA regulate DMI via a direct effect in the anterior prepyriform cortex of the brain (Rudell et al., 2011). Methionine on the other hand if overfed can have a negative impact on DMI (Benevenga, 1974). A meta-analysis conducted by Zanton et al. (2014)
reported that DMI response was affected by supplemental Met source (Smartamine, Mepron, HMTBa, and DL-Met). Cows fed Smartamine had higher DMI, while cows fed Mepron had lower DMI compared to control cows. Supplementation of HMBTa and DL-Met did not affect DMI compared to control cows (Zanton et al., 2014).

Responses to supplementation of RPMet and HMBi on DMI in the literature have been inconsistent. Kung and Rode (1996), Leonardi et al. (2003), and Chen et al. (2011) found that RPMet supplementation did not improve DMI. Limited studies have shown an increase in DMI with RPMet supplementation (Broderick et al., 2009; Zanton et al., 2014), whereas Ordway et al. (2009) and Zanton et al. (2014) reported decreases in DMI. Supplementation of HMBi may increase DMI (Osorio et al., 2014) because of the potential stimulatory effect of HMBi on cellulolytic bacteria and the subsequent increase in ruminal passage rate and DMI (Lee et al., 2015).

Lee et al. (2012b) assessed supplementation of RPMet products in MP-deficient diets. The authors reported that MP-deficient diets below 15% of the NRC requirements decreased DMI. However, the authors observed that a treatment supplemented with a combination of 3 RPAA consisted of Lys (100 g/cow), Met (30 g/cow), and His (50 g/cow) increased DMI (Lee et al., 2012b). In contrast, Lee et al. (2015) found that supplementation with RPLys and RPMet in MP-deficient diets (−256 to −305 MP g/d) did not affect DMI. Recent research suggests that transition cows may benefit from balancing for limiting EAA (Zhou et al., 2016). Supplementing Lys adequate diets with RPMet resulted in increases in DMI (Osorio et al., 2013; Zhou et al., 2016). Improved DMI during the transition period leads to better postpartum performance in dairy cows supplemented with RPMet (Zhou et al., 2016).
Responses to supplementation of NALM on feed intake is scarce, but a recent study by Liang et al. (2019) supplemented mid-lactating cows with 0, 15, 30, or 60 g/d of NALM. Dry matter intake was not affected by NALM supplementation. This finding is in agreement with Amaro et al. (2019), in which dosage rates of 0, 15, 30, and 45 g/d of NALM were fed to 60 multiparous Holstein dairy cows in early lactation (27 ± 4.3 DIM) and the dose did not affect DMI. However, a study by Gresenti (2017) supplemented mid to late lactating cows with 0, 15, 30, or 60 g/d of NALM. Increasing NALM supplementation linearly increased DMI, which the research group speculated may potentially be related to a linear increase in rumen MCP yield (Gresenti, 2017). However, the potential stimulatory effects of NALM supplementation on rumen microbiota needs to be further investigated. Contrasting results from Met supplementation may be due to differences in length of feeding of RPMet, varying Met supplementation amounts or rates (based on % DM), Met products, stage of lactation, or a combination of factors (Osorio et al., 2013).

Research of RPLys and its effect on DMI is limited. However, RPLys supplementation does not appear to affect DMI of lactating dairy cows (Paz et al., 2014). These findings of RPLys and no effect of DMI are in agreement with Blauwiekel et al. (1997) and Robinson et al. (2011). Swanepoel et al. (2010) found that supplementation of RPLys did not influence DMI in mid-lactation cows (77 ± 3.2 DIM). However, the late lactation group (262 ± 6.70 DIM) had a tendency toward lower DMI with addition of RPLys (Swanepoel et al., 2010). Recently, Girma et al. (2019) investigated providing transition cows with an increased level of energy and supplementing RPLys to improve both pre- and post-partal DMI. Supplementation of the TMR with RPLys improved DMI in postpartum cows and RPLys supplementation of the close-up diet increased DMI of cows compared to control animals (Girma et al., 2019). The authors contribute the findings of increased DMI to the endogenous synthesis of carnitine from RPLys.
supplementation. The authors state that supplementing RPLys is an efficient strategy to increase the supply of Lys that serves as the carbon backbone for carnitine synthesis (Vaz and Wanders, 2002; Robinson et al., 2006). Carnitine is the methylated form of Lys (Shug et al., 1982) that buffers acetyl residues from lipid mobilization, reduces ketone body formation, and plasma β-hydroxybutyrate concentrations (Jacobs, 2002). Carnitine is important for oxidation of long-chain fatty acids, regulation of ketosis, support of the immune system, and enhancement of the antioxidant system (Citil et al., 2009; Pirestani et al., 2011). As previously mentioned, DMI is controlled by many factors, so further research needs to be completed to fully understand RPAA (RPLys and RPMet) supplementation and DMI regulation.

Effects on Milk Yield and Composition

The adoption of the multiple component pricing system by the Federal Milk Marketing Administration (2000) changed the method by which producers are compensated for their milk. The system adjusted the pricing structure to pay producers based on the amounts of milk fat, protein and solids in milk. Milk was previously priced based on total fluid milk volume. Economically, milk protein and fat are the most important milk components to dairy farmers. As the value of milk protein continues to increase, there is greater interest among dairy farmers to find alternative feeding strategies such as RPAA supplementation to increase milk protein yields (Vyas and Erdman, 2009). Not only are productive responses important (i.e., increasing milk fat and protein yield), reducing environmental pollution by improving dietary N conversion into milk protein is of concern in the dairy industry (Lapierre et al., 2005). As mentioned previously, Liu et al. (2017) suggest the incorporation of a multiple-limiting AA concept to improve milk
and milk protein yield model predictions, thus allowing modification of diets to increase post-absorptive N efficiency and reduced N excretion by dairy cows.

Encapsulated RPMet products, especially Smartamine and Mepron have been extensively studied in the literature (Patton., 2010). The evidence for one product being more effective than the other is inconclusive, as milk yield responses to supplemental RPMet products is variable. Patton (2010) reported increased milk production for both RPMet products (0.43 kg/d for Mepron and 0.14 kg/d for Smartamine) based on a meta-analysis from 36 studies with 17 studies evaluating Mepron and 18 evaluating Smartamine. Zanton et al. (2014) evaluated the lactational performance of dairy cows to supplemental Met sources. The authors assessed HMTBa provided as either a liquid or Ca salt form (17 papers with 34 control diets and 46 treatment comparisons), Mepron (18 papers with 35 control diets and 42 treatment comparisons), and Smartamine (20 papers with 30 control diets and 39 treatment comparisons). Overall, milk yield did not respond to any form of Met supplementation; however, it tended to increase for HMTBa and Mepron supplementation (Zanton et al., 2014).

Although there is variability in milk yield responses to supplemental Met products, strategic supplementation of RPAAs to low CP diets is becoming more popular. The observed production responses to supplementation of RPMet in cows fed low CP diets (i.e., MP-deficient diets) have been inconsistent and marginal (Arriola Apelo et al., 2014a; Sinclair et al., 2014). Milk yield was not improved when a low CP diet (15.0%) was fed alone or in combination with RPMet supplementation (Arriola Apelo et al., 2014b). However, further research with MP-deficient diets (ranging 5–10% below MP requirement) have sustained milk yield (Lee et al., 2015; Giallongo et al., 2016). Peri-partal RPMet supplementation has been shown to improve post-partal
performance. Osorio et al. (2013) and Zhou et al. (2016) found that cows supplemented with a RPMet source increased milk yield in the subsequent lactation.

A Met analogue, HMTBa, is converted to Met in the body and used as a supplemental Met source in dairy production. Results of published studies assessing the effects of supplementing Met sources, including HMTBa, on performance variables are inconsistent as well. A recent meta-analysis was performed to summarize the accumulated results of HMTBa supplementation on animal performance (Feng et al., 2018). Supplementation of HMTBa had no effect on milk yield, which was in agreement with the meta-analysis done by Zanton et al. (2014). Noftsger et al. (2005) reported no difference in milk production when cows were supplemented with HMBi at 0.13% DM. However, St-Pierre and Sylvester (2005) showed an increase in milk yield (2.9 kg/d) to cows supplemented with HMBi at 0.15% DM. Lee et al. (2015b) found that supplementing HMB (the unprotected form), numerically decreased milk production when % DM inclusion rate of dietary HMB increased. Alternatively, HMB supplementation increased milk production in a quadratic manner (Piepenbrink et al., 2004).

Supplementation of NALM to mid lactation dairy cows improved milk yield in a quadratic manner, with 30 g/d of NALM seeming to be the optimal supplementation rate for milk yield under the experimental conditions (Liang et al., 2019). Amaro et al. (2019) found that milk yield of early lactation Holstein dairy cows tended to be greater ($P = 0.07$) with intermediate concentrations (15 and 30 g/d) of NALM compared to control animals. The authors noted that NALM supplementation at 30 g/d resulted in the highest milk yield (Amaro et al., 2019), which is in agreement with Liang et al. (2019). Encapsulated RPMet, Met analogues, and Met derivatives can impact milk yield, but their effects tend to be more prominent in altering milk composition (milk fat and protein).
Supplementing RPLys to pre-calving cows during the close-up period (-21 to 0 d) at a rate of 40 g/cow/d had no effect on post-partum milk yield (Girma et al., 2019). These results agree with Swanepoel et al. (2010) who observed that supplementing dairy cows with RPLys (41 g/cow/d) did not affect milk yield in early lactation. Paz and Kononoff (2014) did not observe a difference in milk yield when RPLys was supplemented to diets with altering inclusion rates of low-fat distillers dry grains. Cows supplemented with RPLys (37 g/cow/d) after peak lactation showed no improvement in milk yield (Bernard et al., 2014). In contrast, Robinson et al. (2011) observed a 2 kg increase in milk yield when RPLys was supplemented to deliver 15 to 21 g of MP Lys.

Historically, HMB/HMBTα and HMBi supplementation tends to exert their effects on milk fat production in lactating dairy cows (Zanton et al., 2014). Supplementation of HMTBα had no effect on milk yield, fat percent, protein percent, or protein yield, but increased milk fat yield (Feng et al., 2018). Zanton et al. (2014) reported HMTBα supplementation increased milk fat by 5.38 g/g of MP Met from HMTBα, whereas Feng et al. (2018) reported HMTBα supplementation increased milk fat by 5 g/g of MP Met. A meta-analysis by Zanton et al. (2014) concluded HMTBα supplementation increases milk fat yield by 45 g/d and milk protein yield by 13 g/d. St-Pierre and Sylvester (2005) observed a 166 g/d increase of milk fat yield with HMBi supplementation.

The beneficial effects of HMTBα supplementation on milk fat have been investigated (Polan et al., 1970; Wang et al., 2010). Patton et al. (1970) reported that in vitro HMTBα supplementation can stimulate polar lipid formation and the authors suggest that the polar lipid class may increase with rumen protozoal growth. Increased ruminal biohydrogenation intermediates of the alternative pathway (e.g., trans-10 C18:1) exert antilipogenic effects in the mammary gland of ruminants which can lead to milk fat depression (Shingfield and Griinari,
Baldin et al. (2018) witnessed that HMTBa supplemented to either high-producing (44.1 ± 4.50 kg/d) or low-producing (31.4 ± 4.30 kg/d milk yield) lactating dairy cows at 0.1% of diet DM maintained lower concentrations of the biohydrogenation intermediates, trans-10 C18:1 in milk samples. This finding suggests that HMTBa may stabilize ruminal biohydrogenation and help prevent the shift to the alternative biohydrogenation pathway (Baldin et al., 2018). Therefore, it is speculated that supplemental HMTBa exert their effects on milk fat production by shifting ruminal biohydrogenation intermediates.

Physically encapsulated RPMet products and Met derivatives generally do not increase milk fat concentrations, because, unlike Met analogues, they lack a ruminal mode of action. However, encapsulated forms of RPMet have been shown to increase milk fat yield. Chen et al. (2011) found trends ($P = 0.08$) for increased milk fat concentration and yield when lactating dairy cows were supplemented with approximately 15 g of RPMet (Smartamine M) at 0.06% on a DM basis. Recently, Toledo et al. (2017) evaluated the effects of daily top-dressing a TMR with 21.2 g of RPMet from 30 ± 3.0 to 126 ± 3.0 DIM on productive performance in lactating dairy cows. The group found that top-dressing RPMet to multiparous cows increased milk fat percentages (3.45 vs. 3.14%), although no effects were seen in primiparous cows. Based on limited data in the literature, NALM supplementation to either mid or early lactating dairy cows has failed to exhibit beneficial effects on milk fat concentrations (Amaro et al., 2019; Liang et al., 2019).

Lactational performance of dairy cows in response to feeding RPLys has been researched over the past 30 yr, but sufficient data are lacking for beneficial effects of single AA supplementation of Lys in dairy rations. Supplementing combinations of Met and Lys is a much more common practice in the dairy industry. Swanepoel et al. (2010) reported reduced milk fat synthesis to feeding a RPLys product to high-producing dairy cows. Trináctý et al. (2009) fed a
RPLys tablet to high-producing Holstein dairy cows (33.5 kg/d); milk protein concentration increased, while milk fat concentration and fat yield declined. However, according to Robinson (2010) the results described above for Lys supplementation on milk fat are inconsistent with the literature of post-ruminal Lys supplementation. A review of literature by Robinson (2010) reported that RPLys supplementation generally resulted in minimal negative DMI and production responses, possibly due to an AA imbalance or poor AA delivery to the intestinal absorptive site. Productive benefits of RPLys supplementation is disappointing and overall lactational performance effects can only be judged as negligible (Robinson, 2010).

Less convincing evidence exists for the effect of L-Lys fed to lactating cows, but when L-Lys was deleted from a mixture of abomasal infused AAs, milk and milk protein production was reduced in the Lys-deficient infusion (Weeks et al., 2006). The exact relationship of L-Lys to AA deficiencies has yet to be established (Patton et al., 2014). However, increased L-Lys supply appears important in early lactation when microbial synthesis is reduced because of lower DMI, to help drive milk and milk protein production (Robinson et al., 2011).

Although RPLys has minimal effects on production performance, increases in milk protein yield are one of the most likely observed responses due to supplementation with RPMet (Zanton et al., 2014). As previously described, Zanton et al. (2014) evaluated the lactational performance in dairy cows receiving supplemental Met sources (HMTBa, Mepron, and Smartamine) or post-ruminal infusion of DL-Met. Milk protein yield was increased due to supplementation from all Met sources or from infusion of DL-Met and protein concentration was greater for all supplements or infusion of DL-Met, except for cows supplemented with HMTBa. Average milk protein yield responses ranged from 13 g/d for cows supplemented with HMTBa to 35 g/d for Mepron (Zanton et al., 2014). These results are in agreement with the meta-analysis by Patton.
(2010) who noted similar milk protein production responses when cows were supplemented with RPMet (Mepron and Smartamine). Osorio et al. (2013) observed an increase in protein yield post-partal for cows supplemented with Smartamine and MetaSmart (1.24 and 1.23 kg/d) versus control cows (1.11 kg/d) during the transition period (-21 to 30 DIM). Supplementing RPMet can also result in an increase in milk protein percentage (Osorio et al., 2013), but one study (Benefield et al., 2009) reported a negative effect on milk protein concentration. Milk protein concentration has been used to indicate the effectiveness of a Met source, leading to changes in milk protein synthesis (Schwab et al., 2001). This is based on the idea that if milk yield is unchanged, an increase in milk protein concentration will allow for an inference to be made on milk protein yield and therefore efficacy of Met supplementation (i.e., bioavailability) (Zanton et al., 2014). The take-home message is that if Met can escape ruminal degradation and be made available post-ruminally, whether by supplementing RPMet products or Met analogues, milk protein can be increased. Methionine, if not utilized by the cow for milk and milk component production, can also potentially play a pivotal role in both cow and calf immunity.

**Effects on Immunity**

It is well-known that Lys and Met are the most limiting EAA for milk production, but these EAAs also play a crucial role in immune function. Methionine and its derivate metabolites (e.g., glutathione, taurine, polyamines) are immunonutrients in nonruminants (humans), which aid and improve immune functionality and activity (Vailati-Riboni et al., 2017). Recently, Vailati-Riboni et al. (2017) supplemented dairy cows with RPMet at 0.08% DM (8 to 12 g/d DL-Met) during the transition period (-21 to 30 DIM) to determine periparturient immune response using an ex
vivo whole blood approach and the inflammatory response of the blood immune cells were tested with a LPS challenge (Vailati-Riboni et al., 2017). Overall, Met supplemented cows had a lower post-partum IL-1β (pg/µL) concentration compared to the control cows. The Met treated cows had a greater phagocytosis capacity of neutrophils and oxidative burst activity by both monocytes and neutrophils. The authors suggest that the above results indicate that Met supplementation improved immune functional activity, while dampening the innate immune response to a LPS challenge (Vailati-Riboni et al., 2017). They also mention that Met probably acts on the oxidative status of the animal or indirectly through an immunologically active metabolite (e.g., taurine). Taurine is the most plentiful AA in the cytosol of neutrophils that can trap hypochlorous acid to form taurine monochloramine (Vailati-Riboni et al., 2017). Greater oxidative burst in neutrophils could be attributed to higher taurine monochloramine concentrations, leading to peri-partal immune cell hyper-responsiveness. Therefore, Met may act as an immunomodulator of the immune response in transition cows when cows undergo a period of immunosuppression (Vailati-Riboni et al., 2017).

Zhou et al., (2017) investigated differences in liver functionality indexes (LFI) in peri-partal dairy cows fed RPMet or choline. The LFI is a composite index that is used to determine changes in plasma concentrations of blood biomarkers (albumin, cholesterol, and bilirubin) (Bertoni and Trevisi, et al., 2013). The researchers retrospectively assigned cows to low (LFI < 0) and high (LFI > 0) groups. Animals with a high LFI had greater liver functionality and are considered to be at a lower risk for developing health/metabolic disorders during the transition period. The liver regulates the metabolic activity aimed at mobilizing and directing nutrient flow to the mammary gland and the gastrointestinal tract (Seymour, 2016). Therefore, any reduction in liver function can impair lactational performance, reproductive efficiency, and immune status.
A larger number of Met-supplemented cows were in the high LFI group. The high LFI cows had an overall greater liver functionality and decreased state of oxidative stress, indicating that the high LFI cows had a better immunometabolic status during the transition period (Zhou et al., 2017).

In dairy cows, Met can also have a large impact on cow health through a variety of reasons other than immunomodulation or liver functionality (Figure 1). Methionine serves as a source of cysteine for glutathione synthesis via homocysteine produced in the Met cycle (Martinov et al., 2009). Liang et al. (2019) supplemented RPMet to transition cows (-28 d to 30 d) at a rate of 0.10% DM to determine if RPMet alters glutathione metabolism in peri-partal Holstein cows. Adipose tissue biopsies were collected on d −10, 10, and 30, relative to parturition, from 7 cows in each group to measure various proteins and genes related to glutathione metabolism. Overall, increased mRNA abundance of 4 key glutathione metabolism-related genes and upregulation of 4 protein abundance components related to glutathione metabolism were found (Liang et al., 2019). The authors state that RPMet supplementation may alter glutathione metabolism in adipose tissue (Liang et al., 2019). Altered glutathione metabolism in adipose tissue may contribute to reduced oxidative stress and inflammation during the transition period. However, further research is needed to assess the impacts of adipose tissue glutathione metabolism on immunity of dairy cattle.

Methionine plays a role in the liver as a lipotropic agent (Martinov et al., 2009). Methionine is needed for synthesis of SAM, which is a key methyl donor necessary for the packaging of very low-density lipoproteins (VLDL) for exportation out of the liver (Osorio et al., 2014). Triglycerides, if not exported from the liver, can accumulate causing fatty liver disease in ruminants. Sun et al. (2016) observed increased plasma non-esterified fatty acids (NEFA) and
plasma VLDL concentrations in post-partum transition cows supplemented with RPMet. This finding suggests that Met may affect hepatic lipid metabolism and exportation of VLDLs from the liver (Sun et al., 2016).

Transition cows benefit from Met supplementation through lipotropic agent activity, antioxidant substrate formation, immunomodulation and immune functionality. In addition, maternal nutritional status may also benefit neonatal innate immune function (Jacometo et al., 2016). Abdelmegeid et al. (2017) performed an in vitro study with neonatal Holstein calf polymorphonuclear leukocytes (PMNL). Supplementation with either Met, choline, or taurine altered the inflammatory response and affected oxidative stress, suggesting that Met and other nutrients may be needed for an adequate immune response in calves (Abdelmegeid et al., 2017). Jacometo et al. (2018) supplemented RPMet at 0.08% DM during the close-up period to multiparous Holstein cows. Calves born to RPMet supplemented cows showed positive changes in PMNL molecular pathways compared to control calves. Although the data are inconclusive, maternal RPMet supplementation may play a role in neonatal calf immunity, but further research is needed to identify the link between maternal AA supply and calf immunity.

Methionine’s role in ruminant immunity has been investigated more extensively in the literature compared with that of Lys. However, Lys deficiency can impair both antibody responses and cell-mediated immune responses in monogastric animals (Chen et al., 2003; Wu, 2013). Lysine deficiency limits animal immunity-related protein synthesis which includes antibodies and cytokines that are necessary for normal immune function (Liao et al., 2015). Han et al. (2018) reported that Lys deficiency in piglets impaired inflammatory responses in the liver, kidney, and spleen by mediating serum antibody concentrations (i.e., IgG and IgM) and inflammatory cytokines. Additionally, toll-like receptors, which are needed to active the
inflammatory response were altered by Lys restriction in piglets (Han et al., 2018). It is apparent that Lys is necessary for immune function in monogastrics (i.e., humans, swine, and fowl), but data are lacking for the potential beneficial effects of Lys in ruminant immunology.

**Intestinal Absorption of AA**

The absorption of AAs through the gastrointestinal tract of animals is an important biological function because growth, lactation, reproduction, and maintenance requirements depend on the adequate supply of AAs for protein synthesis. Amino acids that reach the small intestine can be incorporated into proteins, converted into other AA for biosynthetic processes, oxidized to CO₂, or transported through enterocytes into the mesenteric portal vein (Stoll and Burrin, 2006). Protein reaching the small intestine of the ruminant can come from a variety of sources: including rumen undegradable protein (RUP), MCP, and endogenous proteins. Ruminants use similar intestinal and pancreatic proteases as monogastric animals for protein breakdown. Absorption rates of AAs differ by anatomical location of the small intestine (i.e., duodenum, jejunum, and ileum), enterocyte maturity, and the site of AA absorption varies by species. In monogastrics, the jejunum is believed to hold the greatest capacity for AA absorption (Webb, 1990). In ruminants, the majority of AA absorption increases with distance from the pylorus and the majority of absorption occurs in the jejunum and ileum (William, 1969; Phillips et al., 1976).

Amino acid absorption site and rate can also be influenced by individual AA. Williams (1969) observed that sheep absorbed the majority of their AAs in the ileum; however, the order of absorption varied with anatomical location, suggesting that AA affinities for transport systems vary among intestinal sites. Phillips et al. (1976) reported that the largest amount of threonine
and valine absorption occurs in the ileum and Met absorption was similar between the jejunum and ileum in sheep. Webb (1990) reported that total Met and Lys uptake was higher by ileal brush border membrane vesicles than by jejunal brush border membrane vesicles. Webb (1990) also reported that in ileal and jejunal tissue, Met transporters had lower affinities and higher capacities for Met than the Lys transporter. This suggests that the mode of transportation and mechanism of AA transport through the enterocytes can differ according to the individual AA being absorbed.

Amino acid transport is affected by size, charge, and AA structural configuration (i.e., R-group side chains). Spencer et al. (1962) suggested that all transport carriers require the presence of an amino/imino group and a carboxyl group. Hydrophilic small weight neutral amino acids are taken up less readily than the larger weight hydrophobic amino acids and basic amino acid transport is intermediate (Webb, 1990). The various carriers are located on the basolateral membrane, brush border, or on both sides of the small intestine (Webb, 1990). To date many AA transport systems have been identified in the small intestine. However, the exact number is unknown, but the classification of amino acid transport systems is based on substrate preference and is determined by kinetic and inhibition analysis measurements (Christensen, 1984).

The major mechanisms for AA transport from the intestinal lumen to the enterocytes occur through simple diffusion, facilitated diffusion, or active transport (Wilson and Webb, 1990). Active transport requires energy and is believed to be Na⁺-dependent, while facilitated diffusion mechanisms are Na⁺-independent systems. The relative significance of each route is highly dependent on the concentration of substrate present. The Na⁺-dependent systems require metabolic energy to transport AAs against a concentration gradient. The Na⁺ gradient which has high extracellular Na⁺ and low intracellular Na⁺ concentrations is regulated by Na⁺/K⁺-ATPase
located in the basolateral membrane of the enterocyte (Webb, 1990). The Na\textsuperscript{+}/K\textsuperscript{+}-ATPase pump co-transport Na\textsuperscript{+} and an AA into the cell and K\textsuperscript{+} out of the cell (Webb and Matthews, 1994). The Na\textsuperscript{+}-independent systems do not require metabolic energy to move along a concentration gradient. Simple diffusion is the predominant form of transport, but when substrate concentrations are low, facilitated diffusion and active transport will account for more transport (Webb, 1990).

**Met Metabolism**

Metabolism of Met is divided into 3 pathways: transmethylation, remethylation, and transsulfuration. Transmethylation is a sequence of reactions present in Met metabolism and is universally present in cells. Transmethylation (i.e., Met metabolism) begins when Met is converted to SAM by Met adenosyltransferase (Brosnan and Brosnan, 2006). Numerous methylases comprise the user group for SAM that donates its methyl group to an acceptor to produce S-adenosylhomocysteine (Brosnan and Brosnan, 2006). The S-adenosylhomocysteine is hydrolyzed to adenosine and homocysteine in a reaction catalyzed by S-adenosylhomocysteinase (Martinov et al., 2009).

Remethylation occurs when homocysteine is methylated back to Met by Met synthase, which is ubiquitous in the body (Brosnan and Brosnan, 2006). Betaine homocysteine methyltransferase which is present in the liver and kidneys of some species can also methylate homocysteine to Met. Methionine synthase uses 5-methyl-tetrahydrofolate as a methyl donor, while betaine homocysteine methyltransferase uses betaine, from the diet or betaine produced during choline oxidation (Stead et al., 2006). Betaine homocysteine methyltransferase and Met synthase solely
affect remethylation. The combined steps of transmethylation and remethylation makeup what is called the Met cycle and these steps occur in most cells of the body (Brosnan and Brosnan, 2006). Methionine catabolism does not occur during transmethylation or remethylation.

In the transsulfuration pathway, homocysteine is converted to cysteine by cystathionine β-synthase and cystathionine γ-lyase. The transsulfuration pathway exists in most tissues except muscle (including heart) and endothelium (Martinov et al., 2009). The conversion of Met to cysteine is an irreversible process. Methionine metabolism plays a vital role in folate assimilation. Methionine synthase converts 5-methyl-tetrahydrofolate to tetrahydrofolate, making it free to support DNA synthesis and various biological functions (Brosnan and Brosnan, 2006). The transsulfuration pathway is responsible for the majority of Met catabolism in the body. The transsulfuration is also vital for the production of glutathione and taurine through its synthesis of cysteine. Glutathione synthesis is often limiting based on cellular cysteine concentrations and the transsulfuration pathway provides half of the cellular cysteine requirements (Mosharov et al., 2000).

It is important to remember that Met metabolism is dependent upon the availability of coenzymes and enzymes. For animals to properly metabolize Met, dietary requirements for vitamins and minerals must be met. Dietary intake of B vitamins, specifically B2, B9, and B12 are necessary for proper Met metabolism in the body. Methionine synthase uses the derivative of vitamin B9 (folic acid), using 5-methyl-tetrahydrofolate as a methyl donor. 5-methyl-tetrahydrofolate donates a methyl group to homocysteine to regenerate Met and the enzyme Met synthase catalyzes this step and utilizes vitamin B12 (cobalamin) as a cofactor (Locasale, 2013). Vitamin B2 (riboflavin) is a cofactor for 5-methyl-tetrahydrofolate synthesis. In monogastrics, Met metabolism can be altered if the diet is limiting in B-vitamins. Because rumen microbes can
synthesize B vitamins, ruminants are usually able to meet B vitamin requirements once rumen microbes reach the small intestine (NRC, 2001).

**Mammary Gland AA Metabolism**

Knowledge of mammary gland AA metabolism is just as important as body or hepatic AA metabolism, as the mammary gland is responsible for milk protein synthesis and can impact lactational performance. Historically, AA metabolism is classified by the balance between arteriovenous uptake and milk casein-AA secretion (Manjarin et al., 2014), while catabolism is represented by excessive AA uptake which is not available for milk protein synthesis. Complex intracellular interactions exist within mammary tissue. Therefore, AA metabolism in the mammary gland may give insight on mechanisms responsible for improved AA utilization in lactating dairy cows. Thus, if molecular mechanisms can be targeted by AA supplementation, then lactational responses may be regulated at the cellular level and add to the complexity of lactation.

**Lys**

Lysine has one of the highest fractional extraction rates by the mammary gland in bovids, ovids, and suids (Trottier et al., 1997; Mabjeesh et al., 2000; Manjarin et al., 2014). The primary metabolic fate of Lys in the mammary gland is for milk protein synthesis. Mabjeesh et al. (2000) described that the uptake of Lys in the mammary gland of dairy cows and goats is greater than milk Lys output. Hurley et al. (2000) reported that a small proportion of Lys is oxidized by
mammary tissue. Lapierre et al. (2009) assessed the responses to mammary and splanchnic metabolism to altered Lys supply in dairy cows. Six catheterized dairy cows received a control diet with low CP (15.0% DM) plus an abomasal infusion of AAs (560 g/d) without or with Lys (at 50.3 g/d) (Lapierre et al., 2009). The researchers found that limiting Lys did not alter the uptake of Lys by the mammary gland. The mammary uptake to milk output ratio of Lys decreased from 1.37 to 1.12 without Lys in the infusion, but Lys was still in excess of mammary supply (Lapierre et al., 2009). The excessive uptake of Lys points toward Lys serving roles, other than milk protein synthesis. The swine NRC (2012) estimates the rate of post-absorptive Lys utilization into milk protein to be 67%, leaving 33% of Lys to be utilized for other processes, including oxidative losses (Manjarin et al., 2014). According to Manjarin et al., (2014), Lys must be involved in other roles, such as utilization of Lys into nonessential AA synthesis and the utilization rate of 67% is a gross underestimation for the amount of Lys used for milk protein synthesis.

**Met**

Methionine extraction by the mammary gland, unlike Lys, closely matches the amounts required for milk protein synthesis (Bequette et al. 1998). Methionine, as previously stated has many biological functions (Figure 1) and Met can be utilized to synthesize phospholipids, carnitine, creatine, and polyamines (Wu, 2013a). To date there is a clear deficit in the literature on the understanding of Met’s role in mammary tissue except for its incorporation into milk proteins (Manjarin et al. 2014). According to Manjarin et al. (2014), none of the catabolic pathways of Met known to occur in other tissues and organs has been studied nor identified in
mammary tissue. The swine NRC (2012) used a coefficient of utilization efficiency for Met of 66%. The utilization efficiency for Met of 66% is most likely underestimated based on the idea that mammary extraction of Met matches the amount required for milk protein output in ovids and suids (Bequette et al. 1998; Manjarin et al., 2014). Guan et al. (2004) estimated the efficiency of Met incorporation into porcine milk protein as being closer to 95%. Lysine and Met catabolic pathways in mammary tissues need to be further investigated in cattle to get a better understanding of the function these EAAs play in the mammary gland and on lactation.

**Mammalian Target of Rapamycin (mTOR) Pathway**

The mammalian target of rapamycin (mTOR) pathway regulates cell cycle progression, cell growth, and protein synthesis through its ability to integrate nutrient and growth factor signals to increase protein translation (Manjarin et al., 2014). The effect of individual AA supplementation on translational protein synthesis has become an important research focus in recent years. The mTOR pathway in the mammary gland has been fully described, but the molecular mechanisms that AA influence for mTOR activation to increase protein synthesis in monogastrics and ruminants is unclear (Manjarin et al., 2014). It is believed that AA availability regulates the mTOR signaling pathway, by controlling translational initiation rates and by suppressing the inhibitory activity of eIF4E binding protein 1 (Arriola Apelo et al., 2014a). Amino acids may also increase elongation rates by stimulating eukaryotic elongation factor 2 and enhance ribosomal activity through activation of ribosomal protein S6 (Arriola Apelo et al., 2014a). Eukaryotic elongation factor 2 has been thought to be a limiting factor for milk protein synthesis (Christophersen et al., 2002).
Recently, Ma et al. (2019) sought to evaluate the effect of supplementing RPMet around parturition on the quantity and phosphorylation of mTOR related signaling proteins in post-partum bovine mammary tissue. The researchers found lower phosphorylation of mTOR complex 1 and were unable to find the exact mechanisms responsible for increased protein synthesis due to RPMet supplementation (Ma et al., 2019). However, they attributed the increased milk protein percentage post-partal to increased DMI which may increase AA flux into the mammary gland. Increased protein synthesis could also be attributed to increases in AKT serine/threonine kinase 1 phosphorylation status and a cascade of intracellular events, leading to upregulation of AA and glucose transporters (Ma et al., 2019). It is evident that the exact molecular mechanisms that AA influence for mTOR activation is unclear and further research needs to be conducted. The findings from such studies could lead to a better understanding of why production responses to supplementation of RPAAAs are inconsistent and how molecular mechanisms could potentially influence milk protein responses in lactating dairy cows.

**BA**

**Current Approaches and Limitations**

Bioavailability of AA in dairy cows has been assessed in vivo by a few techniques which include the production response approach (Schwab et al., 2001; Fleming et al., 2019), the area-under-the-curve method (Graulet et al., 2005; Fleming et al., 2019), the plasma free AA dose-response technique (Rulquin and Kowalczyk, 2003; Borucki Castro et al., 2008; Hanigan et al., 2009), and the revised plasma free Lys dose-response technique (Whitehouse et al., 2017). The
most recent technique described in the literature is the stable isotope-based approach which is used for the assessment of intestinally absorbed AAs from individual feed ingredients (Estes et al., 2018). Although each approach has been described in the literature, caution should be taken when reviewing each BA method, because each approach has limitations as is discussed below.

The major limitation for the production response approach is being able to ensure an AA deficiency, for all treatment animals on the trial, over the entire range of the treatment dosages (Whitehouse et al., 2017). The complexity of AA interactions in the mammary gland, specifically with the mTOR pathway, also limits the utility of the production response approach. If animals respond to AA supplementation with a positive linear response in milk protein concentration or yield, this could either be due the AA exerting its effect directly in the mammary gland, through activation of a molecular pathway, or nutrient interactions to stimulate milk protein synthesis. The AA deficiency is necessary in order to obtain a linear response to AA supplementation in either milk protein concentration (%) or milk protein yield (kg/d) (Whitehouse et al., 2017).

For the area-under-the-curve technique, animals receive a single pulse dose of the RPAA in high quantities not normally encountered by rumen microbes, which may limit or alter the ruminal RPAA degradation, thus affecting the amount of AA that reaches the small intestine for absorption (Whitehouse et al., 2017). The limitation for the plasma dose-response technique was that all trials that used this technique, prior to the revised Lys technique, failed to assign dietary treatments simultaneously within the same Latin square, thus ignoring potential animal variation (Whitehouse et al., 2017).

A stable isotope non-invasive method for measuring BA of AA has been studied in ruminants (Borucki Castro et al., 2008; Estes et al., 2017). There are 2 isotope dilution techniques that
either utilize a bolus or constant rate infusion of the AA isotope of interest (Estes, 2016). The isotopic measurements are used to estimate the total AA flux from the labeled AA infused dosage and subsequent isotopic enrichment of the samples selected to represent the compartment (i.e., pool) of interest (Estes, 2016). The bolus approach uses a single isotope dose delivered into the compartment of interest (Estes, 2016). The constant infusion approach uses a similar principal, but the infusion is continued until enrichment in the compartment of interest reaches a steady state to calculate a rate of total entry into the compartment (Estes, 2016).

Borucki Castro et al. (2008) assessed the efficacy of a whole-body Lys net flux method to determine BA of Lys in dairy cows fed 3 different forms of soybean meal. The whole-body net flux method (i.e., isotopic method) was tested against the plasma dose-response technique and duodenal flow/intestinal digestion method. Borucki Castro et al. (2008) found that the plasma response method recovered 97% of the omasally infused Lys and the isotope method recovered 100% of the omasally infused Lys, suggesting that there was no loss of the infused dose. The researchers concluded that the isotope method is sensitive and accurate in determining Lys availability in dairy cattle, which could be beneficial in eliminating the use of cannulated animals for BA studies (Borucki Castro et al., 2008). However, the plasma response curve yielded similar results to the isotopic method and further research is needed to assess the validity of an isotope approach.

**RPMet**

For RPMet products, the BA is generally calculated based on a positive or negative dietary control. Most often, Smartamine M is the positive control of reference value with a known BA of
80%. However, the equation to derive BA of AA will be dependent on the method used to measure BA (e.g., production response approach, the area-under-the-curve method, the plasma free AA dose-response technique, or the stable isotope-based approach). Before a reference BA value for Smartamine M was established, Berthiaume et al. (2000) stated that RPMet BA should be based on a product’s AA concentration, ruminal stability, and intestinal digestibility. Koenig and Rode (2001) defined BA of RPMet as a combination of effective ruminal degradability and intestinal disappearance with the calculation being equal to: \((100 – \text{effective ruminal degradability}) \times \text{intestinal digestibility}\). Koenig and Rode (2001) estimated the BA of Mepron to be a 23.6% based on the above calculation. Recently, Abdi-Benemar (2016) reported a BA of 61% for Mepron, which is closer to that of Smartamine M.

Bioavailability of Smartamine M was first estimated to be 80% from nylon bag studies and determination of its ruminal stability exceeding 90% at 24 h and intestinal release values close to 90% (Schwab and Ordway, 2003). Robert and Williams (1997), estimated that the BA of Smartamine M falls somewhere between 75 to 97%, with an average BA value of 88%. Graulet et al. (2005) determined the BA of Smartamine M to be 74%, while Schwab (1995) found the BA to be 80%.

The BA of HMBi is between 40 and 58%, based on blood kinetics (Robert et al., 2001; Robert et al., 2002) and milk true protein indices (Schwab et al., 2001). Bioavailability determined by blood Met concentration was 53 and 74% for HMBi and Smartamine M (Graulet et al., 2005). The measurements from Graulet et al. (2005) indicate BA of HMBi to be 71% of that from Smartamine M, whereas another study indicated that BA of HMB was 50% of that of Smartamine M (Robert et al., 2001).
RPLys

Limited data is available in the literature on the BA of RPLys products. However, the most studied RPLys product is AjiPro-L and multiple generations of AjiPro-L have been developed (i.e., generations 1-3). Recently, Whitehouse et al. (2017) estimated the BA of AjiPro-L 2G and AjiPro-L 3G using the plasma free Lys dose-response method. The slope for the AjiPro-L 3G was numerically greater than that for the AjiPro-L 2G, which resulted in a 12% improvement in the BA (Whitehouse et al., 2017). Tucker et al. (2015) compared the BA of 3 commercially available RPLys products using the plasma free Lys dose-response method (MetaboLys; H.J. Baker & Bro., Inc., Westport, CT and Lysine USA; Kemin Industries Inc., Des Moines, IA against the second generation AjiPro-L; Ajinomoto Heartland, Inc.). The BA of MetaboLys and Lysine USA were estimated as 18.3 and 38.2% of the second generation AjiPro-L BA, respectively (Tucker et al., 2015).

CONCLUSIONS

Methionine and Lys are recognized as the 2 most limiting AA for milk protein synthesis and milk production in dairy cows. With this in mind, considerable advancements have been made in both protein and AA nutrition of dairy cows. These include the development of RPAA sources with varying encapsulation coatings, the development of Met analogues, and the development of cost-effective Met and Lys derivatives. As prices of feed protein continue to rise and as environmental issues remain a concern, producers and nutritionists will need to evaluate the potential of feeding lower CP diets with strategic AA supplementation. Incorporation of a
multiple limiting AA concept in an animal requirement model will likely add to the complexity of feeding dairy cattle. Although not necessary for animal requirement models, molecular and cellular mechanisms (i.e., mTOR pathway) can identify sources of variation in response to AA supplementation of dairy cows and potential discrepancies in BA data. A sound approach to determine BA of AA in lactating dairy cattle may not be practical, but the prospect of an in vivo stable isotope-based method could help with precision feeding of cattle.

There is an overall insufficiency of information on Met and Lys derivatives in lactating cattle. The dissertation emphasis will be on Met (NALM) and Lys (NALL) acetylated derivatives supplemented to lactating dairy cows. Their impacts on lactational performance (NALM and NALL) and BA of NALM in lactation cows will be assessed. It was hypothesized that increased Met and Lys supply from the AA derivatives being fed (NALM and NALL) would improve lactational performance by increasing milk yield; however, the degree of response would depend upon the AA derivative supplemental dose in the dietary treatments. It was anticipated that NALM will act as an effective source for delivering BA Met equivalents to the small intestine to be absorbed, transported into the bloodstream, and utilized by the animal for various biological functions.
REFERENCES


Novus International Inc. 2015. GPS safety summary Alimet®.
https://novusint.my.salesforce.com/sfc/p/#E0000000Xz5h/a/44000000MEPp/rSkGZ5ojfXn_.clwu_cDyzaeuVaaR5qa.MpBsBEgym0.

Novus International Inc. 2015. GPS safety summary MFP®.
https://na98.salesforce.com/sfc/p/#E0000000Xz5h/a/E00000008n5E/DyWOSlZyMjbL6VcG9u8MseXdqsLWWW8rFMEilkRLEhk.


**Figure 1.** Biological functions of Met and hepatic Met metabolism. BHMT = Betaine-homocysteine methyltransferase; CBS = cystathionine-β-synthase; CSE = cystathionase; DMG = dimethylglycine; GSH = glutathione; GNMT = glycine N-methyltransferase; HCY = homocysteine; MAT1A = Met adenosyltransferase 1A; MTR = Met reductase; MT = methyltransferase; 5-MTHF = 5-methyltetrahydrofolate; SAH = S-adenosylhomocysteine; SAHH = SAH hydrolase; SAM = S-adenosyl-Met and THF = tetrahydrofolate. Adapted from Zhang and White, (2017).
Influence of supplementing a methionine derivative, N-acetyl-L-methionine in dairy diets on production and ruminal fermentation by lactating cows during early to mid-lactation


ABSTRACT

The present study investigated production responses and ruminal fermentation characteristics of lactating dairy cows when supplemented with N-acetyl-L-Met (NALM) as a source of rumen-protected Met in metabolizable protein (MP) -deficient (MPD) or MP-adequate diet (MPA). Eight lactating dairy cows (53 ± 10.4 d-in-milk, on average) were blocked by parity and days-in-milk, and the experiment was performed in a replicated 4 × 4 Latin square design. Within each square, cows were randomly assigned to a sequence of 4 diets during each of the four 21-d periods (14 d of treatment adaptation and 7 d of data collection and sampling). A 2 × 2 factorial arrangement was used; MPD or MPA was combined without or with NALM: MPD without NALM, MPD with NALM (MPD+NALM), MPA without NALM, and MPA with NALM (MPA+NALM). A NALM product was supplemented in the MPD+NALM and the MPA+NALM at 30 g/cow/d. Supplementation of NALM did not affect dry matter intake (DMI) and milk yield regardless of MP concentration. In addition, supplementing NALM resulted in a similar milk true protein concentration and yield. In contrast, NALM supplementation increased milk fat concentration and yield and 3.5% fat-corrected milk (FCM) yield and tended to increase energy-corrected milk (ECM) yield regardless of MP difference. Additionally, trends were
observed for increased 3.5% FCM yield/DMI and ECM yield/DMI, and the positive effects were greater under the MPA than the MPD diet, resulting in trends toward interactions between MP and NALM. Dietary treatments had similar effects on ruminal fermentation characteristics and microbial protein yield. Plasma concentration of Met increased under the MPD but not the MPA diet, leading to an MP × NALM interaction. Overall results in the current study suggest that NALM exerted minor influence on ruminal metabolism, but increased milk fat concentration, resulting in increases in milk fat yield and feed efficiency. Yet, potential effects of NALM on intermediary metabolism between the gastrointestinal tract, the liver, and the mammary gland need to be explored to understand utilization efficiency for production of dairy cows.

**Key Words:** N-acetyl-L-methionine, feed efficiency, lactational performance, milk fat concentration

**INTRODUCTION**

Dietary sources of N in lactation diets are inefficiently utilized by dairy cows, as they excrete substantially more N in manure over milk, which increases milk production costs and environmental N excretion (Noftsger et al., 2005). Optimizing the balance of total AA in MP has been used as the most promising means to maximize lactational performance with minimal dietary CP supply (Lee et al., 2012a). Studies have suggested that Lys and Met are the most limiting AA, especially in high producing dairy cows fed alfalfa and corn silage-based commercial diets typically used in the United States, and optimal concentrations of these limiting AA in MP are required to increase milk protein synthesis (Schwab et al., 1976). Thus, balancing for these AA is of practical significance as an effective approach to enhance milk protein
production. With an increasing economic value of milk protein, there has been strong interest by dairy producers and industries in nutritional modifications such as supplementation of rumen-protected AA that will increase milk protein yield (Arriola Apelo et al., 2014a).

Considerable research has been conducted over the past 3 decades to develop technologies to protect AA from ruminal degradation, and consequently ruminally protected Met (RPMet) products in different chemical forms have been developed and extensively used to alleviate Met deficiencies in lactating dairy cows (Overton et al., 1998; Patton, 2010). However, attempts to increase milk protein yield by increasing the supply of Met by addition of RPMet have given mixed results mainly due to complex inter-organ metabolisms in the rumen, the liver, and the mammary gland (Bequette et al., 2003) and substantial variations on DMI and CP concentration in feeds (Sinclair et al., 2014; Zanton et al., 2014).

The N-acetyl-L-Met (NALM) is a Met derivative produced via protection of L-Met α-amino group with an N-acetyl group. The cost of production for N-acetyl DL-Met by addition of an α-amino N-acetyl group to DL-Met is economically infeasible, but a cost-effective method for NALM production was recently developed (CJ CheilJedang, Seoul, South Korea) by addition of an α-amino N-acetyl group to L-Met. The NALM has been shown to be bioavailable and capable of replacing dietary Met in animals and humans according to Rottruck and Boggs (1975). In addition, NALM was readily incorporated into tissue proteins and supported growth of rats (Rottruck and Boggs, 1975). The NALM has also been used in foods as an approved food additive (Baxter et al., 2002). Windschitl and Stern (1988) reported that Met flowing out of continuous cultures increased with N-acetyl DL-Met administration without a change in bacterial Met flow, which was attributed to greater escape (67%) of the N-acetyl DL-Met from bacterial
degradation. In the literature, however, there is no research reported on the effects of supplementing NALM in lactation diet on production and ruminal fermentation in dairy cows.

We conducted the present study to investigate lactational performance and nutrient utilization by lactating dairy cows supplemented without or with NALM in MP-deficient (MPD) or MP-adequate diet (MPA), according to NRC (2001). We hypothesized that increased Met supply from NALM would increase milk protein synthesis and milk yield, but the degree of the response would depend upon the MP supply in the diets.

MATERIALS AND METHODS

The dairy cows used in the present study were cared for according to the Live Animal Use in Research Guidelines of the Institutional Animal Care and Use Committee at Utah State University. The study was conducted at the Caine Dairy Research Center (Wellsville, UT), Utah State University from May to July, 2016.

Cows, Experimental Design, and Diets

Eight multiparous lactating Holstein cows were used during this trial. Cows began the experiment averaging 53 ± 10.4 DIM, producing 39.2 ± 5.13 kg/d of milk yield, consuming 24.2 ± 3.51 kg/d of DMI and an average BW of 696 ± 66.2 and 721 ± 57.3 kg at the beginning and the end of the experiment, respectively. The experiment was performed in a replicated 4 × 4 Latin square design. Within each square, cows were randomly assigned to a sequence of 4 diets during each of the four 21-d periods (14 d of treatment adaptation and 7 d of data collection and
sampling). Although primary production parameters were measured in the present study, caution needs to interpret the data due to the short-term observation. Within each square, cows were randomly assigned to a sequence of 4 dietary treatments with a $2 \times 2$ factorial arrangement: MPD without NALM diet (MPD−NALM); MPD with NALM diet (MPD+NALM); MPA without NALM diet (MPA−NALM), and MPA with NALM (MPA+NALM; Table 1-1).

The developmental NALM product from CJ CheilJedang was used as a supplemental source of RPMet in this study. The NALM product was in powder form, and contained Met concentration of 78.0%, with 99.5% purity. The NALM product was added at a rate of 0.13% DM, and the rate was determined for cows to consume 30 g/cow/d of Met in order to supply 15.6 g of digestible Met to the small intestine according to manufacturer’s recommendation. This rate was also based on the requirements of digestible Met for cows fed the MPD diet. The recommendation of required digestible Lys and Met were assumed as 6.6 and 2.2% of the MP requirements, respectively (NRC, 2001). The NALM product was top-dressed onto corresponding experimental diets.

The chemical composition of alfalfa hay was 21.6, 38.9, and 27.9% DM for CP, NDF, and ADF, respectively, whereas corn silage contained 5.32, 41.5, and 23.9% DM for CP, NDF, and ADF, respectively. The diets had a forage-to-concentrate ratio of 61:39 (DM basis) on average and were typical of high-producing dairy cows in the Intermountain West (i.e., Utah, Idaho, Wyoming, Montana, and parts of Arizona and Nevada) with 44% of the forage coming from good-quality alfalfa hay. Diets were formulated based on the NRC (2001) recommendations to provide sufficient NE$_L$, MP, vitamins, and minerals with an estimated intake of 25.0 kg/d to produce 40 kg/d of milk with 3.5% fat and 3.0% true protein.
Cows were housed individually in tie stalls fitted with rubber mattresses covered with straw, allowing free access to water. Cows were individually fed twice daily for ad libitum intake at a level of 110% expected daily intake with 70% of allotted feed fed at 0600 h and 30% fed at 1500 h. Feed offered and refused was recorded daily, and samples were taken during the sampling week to determine DMI. Cows were milked twice daily at 0400 and 1600 h, and milk production was recorded throughout the entire experiment.

Energy Partitioning Calculations

Energy partitioning was determined during treatment periods using data of milk yield, milk composition, and BW of experimental animals. Cows were weighed for 2 consecutive d after the a.m. milking and before the a.m. feeding at the beginning (d 1 and 2) and end (d 20 and 21) of each period. These weights were used to calculate the mean BW of cows for each experimental period. Energy used for maintenance was calculated as BW\(^{0.75}\) × 0.08 (NRC, 2001). Energy of BW change was assumed to be 5.114 Mcal/kg of gain or 4.924 Mcal/kg of loss (NRC, 2001). Milk energy was calculated as (0.0929 × milk fat concentration) + (0.0563 × milk true protein concentration) + (0.0395 × milk lactose concentration) (NRC, 2001). Estimated NE\(_L\) value was calculated by total net energy utilization (maintenance, BW gain, and milk) divided by DMI (Neal et al., 2014).

Feed and Milk Samplings and Analyses
Samples of alfalfa hay and corn silage were taken weekly to determine DM, and diets were adjusted accordingly for change in DM concentration. Samples were composited by month, ground to pass a 1-mm screen (standard model 4; Arthur H. Thomas Co., Swedesboro, NJ), and stored for chemical analysis. Samples of TMR and orts were collected from individual cows on d 15 to d 21, composited, dried at 60°C for 48 h, and ground as previously described. The DM concentrations of samples were used to calculate intakes of DM and nutrients.

Analytical DM concentration of samples was determined by oven drying overnight at 105°C, and OM was determined by ashing at 550°C for 5 h (AOAC, 2000; method 942.05). Concentration of CP was determined using an automated N combustion analyzer (Elementar, Analysensysteme GmbH, Hanau, Germany; AOAC, 2000; method 968.06). Concentrations of NDF and ADF were sequentially determined using a fiber analyzer (200/220, ANKOM Technology, Macedon, NY) according to the methodology supplied by the company, which is based on the methods described by Van Soest et al. (1991). Sodium sulfite was used in the procedure for NDF determination and pre-treated with heat-stable amylase (Type XI-A from Bacillus subtilis; Sigma-Aldrich Corporation, St. Louis, MO). Ether extract was measured using a fat analyzer (XT20, ANKOM Technology; AOAC, 2000; method 2003.05). In addition, samples of TMR were analyzed for starch by Dairyland Laboratories, Inc. (Arcadia, WI) according to Knudsen (1997).

Milk was sampled for 2 consecutive days (d 15 and 16) during the a.m. and p.m. milkings each period. Individual milk samples were analyzed by the Rocky Mountain DHIA Laboratory (Providence, UT) for fat, true protein, lactose, and MUN. Milk composition was expressed on weighted milk yield of a.m. and p.m. samples. Milk fat and protein yields were calculated by
multiplying milk yield from the respective day by fat and true protein concentration of the milk from an individual cow.

**Blood and Urine Samplings and Analyses**

Blood samples were collected immediately before the morning feeding as well as at 1000 and 1400 h into 10-mL plasma vacuum tubes from the coccygeal artery or vein on d 19 and 20 in each period. Blood samples were centrifuged at 2,300 × g for 20 min, and plasma was collected and stored at –40°C for subsequent AA analysis. The plasma samples were prepared for AA analysis using the EZ:faast GC-FID Free (Physiological) Amino Acid Analysis Kit (Phenomenex Inc., Torrance, CA). Concentrations of plasma free AA were determined in accordance with the user manual provided with the kit. Extraction of free AA from the plasma consisted of a combination of solid-phase extraction, derivatization, and liquid/liquid extraction. The organic phase containing the AA in question were analyzed using a GC (model 5890, Hewlett-Packard Lab) with a capillary column (30 m × 0.32-mm i.d., 1-μm phase thickness, Zebron ZB-FAAP; Phenomenex Inc., Torrance, CA) and flame-ionization detection.

On d 15 to 17, spot urine samples were collected from each cow at 0600 and 1800 h for a total of 6 samples per cow (Holt et al., 2013). Using 4 M HCl urine samples were acidified to pH < 4.0 and composited by cow per period. Samples were frozen and stored at –40°C. At a later date, the samples were thawed in preparation for analysis and diluted with 39 parts diluent to 1 part urine. The diluent utilized consisted of 0.202% sodium 1-heptanesulfonate and 0.086% ammonium dihydrogen phosphate (NH₄H₂PO₄). The solution was brought to a pH of 2.1 using 4 M HCl. Utilization of the ratio of the urinary purine derivatives (PD) to creatinine is an accepted
way to estimate the microbial protein (MCP) flow to the duodenum (Shingfield and Offer, 1998). The PD and creatinine were analyzed using an HPLC instrument (Waters Corp., Milford, MA) according to the procedures set forth by Shingfield and Offer (1999). In order to estimate urine volume, creatinine was used as a marker (Valadares et al., 1999), and an average creatinine output of 28 mg/kg of BW as estimated by Whittet (2004) was assumed. Similar creatinine outputs have been reported (25 to 30 mg/kg of BW daily) (McCarthy et al., 1983; Jones et al., 1990). In order to estimate the relative differences in MCP production, the ratio of urinary PD (allantoin and uric acid) to creatinine was used (Shingfield and Offer, 1998), and the supply of MCP was estimated based on estimates of urinary excretion of PD according to the method of Chen et al. (1990) and Janicek et al. (2008).

Ruminal Fermentation Characteristics

Ruminal fluid samples were obtained using a Geishauser probe at 0, 3, and 6 h after the morning feeding on d 18 and 19 in each period. The fluid was collected with a solid, tube-like probe with rows of small holes on the end (Geishauser, 1993). The first 100 mL of ruminal fluid was discharged to avoid contamination from saliva, and then 10 mL was collected for analysis. The pH of the ruminal fluid was measured within 5 min of collecting the samples using a portable pH meter (Oakton pH 6; Oakton Instruments, Vernon Hills, IL). Five milliliters of the ruminal fluid were mixed with 1 mL of 1% sulfuric acid and stored frozen (−40°C) for ammonia-N (NH₃-N) analysis. Concentration of NH₃-N in the ruminal contents was determined as described by Rhine et al. (1998), using a plate reader (MRXe; Dynex Technologies Inc., Chantilly, VA). Another 5 mL of the ruminal fluid was collected and mixed with 1 mL of 25%
metaphosphoric acid, and then stored at −40°C for VFA content determination. Ruminal VFA were separated and quantified using a GLC (model 6890 series II; Hewlett-Packard Co., Avondale, PA) with a capillary column (30 m × 0.32-mm i.d., 1-μm phase thickness, Zebron ZB-FAAP; Phenomenex Inc., Torrance, CA) and flame-ionization detection. The oven temperature was held at 170°C for 4 min, increased to 185°C at a rate of 5°C/min, then increased by 3°C/min to 220°C, and held at this temperature for 1 min. The injector and the detector temperatures were 225 and 250°C, respectively, and the carrier gas was helium (Eun and Beauchemin, 2007).

**Statistical Analysis**

All data were analyzed as a 4 × 4 replicated Latin square with a factorial arrangement of treatments using the Proc Mixed procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC). Data for intake, milk production, and energy partitioning were analyzed with a model that accounted for effects of square, MP difference (MPD vs. MPA), supplementation of NALM (−NALM vs. +NALM), day nested within period, and the interaction between MP, NALM, and day as fixed effects and cow within square and period within square as random effects with day included as a repeated measure. Data for ruminal pH, VFA, and NH₃-N were analyzed using the model described above except that time after feeding was included as the repeated option. For each variable analyzed, 3 covariance structures (compound symmetry, autoregressive order 1, and unstructured covariance) were evaluated. The covariance structure that resulted in the lowest values for the Akaike information criterion and the Schwartz Bayesian criterion was used (Littell et al., 1998). Kenward-Roger's option was used to calculate the denominator degrees of freedom.
Residual errors were used to test main effects and interactions. Differences were considered significant at $P \leq 0.05$, and trends were discussed at $0.05 < P \leq 0.10$.

**RESULTS AND DISCUSSION**

To the best of our knowledge, this is the first time that the effects of supplementing NALM in lactation dairy diets have been described. The lack of data in the literature regarding NALM led us to indirectly compare our results with data reported for HMBi as well as traditional polymer-protected Met (RPMet) products.

A continuous culture study estimated ruminal undegradability of N-acetyl-DL-Met at 67% (Windschitl and Stern, 1988). Monogastric data involving young pigs supplemented with NALM revealed that no NALM was present in either portal or vena caval plasma at any sampling time (Daabees et al., 1984), which indicates hydrolysis of NALM to Met in the intestinal lumen and mucosal cells. The research reported in this paper represents initial attempts to investigate lactational performance and ruminal fermentation profiles of dairy cows fed with NALM as an alternative RPMet source.

*Characteristics of Diet Composition and Treatment*

Ingredients and chemical composition of experimental diets are presented in Table 1-1. The MPA diet provided adequate MP, whereas the MPD diet was 6% MP-deficient (-181 to -190 g/d). The MPD diet was formulated to contain less MP compared to the MPA diet by reducing the amount of dietary concentrations of canola and soybean meal in the diets. The 6% MP
deficiency was chosen for the MPD based on the result from Giallongo et al. (2016) who fed an MP-deficient diet (5-10%) supplemented with RPMet. All diets supplied NE_L in excess of requirement (NRC, 2001). The MPA diet met or exceeded the MP, RDP, and RUP requirements, while the MPD diet was deficient for MP, RDP, and RUP as expected.

The NRC (2001) model suggests that Lys and Met concentrations in MP for optimal milk protein production are 7.2 and 2.4%, respectively. These concentrations are difficult to achieve without feeding very high dietary concentrations of protein. Therefore, it is recommended that the first step in balancing diets for Lys and Met is to maintain a Lys:Met in MP of 3.0 with more practical concentrations of Lys and Met in MP being 6.6 and 2.2%, respectively (Schwab et al., 2005). In this study, supplementing NALM (assuming 67% rumen undegradability) substantially improved Met status, as indicated by the reduction of the predicted (NRC, 2001) digestible Lys-to-digestible Met ratio in MP from 3.5 to 2.7 (Table 1-1). The digestible Lys-to-digestible Met ratio for maximum milk and milk protein yield have traditionally been reported as a 3.00:1 (NRC, 2001, Whitehouse et al., 2009), but optimal ratios as low as 2.69:1 were reported by Van Amburgh et al. (2015) when using the CNCPS model after calculating the optimum efficiency of utilization of Lys and Met using data derived by Lapierre et al. (2007) and from a meta-analysis of 40 published papers (Doepel et al., 2004).

*Feed Intake, Productive Performance, and Net Energy Utilization*

Intake of DM did not differ among dietary treatments, averaging 25.3 kg/d (Table 1-2). The MPD and the MPA diet with supplementation of NALM increased CP intake with a greater increase under the MPA diet, resulting in an MP × NALM interaction. Responses to
supplementation of polymer-coated RPMet and HMBi on feed intake and productive performance in the literature have been quite variable. Kung and Rode (1996) found that rumen-protected AA supplementation did not improve DMI, which corresponds to the findings of other studies performed by Leonardi et al. (2003) and Chen et al. (2011). A limited number of studies have shown an increase in DMI with RPMet supplementation (Broderick et al., 2009; Zanton et al., 2014), while Ordway et al. (2009) and Zanton et al. (2014) reported decreases in DMI. Supplementation of HMBi has also increased DMI (Osorio et al., 2014) because of the potential stimulatory effect of HMBi on cellulolytic bacteria and the subsequent increase in passage rate and DMI (Lee et al., 2015). On the other hand, Lee et al. (2012b) assessed supplementation of RPMet products to MP-deficient diets, as was explored in the current study and reported that MP-deficient diets below 15% of the NRC requirements decreased DMI. However, the authors observed that a treatment supplemented with a combination of 3 rumen-protected AA consisted of Lys (100 g/cow), Met (30 g/cow), and His (50 g/cow) increased DMI (Lee et al., 2012b). An additional study by the same group (Lee et al., 2015) found that supplementation with rumen-protected Lys and RPMet in MP-deficient diets (-256 to -305 MP g/d) did not affect DMI. In the current study, stage of lactation may have interfered with a potential effect of NALM on DMI under the MPA diet, because peak DMI typically tends to lag behind peak milk in early to mid-lactating cows that consume feed in order to meet energy needs.

Feeding the MPA diet increased milk yield by 2.2 kg/d relative to the MPD diet, resulting in more efficient feed utilization for milk production (1.64 vs. 1.57; Table 1-2). Milk yield was not affected by NALM supplementation, while yields of 3.5% FCM and ECM increased or tended to increase ($P = 0.06$) with NALM addition. Noftsger et al. (2005) reported no difference in milk production when cows were supplemented with HMBi at 0.13% DM. However, St-Pierre and
Sylvester (2005) showed an increase in milk yield (2.9 kg/d) to cows supplemented with HMBi at 0.15% DM. The observed production responses to supplementation of RPMet in cows fed low-protein diets (i.e., MP-deficient diets) have been, in most cases, minimal and/or inconsistent (Arriola Apelo et al., 2014a; Sinclair et al., 2014). No negative effect on milk yield was detected by feeding a low CP diet (15%) alone or in combination with RPMet supplementation (Arriola Apelo et al., 2014b). In the current study, a 6% MP-deficient diet decreased milk yield, while previous research with MP-deficient diets (5-10% below MP requirement) have maintained milk yield (Lee et al., 2015; Giallongo et al., 2016). In the present study, there was no improvement in milk yield in response to NALM supplementation in both of the MPD and the MPA diet, thus indicating that NALM was not utilized for milk production. Alternatively, NALM supplementation increased yields of 3.5% FCM and ECM, suggesting a potential role on fatty acid synthesis and delivery to the mammary gland possibly through a methyl donation mechanism in the liver, which will be discussed later in this paper.

There was a tendency toward MP × NALM interaction on milk fat concentration (P = 0.10), because NALM supplementation increased milk fat concentration under the MPA, but not the MPD diet (Table 1-2). However, milk true protein concentration was similar across the diets. Ordway et al. (2009) showed that HMBi did not increase milk fat concentration, but increased milk protein concentration in comparison to the control diet. In contrast, milk fat concentration increased with HMBi supplementation (Dalbach et al., 2011; Osorio et al., 2013). Supplementing RPMet typically results in an increase in milk protein percentage (Rulquin and Delaby, 1997; Samuelson et al., 2001; Lara et al., 2006), but one study (Benefield et al., 2009) reported a negative impact on milk protein concentration. It has been well established that Met acts as a methyl donor needed in the synthesis of milk fat (Osorio et al., 2016; Sun et al., 2016). The
primary methyl carrier during bovine hepatic methyl metabolism is S-adenosyl-Met (SAM). It works to donate methyl groups for the generation of phosphatidylethanolamine, acting as phospholipids for cell membranes in the packaging of very low-density lipoprotein (VLDL; Purohit et al., 2007). Once packaged, the VLDL consist of triglycerides, apolipoproteins B and E, phospholipids, and cholesterol, and consequently Met plays a vital role in both the development of apolipoproteins and phospholipids. The VLDL are then exported from the liver to the mammary gland for milk fat synthesis (Figure 1-1). Thus, as a potential mode of action, NALM after its digestion and absorption may have been involved in hepatic Met metabolism pertaining to fatty acid trafficking, leading to the increased milk fat production in early to mid-lactation observed in the current study.

It has been reported that aminoacylase I is present in multiple mammalian organs (i.e., small intestine, liver, and kidney; Giardina et al., 1997). The bovine liver contains the enzyme, α-N-acylamino acid hydrolase which is capable of hydrolyzing α-N-acylated AA to yield acetate and the corresponding AA (Gade and Brown, 1981). However, it is unclear how cows utilize the NALM into conversion of milk fat, once NALM was hydrolyzed by aminoacylase I and absorbed in the small intestine as acetate and Met (Baxter et al., 2002) as was illustrated in the Figure 1-1.

Feeding the MPA diet increased milk fat yield compared with the MPD diet, and supplementing NALM further increased milk fat yield regardless of MP level (Table 1-2). Although feeding the MPA diet increased milk protein yield compared with the MPD diet, NALM supplementation did not influence milk protein yield. Noftsger et al. (2005) and St-Pierre and Sylvester (2005) demonstrated an increase in milk protein yield with HMBi supplementation. Supplementing HMBi and polymer-coated RPMet typically has more impact
on milk protein vs. milk fat yield (Lara et al., 2006; Rulquin et al., 2006; Ordway et al., 2009). This is most likely due to Met being the first-limiting essential AA in lactation dairy diets, and consequently supplementation of HMBi or polymer-coated RPMet is first partitioned to improve milk protein yield in dairy cows. Contradictory effects have been reported (Phipps et al., 2008 and Ordway et al., 2009) for HMBi on increased milk fat yield. Patton (2010) performed a meta-analysis on 35 studies evaluating production performance in lactating dairy cows supplemented with various RPMet products to dairy diets and found increased production of true milk protein both as a percentage (0.07%) and yield (27 g/d). However, NALM supplementation did not increase milk protein yield, and thus NALM may have been used as a source of Met in methyl donation toward VLDL production as previously discussed. Further research is needed to determine the actual contribution of Met in NALM for VLDL formation and its impacts on milk production parameters.

For the diets supplemented with NALM, the increased milk fat concentration and no change in DMI resulted in a tendency toward MP × NALM interaction on 3.5% FCM yield/DMI and ECM yield/DMI ($P = 0.06$; Table 1-2). A 12-wk study performed by Chen et al. (2011) showed that supplementing HMBi to lactating cows averaging 143 DIM increased ECM yield/DMI. Feed efficiency is a reasonable indicator evaluated on dairy farms, as it helps measure the relative ability of cows to convert feed nutrients into milk or milk components. The increased feed efficiencies observed in the current study suggest that NALM supplementation would be economically favorable in early to mid-lactating cows, but only when fed a diet adequate in MP concentration. However, caution should be exercised to extrapolate these benefits of NALM in the current study due to the short length of data collection and, therefore, further investigation is needed to confirm our data with a relatively long period of experiment.
No changes were seen in either BW gain or net energy utilization with supplementation of NALM in the diets (Table 1-2). However, feeding the MPA diet increased milk yield relative to the MPD diet, leading to an increased net energy utilization for milk. Research on RPMet supplementation on BW gain in ruminants is scarce, but some studies have reported increases in BW gain upon RPMet supplementation (Deetz et al., 1985; Oke et al., 1986). Methionine is a limiting AA in protein synthesis for growing cattle (Richardson et al., 1978), and therefore an increase in available Met can support protein deposition in the body (Loest et al., 2002). The role of Met in the formation of phospholipids through conversion of Met to SAM will also support fat deposition in growing cattle (Obeid et al., 2009). In addition, acetate, a component of NALM, is also recognized for its role as a lipogenic precursor (Bauman and Griinari, 2001). No effect on BW gain due to NALM supplementation suggests that energy was partitioned for milk fat production rather than BW gain in early to mid-lactation in the present study.

**Plasma AA Profiles**

Among EAA, feeding the MPA diet increased plasma concentrations of Ile and Leu compared with the MPD diet, but supplementing NALM decreased those AA only under the MPA diet, resulting in an interaction between MP and NALM (Table 1-3). Supplementing NALM decreased plasma concentration of Val under the MPA but not the MPD diet, leading to a MP × NALM interaction. In contrast, Noftsger et al. (2005) did not find any difference in Val concentration when cows were supplemented with HMB, HMBi, or DL-Met. Similarly, St-Pierre et al. (2005) supplemented a control diet with HMB, HMBi, or a combination of HMB and HMBi, and the dietary treatments had no effect on free plasma amino acids, including Val.
Plasma concentration of Met increased under the MPD but not MPA, leading to an MP × NALM interaction. The no effect of NALM supplementation on plasma Met concentration in the MPA diet observed in this study was unexpected, and its mechanism is difficult to explain. The liver plays a central role in Met metabolism (Finkelstein, 1990); approximately 50% of Met metabolism and up to 85% of all methylation reactions occur in the mammalian liver (Mato et al., 2002). Hepatic gene regulation serves as an important regulatory point for controlling Met metabolism (Finkelstein, 2003; Mato et al., 2008). However, effects of RPMet supplementation on genes for Met metabolism in bovine hepatocytes in vivo has not been clearly understood in lactating dairy cattle. Zhang et al. (2016) demonstrated decreased mRNA expression for genes that catalyze regeneration of Met and showed the decreased need for cellular regeneration of Met with increasing Met concentrations from 2-hydroxy-4-(methylthio)-butanoic acid (HMB) and DL-Met on primary bovine hepatocytes. The authors described that increased Met supply from HMB and DL-Met in primary bovine hepatocytes was not directed at increased glucose synthesis, probably due to other metabolic priorities for Met (Zhang et al., 2016). Likewise, in the current study it is speculated that increased Met supply from NALM supplementation in the MPA diet may have been prioritized in the bovine hepatocyte to the primary methyl donor SAM, allowing SAM to be used for increased VLDL synthesis and exportation to the mammary gland for milk fat synthesis (Figure 1-1).

**Ruminal Fermentation Characteristics**

Ruminal pH decreased in the MPD relative to the MPA diet (6.24 vs. 6.37; Table 1-4), but NALM supplementation did not influence ruminal pH. The mean ruminal pH from all dietary
treatments were at least above 6.2, so even the effect of MP difference on overall microbial physiology may have been minor. Concentration of VFA and their compositions were in general not affected by MP level as well as NALM supplementation. Similarly, Noftsger et al. (2005) and Lee et al. (2015) reported no effects of RPMet supplementation on ruminal pH and VFA production. Concentration of NH$_3$-N also did not differ across the dietary treatments, while MCP yield increased due to feeding the MPA diet compared with the MPD diet, but NALM did not affect MCP yield regardless of MP level (Table 1-4). Supplementation of RPMet traditionally has not had an impact on microbial production, with the exception of Met analogues such as HMBi because of its partial ruminal degradation and potential stimulatory effects on MCP synthesis (Robert et al., 2001). Similarly, Lee et al. (2015) reported increased MCP yield with polymer-coated RPMet supplementation. Although NALM is partially degraded in the rumen (approximately 33%, Figure 1-1), the degraded fraction of the NALM would not contribute to MCP synthesis under the experimental condition of our study. Further research is needed to understand the mode of action of NALM on ruminal metabolism, particularly how the aminoacylase I enzyme acts on NALM.

CONCLUSIONS

The current study investigated the production responses and ruminal fermentation profiles of early to mid-lactating dairy cows supplemented without or with a developmental NALM product in the MPD or the MPA diet. The NALM supplementation did not affect DMI, milk yield, and milk protein yield or alter ruminal fermentation characteristics in either the MPD or the MPA diet, suggesting that early to mid-lactating dairy cows tested in this study may have other
metabolic priorities for Met. However, supplementation of NALM regardless of MP difference increased both 3.5% FCM and ECM yields coupled with increased milk fat concentration which was more impactful in the MPA diet, leading to better feed efficiencies with the MPA diet. Thus, supplementing NALM, particularly in the MPA diet, in early to mid-lactation can be a good approach to formulate an optimal dairy feeding program for improving feed efficiency. Yet, the potential effects of NALM on intermediary metabolism between the gastrointestinal tract, the liver, and the mammary gland needs to be explored to understand the metabolic impacts of NALM on lactational and physiological parameters of dairy cows. Overall, the results in the current study suggest that NALM exerted minor influence on ruminal metabolism, but increased milk fat concentration possibly due to accelerated fatty acid delivery to the mammary gland, resulting in sizable increases in milk fat yield and feed efficiency.

ACKNOWLEDGMENTS

This study was supported by CJ CheilJedang (Seoul, South Korea). The authors thank S. Sharp at Utah State University (Logan) for technical assistance and the staff of the Caine Dairy Research Center (Wellsville, UT) for their conscientious care of the experimental cows.

REFERENCES


Geishauser, T. 1993. An instrument for the collection and transfer of ruminal fluid and for the administration of water soluble drugs in adult cattle. Bovine Pract. 27:38–42.


Whitehouse, N., C. Schwab, D. Luchini, T. Tylutki, and B. Sloan. 2009. Comparison of optimal lysine and methionine concentrations in metabolizable protein estimated by the NRC (2001), CPM-Dairy (v.3.0.10) and AMTS.Cattle (v.2.1.1) models. J. Dairy Sci. 92(Suppl. 1):103 (Abstr.).


Table 1-1. Ingredient and chemical composition of the experimental diets without or with N-acetyl-L-Met (NALM) at 2 different levels of MP fed to lactating Holstein dairy cows (n = 4)

<table>
<thead>
<tr>
<th>Item</th>
<th>Experimental diet(^1)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Item</td>
<td>MPD</td>
<td>MPA</td>
<td>MPD</td>
<td>MPA</td>
<td></td>
</tr>
<tr>
<td>Item</td>
<td>−NALM +NALM</td>
<td>−NALM +NALM</td>
<td>−NALM +NALM</td>
<td>−NALM +NALM</td>
<td></td>
</tr>
<tr>
<td>Ingredient, % of DM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alfalfa hay</td>
<td>27.0 27.0</td>
<td>25.8 25.7</td>
<td>27.0 27.0</td>
<td>25.8 25.7</td>
<td></td>
</tr>
<tr>
<td>Wheat straw</td>
<td>2.49 2.49</td>
<td>2.38 2.38</td>
<td>2.49 2.49</td>
<td>2.38 2.38</td>
<td></td>
</tr>
<tr>
<td>Corn silage</td>
<td>32.5 32.5</td>
<td>31.0 31.0</td>
<td>32.5 32.5</td>
<td>31.0 31.0</td>
<td></td>
</tr>
<tr>
<td>Corn grain (steam-flaked)</td>
<td>11.6 11.6</td>
<td>11.1 11.1</td>
<td>11.6 11.6</td>
<td>11.1 11.1</td>
<td></td>
</tr>
<tr>
<td>Corn grain (high-moisture)</td>
<td>6.17 6.17</td>
<td>5.90 5.89</td>
<td>6.17 6.17</td>
<td>5.90 5.89</td>
<td></td>
</tr>
<tr>
<td>Cottonseed, whole</td>
<td>4.88 4.87</td>
<td>4.66 4.65</td>
<td>4.88 4.87</td>
<td>4.66 4.65</td>
<td></td>
</tr>
<tr>
<td>Canola meal</td>
<td>3.50 3.50</td>
<td>5.68 5.68</td>
<td>3.50 3.50</td>
<td>5.68 5.68</td>
<td></td>
</tr>
<tr>
<td>Soybean meal</td>
<td>3.25 3.24</td>
<td>5.27 5.27</td>
<td>3.25 3.24</td>
<td>5.27 5.27</td>
<td></td>
</tr>
<tr>
<td>Beet pulp, shreds</td>
<td>4.93 4.92</td>
<td>4.71 4.70</td>
<td>4.93 4.92</td>
<td>4.71 4.70</td>
<td></td>
</tr>
<tr>
<td>NALM(^2)</td>
<td>− 0.13</td>
<td>− 0.13</td>
<td>− 0.13</td>
<td>− 0.13</td>
<td></td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>1.08 1.08</td>
<td>1.03 1.03</td>
<td>1.08 1.08</td>
<td>1.03 1.03</td>
<td></td>
</tr>
<tr>
<td>Vitamin and mineral mix(^3)</td>
<td>2.54 2.54</td>
<td>2.43 2.42</td>
<td>2.54 2.54</td>
<td>2.43 2.42</td>
<td></td>
</tr>
<tr>
<td>Chemical composition, % of DM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM, %</td>
<td>60.9 ± 0.53 60.7 ± 0.32</td>
<td>61.0 ± 0.72 62.0 ± 1.50</td>
<td>60.9 ± 0.53 60.7 ± 0.32</td>
<td>61.0 ± 0.72 62.0 ± 1.50</td>
<td></td>
</tr>
<tr>
<td>OM</td>
<td>92.1 ± 0.60 91.6 ± 0.71</td>
<td>92.0 ± 0.84 92.0 ± 0.66</td>
<td>92.1 ± 0.60 91.6 ± 0.71</td>
<td>92.0 ± 0.84 92.0 ± 0.66</td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>15.2 ± 0.14 15.4 ± 0.21</td>
<td>16.1 ± 0.49 16.8 ± 0.35</td>
<td>15.2 ± 0.14 15.4 ± 0.21</td>
<td>16.1 ± 0.49 16.8 ± 0.35</td>
<td></td>
</tr>
<tr>
<td>RDP(^4)</td>
<td>9.52 9.73</td>
<td>9.82 10.3</td>
<td>9.52 9.73</td>
<td>9.82 10.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.68</td>
<td>5.92</td>
<td>6.23</td>
<td>6.50</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td></td>
</tr>
<tr>
<td>RUP&lt;sup&gt;4&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDF</td>
<td>37.8 ± 1.37</td>
<td>37.0 ± 1.88</td>
<td>37.4 ± 1.06</td>
<td>38.2 ± 0.65</td>
<td></td>
</tr>
<tr>
<td>ADF</td>
<td>23.7 ± 0.85</td>
<td>23.4 ± 0.88</td>
<td>23.4 ± 0.84</td>
<td>24.0 ± 0.74</td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>17.5 ± 0.57</td>
<td>19.0 ± 1.91</td>
<td>18.9 ± 0.99</td>
<td>16.6 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>Ether extract</td>
<td>1.66 ± 0.61</td>
<td>1.91 ± 0.86</td>
<td>1.86 ± 0.39</td>
<td>1.68 ± 0.73</td>
<td></td>
</tr>
<tr>
<td>NFC&lt;sup&gt;5&lt;/sup&gt;</td>
<td>37.4 ± 0.14</td>
<td>37.0 ± 0.21</td>
<td>36.7 ± 0.49</td>
<td>35.4 ± 0.35</td>
<td></td>
</tr>
<tr>
<td>NE&lt;sub&gt;L&lt;/sub&gt;&lt;sup&gt;4&lt;/sup&gt;, Mcal/kg</td>
<td>1.67</td>
<td>1.67</td>
<td>1.68</td>
<td>1.68</td>
<td></td>
</tr>
</tbody>
</table>

Protein supply,<sup>6</sup> g/d

|            |        |        |        |        |
| RDP supply | 2,342  | 2,443  | 2,494  | 2,616  |
| RDP balance| -128   | -27    | 24     | 146    |
| RUP supply | 1,397  | 1,499  | 1,595  | 1,651  |
| RUP balance| -173   | -71    | 25     | 81     |
| MP supply  | 2,781  | 2,791  | 3,049  | 3,059  |
| MP requirements | 2,972 | 2,972 | 2,998 | 3,004 |
| MP balance | -190   | -181   | 50     | 55     |

Lys and Met balance,<sup>7</sup> g/d

|            |        |        |        |        |
| dLys requirement | 184 | 184 | 201 | 202 |
| dLys supplied by the diet | 191 | 191 | 209 | 209 |
| Lys balance | 7.0    | 7.0    | 8.0    | 7.0    |
| dMet requirement | 61   | 61    | 67     | 67     |
| dMet supplied by the diet | 55   | 55    | 60     | 60     |
| dMet from Met derivative | 0    | 15.6   | 0      | 15.6   |
| Met balance | -6.0   | 9.6    | -7.0   | 8.6    |
| dLys:dMet<sup>8</sup> | 3.47 | 2.71 | 3.48 | 2.76 |

1MPD−NALM = diet deficient in MP (MPD) without NALM; MPD+NALM = MPD with NALM; MPA−NALM = diet adequate in MP (MPA) without NALM; and MPA+NALM = MPA with NALM.

2A developmental NALM product (CJ CheilJedang, Seoul, Korea) was a source of rumen-protected Met, and it contained 78.0% Met.

3Formulated to contain (per kg DM): 226.7 mg of Se (from sodium selenite), 9278.7 mg of Cu (from copper amino acid complex), 40,537.4 mg of Zn (from zinc amino acid complex), 38,653.4 mg of Mn (from manganese amino acid complex), 552.6 mg of Co (from cobalt carbonate), 1,234,585.2 IU of vitamin A, 152,808.1 IU of vitamin D, 3,815.1 IU of vitamin E, and 295 mg of Rumensin (Elanco Animal Health, Greenfield, IN).

4Based on tabular value (NRC, 2001).

5NFC = 100 – CP – NDF – ether extract – ash.

6All values were estimated using NRC (2001) based on actual DMI, milk yield, milk composition, and BW of the cows throughout the trial.

7Digestible Lys (dLys) and digestible Met (dMet) supply from the diets were estimated using NRC (2001); supply of dMet from Met derivative was estimated with Met concentration and absorption (67%) data provided the manufacturer. Requirements of dLys and dMet were calculated as 6.6 and 2.2% (respectively) of MP requirements.

8Digestible Lys from diet-to-digestible Met from diet and NALM ratio.
Table 1-2. Intake, milk production, feed efficiency, and net energy utilization of lactating Holstein dairy cows supplemented without or with N-acetyl-L-Met (NALM) at 2 different levels of MP

<table>
<thead>
<tr>
<th>Item</th>
<th>Diet¹</th>
<th></th>
<th></th>
<th>Significance of effect²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MPD</td>
<td>MPA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>−NALM</td>
<td>+NALM</td>
<td>−NALM</td>
<td>+NALM</td>
</tr>
<tr>
<td>DM intake, kg/d</td>
<td>24.6</td>
<td>25.6</td>
<td>25.4</td>
<td>25.4</td>
</tr>
<tr>
<td>Yield, kg/d</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk</td>
<td>39.3</td>
<td>39.7</td>
<td>41.3</td>
<td>42.0</td>
</tr>
<tr>
<td>3.5% FCM³</td>
<td>39.0</td>
<td>39.9</td>
<td>40.4</td>
<td>43.4</td>
</tr>
<tr>
<td>ECM⁴</td>
<td>38.7</td>
<td>39.7</td>
<td>40.2</td>
<td>42.9</td>
</tr>
<tr>
<td>Milk composition, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat</td>
<td>3.44</td>
<td>3.52</td>
<td>3.34</td>
<td>3.70</td>
</tr>
<tr>
<td>True protein</td>
<td>2.77</td>
<td>2.82</td>
<td>2.78</td>
<td>2.83</td>
</tr>
<tr>
<td>Lactose</td>
<td>4.84</td>
<td>4.87</td>
<td>4.88</td>
<td>4.85</td>
</tr>
<tr>
<td>Milk component yield, kg/d</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat</td>
<td>1.36</td>
<td>1.41</td>
<td>1.39</td>
<td>1.56</td>
</tr>
<tr>
<td>True protein</td>
<td>1.08</td>
<td>1.11</td>
<td>1.14</td>
<td>1.18</td>
</tr>
<tr>
<td>Lactose</td>
<td>1.91</td>
<td>1.93</td>
<td>2.02</td>
<td>2.04</td>
</tr>
<tr>
<td>Feed efficiency</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk yield/DMI</td>
<td>1.59</td>
<td>1.55</td>
<td>1.61</td>
<td>1.66</td>
</tr>
<tr>
<td>3.5% FCM yield/DMI</td>
<td>1.58ᵇ</td>
<td>1.57ᵇ</td>
<td>1.57ᵇ</td>
<td>1.72ᵃ</td>
</tr>
<tr>
<td>ECM yield/DMI</td>
<td>1.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.55&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.70&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>----------------</td>
<td>-----------------</td>
<td>-----------------</td>
<td>-----------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>BW</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial, kg</td>
<td>704</td>
<td>698</td>
<td>702</td>
<td>704</td>
</tr>
<tr>
<td>Mean, kg</td>
<td>705</td>
<td>705</td>
<td>710</td>
<td>713</td>
</tr>
<tr>
<td>Gain, kg/d</td>
<td>0.06</td>
<td>0.30</td>
<td>0.40</td>
<td>0.40</td>
</tr>
</tbody>
</table>

Net energy utilization,

Mcal/d

<table>
<thead>
<tr>
<th>Maintenance</th>
<th>10.9</th>
<th>10.9</th>
<th>11.0</th>
<th>11.0</th>
<th>0.25</th>
<th>0.03</th>
<th>0.73</th>
<th>0.57</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW gain</td>
<td>0.24</td>
<td>1.16</td>
<td>1.55</td>
<td>1.55</td>
<td>1.322</td>
<td>0.24</td>
<td>0.51</td>
<td>0.51</td>
</tr>
<tr>
<td>Milk</td>
<td>26.2</td>
<td>26.1</td>
<td>26.8</td>
<td>28.5</td>
<td>1.67</td>
<td>0.03</td>
<td>0.20</td>
<td>0.16</td>
</tr>
<tr>
<td>Total&lt;sup&gt;5&lt;/sup&gt;</td>
<td>37.4</td>
<td>38.2</td>
<td>39.3</td>
<td>41.1</td>
<td>2.19</td>
<td>0.03</td>
<td>0.21</td>
<td>0.65</td>
</tr>
<tr>
<td>NE&lt;sub&gt;L&lt;/sub&gt;, Mcal/kg DMI</td>
<td>1.49</td>
<td>1.55</td>
<td>1.55</td>
<td>1.60</td>
<td>0.074</td>
<td>0.13</td>
<td>0.18</td>
<td>0.94</td>
</tr>
</tbody>
</table>

<sup>a-d</sup>Means within a row with different superscripts differ ($P < 0.05$).

<sup>1</sup>MPD−NALM = diet deficient in MP (MPD) without NALM; MPD+NALM = MPD with NALM; MPA−NALM = diet adequate in MP (MPA) without NALM; and MPA+NALM = MPA with NALM.

<sup>2</sup>MP = level of MP in the diet (MPD vs. MPA); NALM = supplementation of NALM (−NALM vs. +NALM); and INT = interaction between MP and NALM.

<sup>3</sup>3.5% FCM = [0.4324 × milk yield (kg/d)] + [16.216 × fat yield (kg/d)].

<sup>4</sup>ECM = 0.327 × milk yield (kg/d) + 12.95 × fat yield (kg/d) + 7.2 × protein yield (kg/d).

<sup>5</sup>Net energy used for maintenance, BW gain, and milk.
Table 1-3. Plasma AA concentrations (µmol/L) of lactating Holstein dairy cows supplemented without or with N-acetyl-L-Met (NALM) at 2 different levels of MP

<table>
<thead>
<tr>
<th>Item</th>
<th>MPD</th>
<th>MP</th>
<th>Significance of effect</th>
<th>SEM</th>
<th>MP</th>
<th>NALM</th>
<th>INT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−NALM</td>
<td>+NALM</td>
<td>−NALM</td>
<td>+NALM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>His</td>
<td>10.1</td>
<td>11.6</td>
<td>10.2</td>
<td>9.7</td>
<td>5.60</td>
<td>0.51</td>
<td>0.70</td>
</tr>
<tr>
<td>Ile</td>
<td>85.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>90.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>102&lt;sup&gt;a&lt;/sup&gt;</td>
<td>91.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.39</td>
<td>&lt; 0.01</td>
<td>0.35</td>
</tr>
<tr>
<td>Leu</td>
<td>119&lt;sup&gt;b&lt;/sup&gt;</td>
<td>122&lt;sup&gt;b&lt;/sup&gt;</td>
<td>144&lt;sup&gt;a&lt;/sup&gt;</td>
<td>123&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.5</td>
<td>&lt; 0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>Lys</td>
<td>61.2</td>
<td>65.8</td>
<td>68.0</td>
<td>65.0</td>
<td>17.61</td>
<td>0.40</td>
<td>0.83</td>
</tr>
<tr>
<td>Met</td>
<td>18.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>17.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.85</td>
<td>0.06</td>
<td>0.49</td>
</tr>
<tr>
<td>Phe</td>
<td>71.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>73.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>76.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>70.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.23</td>
<td>0.44</td>
<td>0.24</td>
</tr>
<tr>
<td>Thr</td>
<td>31.6</td>
<td>33.1</td>
<td>32.1</td>
<td>29.0</td>
<td>7.38</td>
<td>0.25</td>
<td>0.59</td>
</tr>
<tr>
<td>Val</td>
<td>248&lt;sup&gt;b&lt;/sup&gt;</td>
<td>266&lt;sup&gt;b&lt;/sup&gt;</td>
<td>283&lt;sup&gt;a&lt;/sup&gt;</td>
<td>241&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.3</td>
<td>0.74</td>
<td>0.43</td>
</tr>
<tr>
<td>Ala</td>
<td>196&lt;sup&gt;b&lt;/sup&gt;</td>
<td>195&lt;sup&gt;b&lt;/sup&gt;</td>
<td>200&lt;sup&gt;a&lt;/sup&gt;</td>
<td>181&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.3</td>
<td>0.36</td>
<td>0.04</td>
</tr>
<tr>
<td>Asp</td>
<td>37.9</td>
<td>49.8</td>
<td>46.9</td>
<td>38.1</td>
<td>9.48</td>
<td>0.77</td>
<td>0.75</td>
</tr>
<tr>
<td>Glu</td>
<td>24.8</td>
<td>23.3</td>
<td>24.2</td>
<td>22.6</td>
<td>2.50</td>
<td>0.47</td>
<td>0.09</td>
</tr>
<tr>
<td>Gly</td>
<td>198&lt;sup&gt;a&lt;/sup&gt;</td>
<td>198&lt;sup&gt;a&lt;/sup&gt;</td>
<td>188&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>168&lt;sup&gt;c&lt;/sup&gt;</td>
<td>20.9</td>
<td>&lt; 0.01</td>
<td>0.07</td>
</tr>
<tr>
<td>Pro</td>
<td>78.0&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>75.3&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>80.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>70.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.68</td>
<td>0.54</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Tyr</td>
<td>138</td>
<td>166</td>
<td>168</td>
<td>159</td>
<td>42.2</td>
<td>0.35</td>
<td>0.44</td>
</tr>
<tr>
<td>EAA&lt;sup&gt;3&lt;/sup&gt;</td>
<td>645&lt;sup&gt;b&lt;/sup&gt;</td>
<td>682&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>720&lt;sup&gt;a&lt;/sup&gt;</td>
<td>648&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.6</td>
<td>0.34</td>
<td>0.42</td>
</tr>
<tr>
<td>NEAA&lt;sup&gt;4&lt;/sup&gt;</td>
<td>793</td>
<td>707</td>
<td>694</td>
<td>638</td>
<td>64.3</td>
<td>0.19</td>
<td>0.27</td>
</tr>
</tbody>
</table>

<sup>a-c</sup> Means within a row with different superscripts differ (P < 0.05).
1MPD−NALM = diet deficient in MP (MPD) without NALM; MPD+NALM = MPD with NALM; MPA−NALM = diet adequate in MP (MPA) without NALM; and MPA+NALM = MPA with NALM.

2MP = level of MP in the diet (MPD vs. MPA); NALM = supplementation of NALM (−NALM vs. +NALM); and INT = interaction between MP and NALM.

3Essential amino acid analyzed.

4Nonessential amino acid analyzed.
Table 1-4. Ruminal fermentation characteristics of lactating Holstein dairy cows supplemented without or with N-acetyl-L-Met (NALM) at 2 different levels of MP

<table>
<thead>
<tr>
<th>Item</th>
<th>Diet</th>
<th>SEM</th>
<th>Significance of effect</th>
<th>MP</th>
<th>NALM</th>
<th>INT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MPD NALM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MPD+NALM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ruminal pH</td>
<td>6.26</td>
<td>6.21</td>
<td>6.35</td>
<td>6.39</td>
<td>0.101</td>
<td>0.03</td>
</tr>
<tr>
<td>Total VFA, mM</td>
<td>146</td>
<td>154</td>
<td>147</td>
<td>144</td>
<td>6.6</td>
<td>0.53</td>
</tr>
<tr>
<td>Individual VFA, mol/100 mol</td>
<td>Acetate (A)</td>
<td>62.4</td>
<td>62.0</td>
<td>62.0</td>
<td>62.4</td>
<td>3.79</td>
</tr>
<tr>
<td></td>
<td>Propionate (P)</td>
<td>23.3</td>
<td>23.7</td>
<td>23.2</td>
<td>23.3</td>
<td>2.99</td>
</tr>
<tr>
<td></td>
<td>Butyrate</td>
<td>10.7</td>
<td>10.6</td>
<td>11.0</td>
<td>10.7</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>Valerate</td>
<td>1.57</td>
<td>1.66</td>
<td>1.47</td>
<td>1.62</td>
<td>0.403</td>
</tr>
<tr>
<td></td>
<td>Isobutyrate</td>
<td>0.79</td>
<td>0.89</td>
<td>0.95</td>
<td>0.87</td>
<td>0.128</td>
</tr>
<tr>
<td></td>
<td>Isovalerate</td>
<td>1.27b</td>
<td>1.27b</td>
<td>1.41a</td>
<td>1.20b</td>
<td>0.179</td>
</tr>
<tr>
<td></td>
<td>A:P</td>
<td>2.77</td>
<td>2.70</td>
<td>2.75</td>
<td>2.74</td>
<td>0.210</td>
</tr>
<tr>
<td>NH₃-N, mg/100 mL</td>
<td>9.34</td>
<td>9.05</td>
<td>9.28</td>
<td>9.82</td>
<td>1.425</td>
<td>0.70</td>
</tr>
<tr>
<td>MCP,g/d</td>
<td>2,540</td>
<td>2,649</td>
<td>2,954</td>
<td>2,837</td>
<td>237.7</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

a,b Means within a row with different superscripts differ (P < 0.05).

¹MPD−NALM = diet deficient in MP (MPD) without NALM; MPD+NALM = MPD with NALM;
MPA−NALM = diet adequate in MP (MPA) without NALM; and MPA+NALM = MPA with NALM.
\(^2\)MP = level of MP in the diet (MPD vs. MPA); NALM = supplementation of NALM (−NALM vs. +NALM); and INT = interaction between MP and NALM.

\(^3\)Ammonia-N.

\(^4\)Microbial protein production (g/d) = (\{\text{urinary total purine derivatives (allantoin + uric acid)} - (0.385 \times BW^{0.75})/0.85\} \times 70 \times 6.25)/(0.13 \times 0.83 \times 1,000) \) (Janicek et al., 2008).
Figure 1-1. Proposed mechanism of N-acetyl-L-Met (NALM) on its potential effects on milk fat and milk production. SAM = S-adenosyl-Met; VLDL = very low-density lipoprotein (Nelson and Cox, 2013)
CHAPTER 2

Effects of supplementing three different forms of rumen-protected lysine on lactational performance of mid to late lactation dairy cows

ABSTRACT

The present study investigated lactational performance of dairy cows when supplemented with Ne-acetyl-L-Lys (εNALL), Nα, ε-acetyl-L-Lys (diNALL), or AjiPro®-L (AP) as a source of rumen-protected Lys (RPLys) to a control diet. Control cows (CL) were provided the basal diet formulated to contain 15.3% CP and supply adequate MP (+ 10 g/d balance). Forty lactating Holstein dairy cows (151 ± 78.0 days-in-milk, on average) were assigned to treatments (n = 10) by parity and days-in-milk, and the experiment was performed in a randomized complete block design. Cows were fed the same CL diet for a 2-wk adaptation period. Cows were then randomly assigned within blocks to 1 of the 4 experimental diets and fed assigned diets throughout the remaining 8 wk of the trial (entire study duration was 70 d). Developmental εNALL, diNALL, and the commercial RPLys product AP were supplemented to the CL diet at 51.5, 63, and 100 g/cow/d to provide 20 g/d of digestible Lys, respectively. Feeding the diNALL and the AP diets decreased DMI. Milk yield was not altered by RPLys supplementation. Milk composition (%) and milk component yields were not affected by RPLys supplementation. In contrast, milk production efficiency (milk yield/DMI) increased with
the diNALL diet in comparison to the CL diet. Body weight change was not affected by RPLys supplementation. The diNALL diet shifted net energy partitioning (% energy intake) toward milk production. Dietary treatments had similar plasma AA, N utilization, and rumen NH₃-N concentrations. Overall, results in the current study suggest that diNALL and AP decreased DMI leading to improved milk production efficiency. However, based on the current findings, the potential effects of RPLys on DMI regulation warrant further investigation as stage of lactation and physiological changes occurring through stage of lactation may have alter DMI.

**Key Words:** N-acetyl-L-lysine, feed efficiency, lactational performance, rumen-protected lysine

**INTRODUCTION**

Lysine is commonly considered the first limiting essential AA (EAA) in dairy cows fed a corn-based ration or rations that include corn by-products, such as dried distillers grains with solubles (Schingoethe et al., 2009). Lysine’s main biological role in dairy cattle is to promote protein synthesis. Lysine may be used for weight gain in growing dairy cattle, milk protein production in lactating dairy cows, incorporation into mammalian tissues for structural integrity, or serve as a carbon backbone for carnitine synthesis (NRC, 2001; Vaz and Wanders, 2002). However, for Lys products to exert their biological effects in dairy cows, they must be able to avoid microbial degradation in the rumen and supply post-ruminal Lys for small intestinal absorption.
An effective method to increase post-ruminal Lys supply and improve post-ruminal Lys absorptive efficiency is to feed rumen-protected Lys (RPLys). Inconsistent post-ruminal polymer degradation and high production costs have limited the development of RPLys products (Swanepoel et al., 2010). However, since the early 1990s rumen stable AA matrixes have been developed and numerous RPLys products have become available for commercial use in animal agriculture (Robinson, 2009b). Available commercial RPLys products use encapsulation, matrix technology, or a combination of both to protect a Lys core from ruminal degradation (Ji et al., 2016). These RPLys products are often coated by a series of lipid or fatty acid calcium-based salts (Ji et al., 2016). Wu and Papas (1997) found that RPLys coating protection efficacy in the rumen depends on coating composition, pellet surface smoothness, inner core pellet strength, and pellet solubility and size. Recently, the production of Lys derivatives, a new form of RPLys may serve as an alternative to physically protected lipid RPLys products.

The N-acetyl-L-Lys (NALL) is a Lys derivative produced via protection of the L-Lys α-amino (Nα, ε-acetyl-L-Lys; diNALL) or ε-amino (Nε-acetyl-L-Lys; εNALL) group with an N-acetyl group. The biological availability of N-substituted Lys products has previously been described (Finot et al., 1978). The biological availability for diNALL and εNALL is 0 and 50% in the rat, respectively (Neuberger and Sanger, 1943; Bjarnason and Carpenter, 1969). According to Finot et al. (1978) the Lys derivatives which are partially utilized as Lys sources in rats are Nε-formyl-L-Lys and εNALL. They belong to the group of Nε-acyl-L-Lys and are negligibly hydrolyzed by homogenates of the intestinal mucosa and liver, and only slightly so by the kidneys of the rat (Finot et al., 1978). Paik
and Benoiton (1963) and Leclerc and Benoiton (1968) found that it is the renal ε-Lys acylase which is responsible for Ne-formyl-L-Lys and εNALL hydrolysis. It is anticipated that εNALL will be biologically available in lactating dairy cows, however, there is no research reported on the effects of supplementing NALL in lactation diets on production and ruminal fermentation in dairy cows.

The objective of the present study was to investigate lactational performance and nutrient utilization by mid to late lactating dairy cows supplemented with 3 different forms of RPLys. We hypothesized that supplementing RPLys to mid to late lactation dairy cows would increase post-ruminal Lys supply and increase milk protein synthesis and milk yield.

MATERIALS AND METHODS

The dairy cows used in the present study were cared for according to the Live Animal Use in Research Guidelines of the Institutional Animal Care and Use Committee at Utah State University. The study was conducted at the Caine Dairy Research Center (Wellsville, UT), Utah State University from July to September, 2017.

Cows, Experimental Design, and Diets

Forty multiparous lactating Holstein dairy cows were used during the trial. Cows were tested in mid to late lactation and began the experiment averaging 3.6 ± 1.21 parity,
151 ± 78.0 DIM and producing 39.6 ± 6.89 kg milk/d. The experiment was performed as a randomized complete block design. Cows were grouped into 10 blocks of 4 cows by parity and DIM. Cows were fed the same control diet (CL) for a 2-wk adaptation period. Cows were then randomly assigned within blocks to 1 of the 4 experimental diets and fed assigned diets throughout the remaining 8 wk of the trial (entire study duration was 70 d). Experimental diets were based on alfalfa hay, corn silage, and dried distillers grains with solubles. Table 2-1 lists the chemical composition of the 4 dietary treatments. The control diet was formulated to contain 15.3% CP and supply adequate MP (+ 10 g/d balance). The dietary treatments fed in the study are as follows; 1) CL = normal CP (15.3%) without RPLys, 2) εNALL = normal CP with εNALL (51.5 g/d), 3) diNALL = normal CP with diNALL (63 g/d), and 4) AP = normal CP with AjiPro®-L (100 g/d).

The developmental NALL products from CJ CheilJedang (Seoul, South Korea) were used as a supplemental source of RPLys in this study. Nε-acetyl-L-Lys and diNALL are protected from rumen degradation with an N-acetyl group attached to either the α- or the ε-amino group. The εNALL and diNALL products were added at a rate of 51.5 and 63 g/d, respectively. The rate was determined for lactating dairy cows based on manufacturer’s recommendation in order to supply 20 g of digestible Lys to the small intestine. The recommendation of required digestible Lys and Met were assumed as 6.6 and 2.2% of the MP requirements, respectively (NRC, 2001). The RPLys products were top-dressed onto corresponding experimental diets at each a.m. feeding. Diets were formulated based on NRC (2001) recommendations to provide sufficient NE\textsubscript{L}, MP,
vitamins, and minerals with an estimated intake of 25.0 kg/d to produce 36 kg/d of milk with 3.5% fat and 3.0% true protein (Table 2-1).

Cows were housed individually in tie stalls fitted with rubber mattresses covered with straw, allowing free access to water. Cows were individually fed twice daily for ad libitum intake at a level of 110% expected daily intake with 60% of allotted feed fed at 0800 h and 40% fed at 1500 h. Feed offered and refused were recorded daily, and feed samples were taken during each week of the trial to determine DMI. Cows were milked twice daily at 0600 and 1600 h, and milk production was recorded throughout the entire experiment.

**Energy Partitioning Calculations**

Energy partitioning was determined during sampling weeks using data of milk yield, milk composition, and BW of experimental animals. Cows were weighed for 2 consecutive d after the a.m. milking and before the a.m. feeding at the end of weeks 2, 4, 6, 8 and 10. These weights were used to calculate the mean BW of cows for each sampling week. Energy used for maintenance was calculated as $BW^{0.75} \times 0.08$ (NRC, 2001). Energy of BW change was assumed to be 5.114 Mcal/kg of gain or 4.924 Mcal/kg of loss (NRC, 2001). Milk energy was calculated as $(0.0929 \times \text{milk fat concentration}) + (0.0563 \times \text{milk true protein concentration}) + (0.0395 \times \text{milk lactose concentration})$ (NRC, 2001). Estimated NE₅ value was calculated by total net energy utilization (maintenance, BW gain, and milk) divided by DMI (Neal et al., 2014).
Feed and Milk Samplings and Analyses

Samples of alfalfa hay, corn silage and dried distiller grains were taken weekly to determine DM, and diets were adjusted accordingly for change in forage DM concentration. Samples were ground to pass a 1-mm screen (standard model 4; Arthur H. Thomas Co., Swedesboro, NJ), and stored for chemical analysis. Samples of TMR and orts were collected from individual cows on d 3 and d 4, at the end of the adaptation period and sampling weeks (3 through 10) composited, dried at 60°C for 48 h, and ground as previously described. The DM concentrations of samples were used to calculate intakes of DM and nutrients.

Analytical DM concentration of samples was determined by oven drying overnight at 105°C, and OM was determined by ashing at 550°C for 5 h (AOAC, 2000; method 942.05). Concentration of CP was determined using an automated N combustion analyzer (Elementar, Analysensysteme GmbH, Hanau, Germany; AOAC, 2000; method 968.06). Concentrations of NDF and ADF were sequentially determined using a fiber analyzer (200/220, ANKOM Technology, Macedon, NY) according to the methodology supplied by the company, which is based on the methods described by Van Soest et al. (1991). Sodium sulfite was used in the procedure for NDF determination and pre-treated with heat-stable amylase (Type XI-A from Bacillus subtilis; Sigma-Aldrich Corporation, St. Louis, MO). Ether extract was measured using a fat analyzer (XT20, ANKOM Technology; AOAC, 2000; method 2003.05). In addition, samples of TMR were
analyzed for starch by Dairyland Laboratories, Inc. (Arcadia, WI) according to Knudsen (1997).

Milk was sampled for 2 consecutive d (d 3 and 4) during the a.m. and p.m. milkings each week. Individual milk samples were analyzed by the Rocky Mountain DHIA Laboratory (Logan, UT) for fat, true protein, lactose, and MUN. Milk composition was expressed on weighted milk yield of a.m. and p.m. samples. Milk fat and protein yields were calculated by multiplying milk yield from the respective day by fat and true protein concentration of the milk from an individual cow.

**Blood Samplings and Analyses**

Blood samples were collected immediately before the morning feeding and at 1400 h into 10-mL EDTA plasma vacuum tubes from the coccygeal artery or vein on d 6 and 7 in each sampling week (4, 6, 8, and 10) and placed on ice. Blood samples were centrifuged at 2,300 × g for 20 min, and plasma was collected and stored at −40°C for subsequent AA analysis. The plasma samples were prepared for AA analysis using the EZ:faast GC-FID Free (Physiological) AA Analysis kit (Phenomenex Inc., Torrance, CA). Concentrations of plasma free AA were determined in accordance with the user manual provided with the kit. Extraction of free AA from the plasma consisted of a combination of solid-phase extraction, derivatization, and liquid/liquid extraction. The organic phase containing the AA in question were analyzed using a GC (model 5890, Hewlett-Packard Co, Avondale, PA) with a capillary column (30 m × 0.32-mm i.d., 1-μm
phase thickness, Zebron ZB-FAAP; Phenomenex Inc., Torrance, CA) and flame-ionization detection.

**Ruminal Fermentation Characteristics**

Ruminal fluid samples were obtained using a Geishauser probe at 3 h after the morning feeding on d 5 and 6 of each sampling week (4, 6, 8, and 10). The fluid was collected with a solid, tube-like probe with rows of small holes on the end (Geishauser, 1993). The first 100 mL of ruminal fluid was discharged to avoid contamination from saliva, and then 10 mL was collected for analysis. The pH of the ruminal fluid was measured within 5 min of collecting the samples using a portable pH meter (Oakton pH 6; Oakton Instruments, Vernon Hills, IL). Five milliliters of the ruminal fluid were mixed with 1 mL of 1% sulfuric acid and stored frozen (−40°C) for ammonia-N (NH₃-N) analysis. Concentration of NH₃-N in the ruminal contents was determined as described by Rhine et al. (1998), using a plate reader (MRXe; Dynex Technologies Inc., Chantilly, VA). Another 5 mL of the ruminal fluid was collected and mixed with 1 mL of 25% metaphosphoric acid, and then stored at −40°C for VFA content determination. Ruminal VFA were separated and quantified using a GC (model 6890 series II; Hewlett-Packard Co.) with a capillary column (30 m × 0.32-mm i.d., 1-μm phase thickness, Zebron ZB-FAAP; Phenomenex Inc., Torrance, CA) and flame-ionization detection. The oven temperature was held at 170°C for 4 min, increased to 185°C at a rate of 5°C/min, then increased by 3°C/min to 220°C, and held at this temperature for 1 min. The injector and
the detector temperatures were 225 and 250°C, respectively, and the carrier gas was helium (Eun and Beauchemin, 2007).

Statistical Analysis

Statistical analyses of all data except BW change, N excretion, AA and VFA were conducted using the Proc Mixed procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC) with a repeated measures model that included the covariate mean for each trait for each cow, plus block, treatment, week, and the interaction of treatment × week. A similar approach was used for BW change, N excretion, AA and VFA data, except the covariate was removed from the model. Overall, treatment differences were examined using least squares means with the lowest standard error. Week and block were included in the final statistical model for all analyses. The PDIFF option of SAS (version 9.4; SAS Institute Inc., Cary, NC) was used to test treatment differences among least squares means, and the SLICE option was used to analyze treatment differences among weekly treatment means. Differences were considered significant at $P \leq 0.05$, and trends were discussed at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Characteristics of Diet Composition and Treatment
Ingredients and chemical composition of experimental diets are presented in Table 2-1. The CL diet had a CP content of 15.3%, which provided an adequate MP supply of 2,910 g/d (+ 10 g/d balance), whereas the RPLys supplemented diets provided an MP supply of 2,930 g/d (+ 30 g/d balance) according to NRC (2001). Corn distillers grains were added to the CL diet at an inclusion rate of 21% of DM in order to achieve a Lys balance of – 20 g/d. High inclusion rates of corn distillers grains above 20% of DM have been shown to increase the amount of rumen biohydrogenation intermediates (Hippen et al., 2004) and may decrease milk fat concentration. Therefore, caution was taken to ensure that the NDF concentration in the diets remained near NRC (2001) recommendation of 25 to 33% of diet DM as total NDF. The RPLys supplemented diets maintained a Lys balance of – 1 g/d (0 g/d Lys balance targeted) by providing 20 g of metabolizable Lys to the diets based on manufacturers recommendation for each Lys treatment. All diets supplied NE\textsubscript{L} in excess of requirement (NRC, 2001).

The concentrations of Lys and Met in MP was 6.6 and 2.2%, respectively. Digestible Lys and Met concentrations in MP (dLys:dMet) were maintained at 3.05:1 for RPLys supplemented diets and 2.73:1 for the CL diet. The dLys:dMet ratio for optimal milk and milk protein yield is reported in the NRC as 3.0:1 (NRC, 2001), but ratios as low as 2.69:1 have been reported (Doepel et al., 2004; Van Amburgh et al., 2015).

*Feed Intake, Productive Performance, and Net Energy Utilization*
Feeding the diNALL and the AP diets reduced intake of DM compared to the CL diet (Table 2-2). Responses to supplementation of RPLys on feed intake in the literature are limited. Paz et al. (2014) reported that RPLys supplementation did not affect DMI in lactating dairy cows, which agrees with Blauwiekel et al. (1997) and Robinson et al. (2011). Swanepoel et al. (2010) found that supplementation of RPLys did not influence DMI in early lactating cows (77 ± 3.2 DIM); however, the mid lactation group (262 ± 6.70 DIM) had a tendency ($P = 0.09$) toward lower DMI with RPLys supplementation. The mechanism by which RPLys reduces DMI in mid lactation cows is unknown, but the authors suggested that RPLys possibly affected animal metabolism, which reduced digestion in the lower digestive tract (Swanepoel et al., 2010).

A variety of factors such as forage quality, TMR nutrient balance, feed delivery, feeding method, TMR palatability, ration DM content, environmental conditions, housing facilities, and managerial practices can impact DMI (Allen and Bradford, 2009), which might explain the lack of response seen with εNALL supplementation. In the current study, stage of lactation and week of trial may have altered DMI in cows fed the diNALL and AP diets. This is because energy requirements decrease during mid lactation and control of feed intake by gut distension gradually diminishes, while control by hepatic oxidation increases (Allen and Bradford, 2009). Therefore, physiological changes occurring through stage of lactation and the physical and chemical characteristics (NDF concentration) of feeds beyond their nutrient composition can alter DMI (Allen and Bradford, 2009).
Supplementation of RPLys did not alter milk yield (Table 2-2). Milk, 3.5% FCM, and ECM yields were not affected by dietary treatments. Swanepoel et al. (2010) observed that dairy cows supplemented with RPLys at a rate of 41 g/cow/d did not affect milk yield in early lactation. Paz and Kononoff (2014) did not detect a difference in milk yield when RPLys was supplemented to diets with altering inclusion rates of low-fat dried distillers grains (15 vs. 30% of diet DM). Furthermore, cows supplemented with RPLys (37 g/cow/d) after peak lactation showed no improvement in milk yield (Bernard et al., 2014). In contrast, Robinson et al. (2011) found a 2 kg increase in milk yield when RPLys was supplemented to deliver 15 to 21 g of MP Lys. In the present study, there was no improvement in milk yield in response to RPLys supplementation, thus indicating that RPLys was not utilized in the mammary gland of mid to late lactating dairy cows for milk production. The RPLys supplemental dosage rate, lack of a digestible Lys delivery to the small intestine for absorption, or reduced DMI may be responsible for the absence of production responses seen in the current study.

Milk composition (%) and milk component yields (kg/d) were not affected by dietary treatments (Table 2-2). Swanepoel et al. (2010) reported reduced milk fat synthesis to feeding a RPLys product to high producing dairy cows. Třináctý et al. (2009) fed RPLys to lactating Holstein dairy cows (33.5 kg/d) and while milk protein concentration increased, milk fat concentration and yield declined. A review of literature by Robinson (2010) stated that RPLys supplementation generally results in minimal production responses, possibly due to an AA imbalance or poor AA delivery to the intestinal absorptive site. The productive benefits of RPLys supplementation on overall
performance effects in lactating dairy cows can only be judged as negative (Robinson, 2010). However, increased Lys supply appear important in early lactation when MCP synthesis is reduced because of lower DMI, to help drive milk and milk protein production (Robinson et al., 2011). Stage of lactation (mid to late) or lack of a digestible Lys deficiency in the diets supplemented with RPLys may be responsible for the absence of production responses seen in the current study.

The diNALL diet reduced intake of DM and maintained milk yield resulting in better feed efficiency for milk production compared to the CL diet (1.54 vs. 1.33; Table 2-2). Wang et al. (2010) supplemented L-Lys-HCl at 0.50% DM to a control diet and detected increased milk yield with no increase in DMI, improving feed efficiency to 1.34 vs. 1.27 for the control diet. As previously mentioned, the biological availability for diNALL and εNALL is 0 and 50% in the rat based on growth rates (Neuberger and Sanger, 1943). The improvement in feed efficiency for milk production with diNALL supplementation demonstrate that, unlike in the rat, diNALL may be biologically available in lactating dairy cows. Additionally, the increased feed efficiency observed in the current study suggest that diNALL supplementation would be favorable in mid to late lactating cows, but only when fed to a diet that has a digestible Lys deficit. Based on the current findings the biological availabilities of diNALL and εNALL in lactating dairy cows warrant further investigation.

No changes were seen in either BW gain or net energy utilization with supplementation of RPLys in the diets (Table 2-2). However, feeding the diNALL diet reduced BW gain relative to the CL diet, leading to an increased net energy partitioning
for milk. No effect on BW gain due to diNALL supplementation suggests that energy was primarily partitioned toward milk production rather than BW gain in mid to late lactation in the current study.

**Plasma AA Profiles**

Plasma AA concentrations (EAA and NEAA) were not affected by RPLys supplementation (Table 2-3). The plasma Lys concentrations observed in the current study (Table 2-3) agree with Swanepoel et al. (2010), who did not detect a difference in plasma Lys concentrations of mid lactating dairy cows supplemented with RPLys at 41 g/cow/d. Since, RPLys was supplemented to the CL diets, an increase in plasma Lys concentration was expected. However, because digestible Lys requirements were met, the absorbed Lys may have been used for biological functions other than milk production, converted to metabolites or not utilized. Lysine is an AA with a high fractional extraction rate by the mammary gland in ruminants (Manjarin et al., 2014). The primary metabolic fate of Lys in the mammary gland is for milk and milk protein synthesis. Mabjeesh et al. (2000) observed that the uptake of Lys in the mammary gland of dairy cows is greater than milk Lys output. The plasma Lys concentration along with changes in production parameters (i.e., milk yield) for the diNALL diet indicates that plasma Lys may have been utilized for improved milk yield. Since, plasma AA profiles were not altered by RPLys supplementation, further research is needed to determine the impact of Lys derivative supplementation on plasma AA profiles in lactating dairy cows.
**Utilization of N**

Intake of N in the current study followed DMI, meaning that cows fed the diNALL and the AP diets consumed less N than those fed the εNALL and CL diets (Table 2-4). Supplementation of RPLys products do not often increase N intake because of either a lower dietary CP or MP concentration in basal diets associated with RPLys or a combination of RPAA supplementation (Leonardi et al., 2003; Broderick et al., 2009). Lee et al. (2015) supplemented a MP-deficient diet (MP balance of −281 g/d; CP of 13.7% DM); with 100 g of RPLys/cow/d (estimated digestible Lys supply = 24 g/d) and reported no increase in N intake compared to the MP-deficient diet. Supplementation of RPLys had no effect on milk N excretion in the current study. Milk N efficiency is primarily driven by feed N intake (Huhtanen and Hristov, 2009). Although the N intake was reduced for the diNALL and the AP diets, no change in milk N excretion after supplementation of RPLys resulted in milk N:N intakes that were similar across all diets (0.32 on average). The N utilization efficiencies reported in the current study are similar to the average N efficiencies (25 to 35%) reported in the literature (Hristov et al., 2004). The lack of response in milk N to dietary treatments is related to DMI and milk yield seen in the current study.

Concentrations of MUN and NH$_3$-N were not affected by dietary treatments. Urea-N (milk, serum, and urine) in cattle is derived from excess NH$_3$-N absorbed through the rumen wall from rumen CP degradation and deamination of AA (Linn and Olson, 1995;
Wang et al., 2008). Wang et al. (2010) supplemented L-Lys-HCl at 0.50% of DM to a control diet that was adequate in energy, but slightly limiting in MP and detected increased urea-N concentration in serum, urine, and milk compared with the control diet. In the present study, supplementation of RPLys maintained MUN and NH$_3$-N concentrations, indicating that the RPLys diets may have provided a more favorable AA balance and resulted in less deamination of absorbed AA.

Urinary and fecal N excretion were not affected by dietary treatments. However, manure N excretion (urinary N excretion, g/d + fecal N excretion, g/d) decreased for the diNALL and the AP diets. Urinary and fecal N are directly correlated with DM and N intakes (Huhtanen et al., 2008). Therefore, because of the reduced N intake of animals fed the diNALL and the AP diets, manure N excretion decreased. The reduced manure N excretion in the current study indicates the potential use of supplementing diNALL to lactating dairy cows to aid in environmental N management on dairy farms, however further lactation trials are needed to determine how NALL products impact N utilization in lactating dairy cows.

**Ruminal Fermentation Characteristics**

Supplementation of RPLys in the εNALL, the diNALL and the AP diets reduced ruminal pH and ranged from 6.15 to 6.23, compared to the CL diet (pH = 6.42; Table 2-5). The mean ruminal pH from all dietary treatments were close to or above 6.2, so RPLys influence on overall microbial composition may be negligible. The effects of
RPLys supplementation on ruminal pH is not well defined in the literature. Recently, Lee et al. (2015) determined that RPLys supplementation to an MP-deficient diet did not alter ruminal pH in mid-lactation dairy cows.

Total and individual VFA concentrations were similar across dietary treatments. According to Lee et al. (2015), rumen fermentation variables (total and individual VFA) were not affected by RPLys supplementation to a MP-deficient diet. A previous study performed by our group (Menchu, 2019), estimated the ruminal escape for the NALL products used in the current study. The ruminal escape (% of dose) averaged 43.5 for both NALL products, indicating that the NALL chemical structure is altered in the rumen environment. The absence of response in VFA profiles to \( \varepsilon \)NALL and diNALL dietary treatments, suggests that NALL lacks a ruminal mode of action and further research is needed to understand the metabolism of NALL in the rumen.

**CONCLUSIONS**

Results of the current study show that feeding the diNALL diet in mid to late lactation dairy cows decreased DMI, leading to greater feed efficiency compared to the CL diet. Milk composition, milk component yields, AA, and VFA profiles were not affected by RPLys supplementation. Net energy shifted toward milk production for the diNALL treatment. Rumen-protected Lys supplementation did not alter utilization of N by lactating Holstein dairy cows in the present study. These findings suggest that the developmental NALL products may be biologically active in lactating dairy cows, with
diNALL showing the most benefit to improve feed efficiency. However, further research is needed to discover the role of Lys derivatives in lactating dairy cows on productive performance, rumen metabolism, and bioavailability.

ACKNOWLEDGMENTS

This study was supported by CJ CheilJedang (Seoul, South Korea). The authors thank S. Sharp at Utah State University (Logan) for technical assistance and the staff of the Caine Dairy Research Center (Wellsville, UT) for their care and handling of the experimental cows.

REFERENCES


Geishauser, T. 1993. An instrument for the collection and transfer of ruminal fluid and for the administration of water soluble drugs in adult cattle. Bovine Pract. 27:38–42.


Table 2-1. Ingredient and chemical composition of the experimental diets supplemented with rumen-protected lysine fed to lactating Holstein dairy cows (n = 40)

<table>
<thead>
<tr>
<th>Item</th>
<th>CL</th>
<th>eNALL</th>
<th>diNALL</th>
<th>AP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredient, % of DM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alfalfa hay</td>
<td>19.6</td>
<td>19.5</td>
<td>19.5</td>
<td>19.5</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>4.91</td>
<td>4.90</td>
<td>4.90</td>
<td>4.89</td>
</tr>
<tr>
<td>Corn silage</td>
<td>26.4</td>
<td>26.4</td>
<td>26.4</td>
<td>26.3</td>
</tr>
<tr>
<td>Corn grain (steam-flaked)</td>
<td>13.9</td>
<td>13.9</td>
<td>13.9</td>
<td>13.9</td>
</tr>
<tr>
<td>Corn distillers grain</td>
<td>21.0</td>
<td>21.0</td>
<td>21.0</td>
<td>20.9</td>
</tr>
<tr>
<td>Cottonseed, whole</td>
<td>4.86</td>
<td>4.84</td>
<td>4.84</td>
<td>4.83</td>
</tr>
<tr>
<td>Beet pulp, shreds</td>
<td>6.54</td>
<td>6.53</td>
<td>6.53</td>
<td>6.52</td>
</tr>
<tr>
<td>Lysine supplement^2</td>
<td>–</td>
<td>0.20</td>
<td>0.25</td>
<td>0.40</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>0.63</td>
<td>0.63</td>
<td>0.63</td>
<td>0.63</td>
</tr>
<tr>
<td>Vitamin and mineral mix^3</td>
<td>2.11</td>
<td>2.10</td>
<td>2.10</td>
<td>2.09</td>
</tr>
<tr>
<td>Chemical composition, % of DM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM, %</td>
<td>57.6±1.88</td>
<td>58.1±1.93</td>
<td>57.8±2.74</td>
<td>58.2±2.80</td>
</tr>
<tr>
<td>OM</td>
<td>91.2±0.38</td>
<td>91.3±0.48</td>
<td>91.7±1.22</td>
<td>91.1±0.58</td>
</tr>
<tr>
<td>CP</td>
<td>15.3±0.20</td>
<td>15.4±0.25</td>
<td>15.4±0.51</td>
<td>15.4±0.36</td>
</tr>
<tr>
<td>RDP^4</td>
<td>8.58</td>
<td>8.64</td>
<td>8.64</td>
<td>8.64</td>
</tr>
<tr>
<td>RUP^4</td>
<td>6.72</td>
<td>6.76</td>
<td>6.76</td>
<td>6.76</td>
</tr>
<tr>
<td></td>
<td>1st Trial</td>
<td>2nd Trial</td>
<td>3rd Trial</td>
<td>4th Trial</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td>NDF</td>
<td>34.6 ± 1.98</td>
<td>34.2 ± 2.46</td>
<td>34.3 ± 1.65</td>
<td>34.2 ± 2.99</td>
</tr>
<tr>
<td>ADF</td>
<td>18.6 ± 1.37</td>
<td>18.0 ± 1.36</td>
<td>18.0 ± 0.90</td>
<td>18.0 ± 1.64</td>
</tr>
<tr>
<td>Starch</td>
<td>19.5 ± 0.65</td>
<td>19.4 ± 1.02</td>
<td>19.4 ± 0.99</td>
<td>19.5 ± 0.54</td>
</tr>
<tr>
<td>Ether extract</td>
<td>3.38 ± 1.06</td>
<td>4.58 ± 0.91</td>
<td>3.96 ± 1.39</td>
<td>4.10 ± 1.29</td>
</tr>
<tr>
<td>NFC</td>
<td>37.9 ± 2.56</td>
<td>37.1 ± 3.21</td>
<td>38.0 ± 3.10</td>
<td>37.4 ± 3.43</td>
</tr>
<tr>
<td>NE_L</td>
<td>1.74</td>
<td>1.74</td>
<td>1.74</td>
<td>1.74</td>
</tr>
<tr>
<td>Protein supply</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MP supply</td>
<td>2,910</td>
<td>2,930</td>
<td>2,930</td>
<td>2,930</td>
</tr>
<tr>
<td>MP requirement</td>
<td>2,900</td>
<td>2,900</td>
<td>2,900</td>
<td>2,900</td>
</tr>
<tr>
<td>MP balance</td>
<td>10</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Lys and Met balance</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dLys requirement</td>
<td>192</td>
<td>193</td>
<td>193</td>
<td>193</td>
</tr>
<tr>
<td>dLys supplied by the diet</td>
<td>172</td>
<td>172</td>
<td>172</td>
<td>172</td>
</tr>
<tr>
<td>dLys from Lys supplement</td>
<td>0</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Lys balance</td>
<td>-20</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>dMet requirement</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>dMet supplied by the diet</td>
<td>63</td>
<td>63</td>
<td>63</td>
<td>63</td>
</tr>
<tr>
<td>Met balance</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>dLys:dMet</td>
<td>2.73</td>
<td>3.05</td>
<td>3.05</td>
<td>3.05</td>
</tr>
</tbody>
</table>
\[ \text{CL} = \text{control diet; \( \varepsilon \text{NALL} = \text{Control + \( \varepsilon \text{NALL} \) (CJ CheilJedang, Seoul, Korea); diNALL=} \text{Control + diNALL (CJ CheilJedang, Seoul, Korea); and AP = Control + AjiPro}^{\circledR}\text{-L v2 (Ajinomoto, Japan).} \]

\[ \text{Developmental NALL products (\( \varepsilon \text{NALL and diNALL} \) (CJ CheilJedang, Seoul, Korea) and a commercial RPLys product (AjiPro}^{\circledR}\text{-L v2) (Ajinomoto, Japan) were used as the sources of rumen-protected lysine.} \]

\[ \text{Formulated to contain (per kg DM): 226.7 mg of Se (from sodium selenite), 9278.7 mg of Cu (from copper amino acid complex), 40,537.4 mg of Zn (from zinc amino acid complex), 38,653.4 mg of Mn (from manganese amino acid complex), 552.6 mg of Co (from cobalt carbonate), 1,234,585.2 IU of vitamin A, 152,808.1 IU of vitamin D, 3,815.1 IU of vitamin E, and 295 mg of Rumensin (Elanco Animal Health, Greenfield, IN).} \]

\[ \text{Based on tabular value (NRC, 2001).} \]

\[ \text{NFC = 100 – CP – NDF – ether extract – ash.} \]

\[ \text{All values were estimated using NRC (2001) based on actual DMI, milk yield, milk composition, and BW of the cows throughout the trial.} \]

\[ \text{Digestible Lys (dLys) and digestible Met (dMet) supply from the diets were estimated using NRC (2001); supply of dLys from RPLys products were estimated by data provided the manufacturer. Requirements of dLys and dMet were calculated as 6.6 and 2.2\% (respectively) of MP requirements.} \]

\[ \text{Digestible Lys from diet-to-digestible Met from diet ratio.} \]
Table 2-2. Dry matter intake, productive performance, body weight (BW) change, and net energy utilization of mid to late lactation Holstein dairy cows supplemented with rumen-protected lysine

<table>
<thead>
<tr>
<th>Item</th>
<th>Diet&lt;sup&gt;1&lt;/sup&gt;</th>
<th>CL</th>
<th>εNALL</th>
<th>diNALL</th>
<th>AP</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intake, kg/d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td></td>
<td>26.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>24.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.41</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Yield, kg/d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk</td>
<td></td>
<td>36.4</td>
<td>36.6</td>
<td>37.1</td>
<td>35.6</td>
<td>1.16</td>
<td>0.83</td>
</tr>
<tr>
<td>3.5% FCM</td>
<td></td>
<td>36.2</td>
<td>36.5</td>
<td>35.8</td>
<td>35.6</td>
<td>1.83</td>
<td>0.98</td>
</tr>
<tr>
<td>ECM</td>
<td></td>
<td>36.5</td>
<td>36.9</td>
<td>36.5</td>
<td>35.7</td>
<td>1.62</td>
<td>0.96</td>
</tr>
<tr>
<td>Milk composition, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat</td>
<td></td>
<td>3.74</td>
<td>3.39</td>
<td>3.32</td>
<td>3.30</td>
<td>0.178</td>
<td>0.29</td>
</tr>
<tr>
<td>True protein</td>
<td></td>
<td>3.05</td>
<td>3.00</td>
<td>2.98</td>
<td>3.05</td>
<td>0.101</td>
<td>0.95</td>
</tr>
<tr>
<td>Lactose</td>
<td></td>
<td>4.88</td>
<td>4.72</td>
<td>4.79</td>
<td>4.85</td>
<td>0.055</td>
<td>0.17</td>
</tr>
<tr>
<td>Milk component yield, kg/d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat</td>
<td></td>
<td>1.29</td>
<td>1.26</td>
<td>1.22</td>
<td>1.23</td>
<td>0.086</td>
<td>0.91</td>
</tr>
<tr>
<td>True protein</td>
<td></td>
<td>1.09</td>
<td>1.09</td>
<td>1.11</td>
<td>1.04</td>
<td>0.034</td>
<td>0.50</td>
</tr>
<tr>
<td>Lactose</td>
<td></td>
<td>1.77</td>
<td>1.76</td>
<td>1.78</td>
<td>1.73</td>
<td>0.061</td>
<td>0.92</td>
</tr>
</tbody>
</table>

Dairy efficiency
<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk yield/DMI</td>
<td>1.33^b</td>
<td>1.46^{ab}</td>
<td>1.54^a</td>
<td>1.46^{ab}</td>
<td>0.046</td>
</tr>
<tr>
<td>3.5% FCM yield/DMI</td>
<td>1.33</td>
<td>1.44</td>
<td>1.49</td>
<td>1.46</td>
<td>0.070</td>
</tr>
<tr>
<td>ECM yield/DMI</td>
<td>1.35</td>
<td>1.45</td>
<td>1.51</td>
<td>1.47</td>
<td>0.061</td>
</tr>
<tr>
<td>BW</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial, kg</td>
<td>745</td>
<td>758</td>
<td>730</td>
<td>735</td>
<td>18.9</td>
</tr>
<tr>
<td>Mean, kg</td>
<td>769</td>
<td>772</td>
<td>730</td>
<td>746</td>
<td>18.8</td>
</tr>
<tr>
<td>Gain, kg/d</td>
<td>1.74</td>
<td>1.08</td>
<td>0.10</td>
<td>1.02</td>
<td>0.661</td>
</tr>
<tr>
<td>Calculated net energy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>values, Mcal/d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maintenance</td>
<td>11.7</td>
<td>11.7</td>
<td>11.2</td>
<td>11.4</td>
<td>0.21</td>
</tr>
<tr>
<td>BW gain</td>
<td>8.89</td>
<td>5.50</td>
<td>0.51</td>
<td>5.22</td>
<td>3.38</td>
</tr>
<tr>
<td>Milk</td>
<td>25.9</td>
<td>24.4</td>
<td>24.1</td>
<td>24.1</td>
<td>1.75</td>
</tr>
<tr>
<td>BW gain + milk</td>
<td>34.7</td>
<td>29.9</td>
<td>24.6</td>
<td>29.3</td>
<td>3.03</td>
</tr>
<tr>
<td>Total^2</td>
<td>46.4</td>
<td>41.6</td>
<td>35.9</td>
<td>40.8</td>
<td>3.05</td>
</tr>
<tr>
<td>NE_L,^3 Mcal/kg of DMI</td>
<td>1.74</td>
<td>1.56</td>
<td>1.43</td>
<td>1.62</td>
<td>0.113</td>
</tr>
<tr>
<td>Net energy partitioning,</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% energy intake</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maintenance</td>
<td>27.1</td>
<td>29.3</td>
<td>34.6</td>
<td>29.0</td>
<td>2.52</td>
</tr>
<tr>
<td>BW gain</td>
<td>12.3</td>
<td>10.3</td>
<td>-10.5</td>
<td>10.7</td>
<td>9.23</td>
</tr>
<tr>
<td>Milk</td>
<td>60.6</td>
<td>60.4</td>
<td>75.8</td>
<td>60.3</td>
<td>7.18</td>
</tr>
<tr>
<td>BW gain + milk</td>
<td>72.9</td>
<td>70.7</td>
<td>65.4</td>
<td>71.0</td>
<td>2.52</td>
</tr>
</tbody>
</table>
Means within a row with different superscripts differ ($P < 0.05$).

1CL= control diet; eNALL = Control + eNALL (CJ CheilJedang, Seoul, Korea);

diNALL= Control + diNALL (CJ CheilJedang, Seoul, Korea); and AP = Control +

AjiPro®-L v2 (Ajinomoto, Japan).

2Net energy used for maintenance, BW change, and milk.

3Calculated NE$_L$ = calculated total net energy, Mcal/d ÷ DMI (kg/d).
Table 2-3. Plasma AA concentrations (μmol/L) of mid to late lactation Holstein dairy cows supplemented with rumen-protected lysine

<table>
<thead>
<tr>
<th>Item</th>
<th>Diet(^1)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CL</td>
<td>εNALL</td>
<td>diNALL</td>
<td>AP</td>
<td>SEM</td>
<td>P</td>
</tr>
<tr>
<td>Arg</td>
<td>48.9</td>
<td>53.1</td>
<td>48.8</td>
<td>54.2</td>
<td>3.09</td>
<td>0.49</td>
</tr>
<tr>
<td>His</td>
<td>51.0</td>
<td>49.9</td>
<td>49.7</td>
<td>47.2</td>
<td>2.70</td>
<td>0.78</td>
</tr>
<tr>
<td>Ile</td>
<td>88.7</td>
<td>86.8</td>
<td>80.5</td>
<td>88.4</td>
<td>5.61</td>
<td>0.71</td>
</tr>
<tr>
<td>Leu</td>
<td>206</td>
<td>197</td>
<td>181</td>
<td>191</td>
<td>12.6</td>
<td>0.58</td>
</tr>
<tr>
<td>Lys</td>
<td>71.6</td>
<td>78.7</td>
<td>66.5</td>
<td>77.6</td>
<td>4.71</td>
<td>0.24</td>
</tr>
<tr>
<td>Met</td>
<td>20.7</td>
<td>20.7</td>
<td>20.3</td>
<td>20.5</td>
<td>1.04</td>
<td>0.99</td>
</tr>
<tr>
<td>Phe</td>
<td>44.0</td>
<td>43.5</td>
<td>41.0</td>
<td>43.7</td>
<td>1.77</td>
<td>0.60</td>
</tr>
<tr>
<td>Val</td>
<td>217</td>
<td>202</td>
<td>192</td>
<td>207</td>
<td>14.2</td>
<td>0.67</td>
</tr>
<tr>
<td>EAA(^2)</td>
<td>748</td>
<td>731</td>
<td>678</td>
<td>731</td>
<td>42.6</td>
<td>0.68</td>
</tr>
<tr>
<td>Ala</td>
<td>251</td>
<td>237</td>
<td>247</td>
<td>235</td>
<td>10.6</td>
<td>0.65</td>
</tr>
<tr>
<td>Gly</td>
<td>232</td>
<td>218</td>
<td>231</td>
<td>221</td>
<td>16.6</td>
<td>0.92</td>
</tr>
<tr>
<td>Ser</td>
<td>81.9</td>
<td>81.5</td>
<td>77.7</td>
<td>81.2</td>
<td>5.12</td>
<td>0.72</td>
</tr>
<tr>
<td>Tyr</td>
<td>55.9</td>
<td>55.3</td>
<td>51.6</td>
<td>57.6</td>
<td>3.58</td>
<td>0.68</td>
</tr>
<tr>
<td>NEAA(^3)</td>
<td>621</td>
<td>609</td>
<td>607</td>
<td>595</td>
<td>28.1</td>
<td>0.93</td>
</tr>
</tbody>
</table>

\(^1\)CL= control diet; εNALL = Control + εNALL (CJ CheilJedang, Seoul, Korea); diNALL = Control + diNALL (CJ CheilJedang, Seoul, Korea); and AP = Control + AjiPro\(^®\)-L v2 (Ajinomoto, Japan).
Essential amino acid analyzed.

Nonessential amino acid analyzed.
Table 2-4. Utilization of N by mid to late lactation Holstein dairy cows supplemented with rumen-protected lysine

<table>
<thead>
<tr>
<th>Item</th>
<th>Diet(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CL</td>
</tr>
<tr>
<td>N intake, g/d</td>
<td>613(^b)</td>
</tr>
<tr>
<td>Milk N,(^2) g/d</td>
<td>189</td>
</tr>
<tr>
<td>Milk N:N intake(^3)</td>
<td>0.31</td>
</tr>
<tr>
<td>MUN, mg/100 mL</td>
<td>11.2</td>
</tr>
<tr>
<td>NH(_3)-N, mg/100 mL</td>
<td>11.4</td>
</tr>
<tr>
<td>Urinary N excretion,(^5) g/d</td>
<td>224</td>
</tr>
<tr>
<td>Fecal N excretion,(^6) g/d</td>
<td>201</td>
</tr>
<tr>
<td>Manure N excretion,(^7) g/d</td>
<td>424(^b)</td>
</tr>
</tbody>
</table>

\(^{a,b}\) Means within a row with different superscripts differ \((P < 0.05)\).

\(^1\)CL = control diet; eNALL = Control + eNALL (CJ CheilJedang, Seoul, Korea); diNALL = Control + diNALL (CJ CheilJedang, Seoul, Korea); and AP = Control + AjiPro\(^®\)-L v2 (Ajinomoto, Japan).

\(^2\)Milk N (kg/d) = milk true protein (kg/d)/6.38 + MUN (kg/d).

\(^3\)Efficiency of use of feed N to milk N.

\(^4\)Ruminal ammonia-N.

\(^5\)Predicted using the equation: 0.026 × MUN, mg/100 mL × BW, kg (Wattiaux and Karg, 2004).
Predicted using the equation: N intake, g/d – urinary N excretion, g/d – milk N, g/d.

Manure N, g/d = urinary N excretion, g/d + fecal N excretion, g/d.
### Table 2-5. Ruminal fermentation characteristics of mid to late lactation Holstein dairy cows supplemented with rumen-protected lysine

<table>
<thead>
<tr>
<th>Item</th>
<th>Diet&lt;sup&gt;1&lt;/sup&gt;</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CL</td>
<td>εNALL</td>
<td>diNALL</td>
<td>AP</td>
<td>SEM</td>
<td>P</td>
</tr>
<tr>
<td>Ruminal pH</td>
<td>6.42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.06</td>
<td>0.02</td>
</tr>
<tr>
<td>Total VFA, mM</td>
<td>96.5</td>
<td>98.2</td>
<td>97.0</td>
<td>98.2</td>
<td>5.5</td>
<td>0.53</td>
</tr>
<tr>
<td>Individual VFA, mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate (A)</td>
<td>57.5</td>
<td>56.5</td>
<td>57.9</td>
<td>58.4</td>
<td>1.03</td>
<td>0.59</td>
</tr>
<tr>
<td>Propionate (P)</td>
<td>25.4</td>
<td>28.4</td>
<td>26.4</td>
<td>26.8</td>
<td>0.89</td>
<td>0.15</td>
</tr>
<tr>
<td>Butyrate</td>
<td>10.4</td>
<td>10.1</td>
<td>9.6</td>
<td>9.8</td>
<td>0.44</td>
<td>0.19</td>
</tr>
<tr>
<td>Valerate</td>
<td>1.53</td>
<td>1.57</td>
<td>1.52</td>
<td>1.50</td>
<td>0.07</td>
<td>0.60</td>
</tr>
<tr>
<td>Isobutyrate</td>
<td>0.87</td>
<td>0.83</td>
<td>0.86</td>
<td>0.92</td>
<td>0.05</td>
<td>0.22</td>
</tr>
<tr>
<td>Isovalerate</td>
<td>0.83</td>
<td>0.84</td>
<td>0.70</td>
<td>0.74</td>
<td>0.07</td>
<td>0.38</td>
</tr>
<tr>
<td>A:P</td>
<td>2.35</td>
<td>2.05</td>
<td>2.24</td>
<td>2.25</td>
<td>0.10</td>
<td>0.25</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Means within a row with different superscripts differ ($P < 0.05$).

<sup>1</sup>CL = control diet; εNALL = Control + εNALL (CJ CheilJedang, Seoul, Korea); diNALL = Control + diNALL (CJ CheilJedang, Seoul, Korea); and AP = Control + AjiPro<sup>®</sup>-L v2 (Ajinomoto, Japan).
CHAPTER 3

Bioavailability and lactational performance of dairy cows supplemented with N-acetyl-L-methionine at different doses

ABSTRACT

The present study investigated the bioavailability and lactational performance of dairy cows supplemented with N-acetyl-L-Met (NALM) at different doses. Six lactating dairy cows (75 ± 20.1 d-in-milk) were blocked by parity and d-in-milk. The experiment was performed as a replicated 3 × 3 Latin square design. Within each square, cows were randomly assigned to a sequence of 3 diets during each of the three 13-d periods (10 d of treatment adaptation and 3 d of data collection and sampling). The 3 dietary treatments are as follows: basal diet (15.7% CP) without NALM (control); control diet with 30 g/d of supplemented NALM (30NALM), and control diet with 60 g/d of supplemented NALM (60NALM). The NALM was rumen dosed for 11 d and then abomasally infused on d 12 and d 13 for quantification of NALM bioavailability (BA). Supplementation of NALM did not affect dry matter intake, milk production parameters, or feed efficiency. Supplementation of NALM led to a quadratic trend (P = 0.06) toward increases in plasma Arg, Gly, and Met concentrations and quadratic increases in plasma His and Ser concentrations. Rumen NALM dosing led to a quadratic increase in the baseline mean plasma Met concentration. Abomasal infusion with NALM increased the area under the
curve (AUC) versus control and both linear and quadratic increases in AUC were observed with NALM dosing. The mean AUC per Met unit ($\mu M \times h/L \div Met (g)$) for the control, 30NALM, and 60NALM were 4.68, 5.89, and 5.07, which corresponded to a Met BA ($AUC per Met unit \div AUC per Met unit_{control}$) of 100, 139, and 115%, respectively. The absence of plasma NALM and increases in plasma Met for both ruminal and abomasal NALM dosing in the current study suggests that NALM supplemented by either rumen placement or abomasal infusion to lactating dairy cows may have been deacetylated before entering the central circulation. In the current study, BA estimation by plasma Met was greater for NALM doses, indicating NALM improved plasma Met concentrations relative to the control. The absence of NALM in plasma, liver, milk, and muscle reveals that the N-acetyl group is cleaved from NALM prior to reaching central circulation and should be considered as a safe and effective BA rumen-protected Met source in lactating dairy cows.

**Key Words:** N-acetyl-L-methionine, bioavailability, lactational performance, area under the curve

**INTRODUCTION**

Protein nutrition of dairy cows in recent years has shifted from the simple use of dietary CP towards meeting the ammonia and AA needs of ruminal fermentation for MCP synthesis (Schwab and Broderick, 2017), while providing specific bypass AAs. Strategic balancing of individual AA in MP has also been used as a method to maximize
lactational performance, meet AA requirements of the cow, and deliver consistent post-ruminal AA, while minimizing total dietary CP supply (Lee et al., 2012a). When excess CP is fed and the AA profile within the CP supply does not match animal AA requirements, increases in environmental N excretion will result (Liu et al., 2017). Because Lys and Met have traditionally been recognized as the most limiting AA for lactating dairy cows (Schwab et al., 1992), research has focused on Met and Lys dietary supply in order to maximize milk production and milk component synthesis. However, balancing rations for only Lys and Met requirements may not improve productive responses, because additive effects of other AAs may be overlooked. Therefore, balancing rations for EAA requirements of dairy cows (not just Lys and Met) and for multiple-limiting AA may help to reduce overfeeding of protein, improve post-ruminal AA absorptive efficiency, reduce N excretion, and improve lactational performance (Liu et al., 2017).

Protection of free AAs from ruminal degradation dates to the 1960s when researchers began to understand the importance of balancing AA in order to meet dairy cow AA requirements (Schwab and Broderick, 2017). Numerous physically protected (coated or encapsulated) and chemical forms (Met analogues) of rumen-protected Met (RPMet) have been developed to escape ruminal degradation and provide post-ruminal digestible Met (dMet) for intestinal absorption (Schwab and Ordway, 2003). Production responses to supplemental RPMet products have been inconsistent and the biological mechanisms contributing to these responses are difficult to determine. However, infusing or
supplementing cows with RPMet products generally result in increased milk fat and protein yields (Zanton et al., 2014).

Recently, a new form of RPMet, a Met derivative, N-acetyl-L-Met (NALM) has been shown to increase milk fat composition and yield (Fagundes et al., 2018). Supplementation of NALM to early and mid-lactation dairy cows improved milk yield (Liang et al., 2019; Amaro et al., 2019). The studies note that NALM supplementation at 30 g/d resulted in the highest milk yield and these studies represent the initial attempts to measure production responses to supplemental NALM in lactating dairy cows.

The NALM is a Met derivative produced by chemical protection of the L-Met α-amino group with a N-acetyl group. In monogastric animals, the L-Met from NALM is metabolically equivalent to free L-Met (Rotruck and Boggs, 1975). Several animal studies conclude that the bioavailability (BA) of L-Met and NALM are similar based on feeding studies measuring growth rates and weight gain (Boggs et al., 1975; Young Jenkins et al., 1978; Friedman and Gumbmann, 1987). To date, there is no research reported on the effects of supplementing NALM to lactating dairy cows on BA.

Therefore, the objective of this study was to evaluate the BA and lactational performance of lactating dairy cows supplemented with NALM at 2 different doses. We hypothesized that Met supply from abomasal infusion of NALM would increase plasma Met concentration and the BA of NALM would be greater for each NALM dose compared to the control.
MATERIALS AND METHODS

The dairy cows used in the present study were cared for according to the Live Animal Use in Research Guidelines of the Institutional Animal Care and Use Committee at Utah State University. The study was conducted at the Caine Dairy Research Center (Wellsville, UT), Utah State University from March to May, 2019.

Cows, Experimental Design, and Diets

Three multiparous and three primiparous lactating Holstein dairy cows fitted with a rumen cannula were used in the trial. Cows began the experiment averaging 75 ± 20.1 DIM with 32.7 ± 5.28 kg/d of milk yield. The experiment was performed in a replicated 3 × 3 Latin square design. Within each square, cows were randomly assigned to a sequence of 3 diets during each of the three 13-d periods (10 d of treatment adaptation and 3 d of data collection and sampling). A washout period of 7 d was assigned before the start of subsequent periods to avoid carryover effects. Within each square, cows were randomly assigned to a sequence of 3 dietary treatments: basal diet (15.7% CP) without NALM (control); control diet with 30 g/d of supplemented NALM (30NALM), and control diet with 60 g/d of supplemented NALM (60NALM; Table 3-1).

The developmental NALM product from CJ CheilJedang (Seoul, South Korea) was used as the supplemental source of RPMet in this study. The NALM product was in
powder form, and contained Met concentration of 78.0%, with 99.5% purity. The NALM product was added at a rate determined for cows to consume 30 and 60 g/cow/d of Met in order to supply 15.6 and 31.2 g of dMet to the small intestine according to manufacturer’s recommendation, respectively. The rates were also based on previous research (Liang et al., 2019) and the 30 g/cow/d rate tested by Fagundes et al., (2018).

The recommendation of required digestible Lys and Met were assumed as 6.6 and 2.2% of the MP requirements, respectively (NRC, 2001; Table 3-2). The NALM product was placed through the rumen cannula at the liquid phase just under the rumen fiber mat daily at 0700 h during the 10 d of treatment adaptation and 1 d of data collection and sampling.

Diets were formulated based on the NRC recommendations to provide sufficient NE_L, ME, MP, minerals, and vitamins with an estimated DMI of 25.0 kg/d to produce 40 kg/d of milk with 3.8% fat and 3.0% true protein (NRC, 2001; Table 3-2). The basal diet was also formulated to balance Lys (2.0 g/d) and Met (0 g/d) recommendations according to NRC (2001). Cows were housed individually in tie stalls fitted with rubber mattresses covered with straw, allowing free access to water. Cows were individually fed twice daily for ad libitum intake at a level of 110% expected daily intake with 60% of allotted feed fed at 0600 h and 40% fed at 1500 h. Feed offered and refused was recorded daily, and feed samples were taken during the sampling week to determine DMI. Cows were milked twice daily at 0600 and 1600 h, and milk production was recorded throughout the entire experiment.
Feed and Milk Samplings and Analyses

Samples of alfalfa hay and corn silage were taken weekly to determine DM, and diets were adjusted accordingly for change in DM concentration. Samples were ground to pass a 1-mm screen (standard model 4; Arthur H. Thomas Co., Swedesboro, NJ), and stored for chemical analysis. Samples of TMR and orts were collected from individual cows on d 11 to d 13, composited by period, dried at 60°C for 48 h, and ground as previously described. The DM concentrations of samples were used to calculate intakes of DM and nutrients.

Analytical DM concentration of samples was determined by oven-drying overnight at 105 °C. Concentration of CP was determined using an automated N combustion analyzer (Elementar, Analysensysteme GmbH, Hanau, Germany; AOAC, 2000; method 968.06). Concentrations of NDF and ADF were sequentially determined using a fiber analyzer (200/220, ANKOM Technology, Macedon, NY) according to the methodology supplied by the company, which is based on the methods described by Van Soest et al. (1991). Ether extract was measured using a fat analyzer (XT20, ANKOM Technology; AOAC, 2000; method 2003.05). In addition, samples of TMR were analyzed for starch by Cumberland Valley Analytical Services (Waynesboro, PA).

Milk was sampled for 3 consecutive d (d 11 to 13) during the a.m. and p.m. milkings each period. Individual milk samples were analyzed by the Rocky Mountain DHIA Laboratory (Providence, UT) for fat, true protein, lactose, and milk urea nitrogen (MUN). Milk composition was expressed on weighted milk yield of a.m. and p.m. samples. Milk
fat and protein yields were calculated by multiplying milk yield from the respective day by fat and true protein concentration of the milk from an individual cow. A subset of milk samples from d 11 to d 13 of each period were freeze-dried, composited by cow, and stored for NALM and Met analysis by Bio Research Institute, CJ CheilJedang (Suwon, South Korea).

**BA, AA, and Plasma NALM Analyses**

To determine the BA of NALM, 15.6 g (30 g oral equivalent) or 31.2 g (60 g oral equivalent) of NALM was suspended in 1 L of water and bolus infused into the abomasum over 5 min at 0700 h on d 12 and 13 of each sampling period. An infusion line (Nalgene 980® braided clear PVC tubing; 6.4 mm i.d., 2.3 mm wall; Fisher Scientific, Waltham, MA) containing a distal flange (Spires et al., 1975) was passed through the sulcus omasi and placed in the abomasum via the abomasal orifice with an insertion and delivery device as described by Gressley et al., (2006). Infusion line placement was assured by hand to confirm the flange was in the abomasum and not the omasum. After the NALM bolus administration, the infusion line was rinsed with 250 mL of water to ensure full delivery of the NALM dose to the abomasum. Control infusion consisted of 1.25 L of water bolus infused into the abomasum.

Indwelling catheters were placed in the jugular vein after the a.m. milking on d 11 of each sampling period and catheter placement altered each period. Catheters were removed after the final blood sampling on d 13 of each period. Blood samples were
collected on d 11 at 0, 2, 4, 6, 8, and 10 h after rumen NALM dosing into 10-mL EDTA plasma vacuum tubes. The blood samples taken on d 11 were used to establish individual basal plasma Met concentration for each test animal before abomasal infusions on d 12 and d 13. Blood samples were collected on d 12 at 0, 1, 2, 3, 4, 5, 7, 9, 11, 13, 15 h and on d 13 at 0, 2, 4, 6, 8 and 10 h after NALM abomasal infusion into 10-mL EDTA plasma vacuum tubes. Samples were placed on ice and centrifuged at 2,300 × g for 20 min, after each sampling timepoint, and plasma was collected and stored at –40°C for subsequent plasma Met, NALM, and AA analyses. The plasma samples were prepared for AA and NALM analysis by filtration using an Amicon Ultra-0.5 Centrifugal Filter Unit (3KDa cutoff; Cat # UFC5003BK, Millipore, Burlington, MA) to remove plasma proteins. Flow-through samples were analyzed for Met and NALM using UPLC (Shimadzu Nexera, Seoul, South Korea).

Methionine BA of the NALM dose was determined from the plasma Met area under the curve (AUC) with respect to time for d 12 and 13 of each sampling period. The AUC was numerically estimated using the trapezoidal rule after subtraction of the mean of the pre-infusion concentrations from all values. The AUC was then expressed per gram of total Met concentration in the diet (AUC per Met unit). The ratio of the NALM dose (30 g or 60 g) AUC per Met unit over the control AUC per Met unit represented the Met BA of the NALM dose to the control. The NALM dose abomasally infused was adjusted for ruminal loss (33%), Met content (78%), purity (99.5%), and therefore the resulting AUC per Met Unit was expressed over the control for Met BA estimation of NALM. The control Met BA is assumed to be 100%, because the dMet (total Met in the basal diet)
supplied in the control diet is available to the animal for absorption. Results of BA were expressed as the mean percentage of the amount of Met supplied by NALM.

**Liver and Muscle Biopsies**

Liver and muscle biopsy samples were collected on d 12 of each sampling period post a.m. feeding. Liver biopsies were collected by the paracostal, percutaneous needle biopsy method as described by Mølgaard et al. (2012). Muscle biopsies were collected by surgical excision of a 1 cm³ *M. longissimus dorsi* sample, dorsal to the last rib under local anesthesia as described by Bradley (1978). Liver and muscle biopsy samples were freeze-dried and stored for tissue NALM and Met analysis by Bio Research Institute, CJ CheilJedang.

**Tissue and Milk NALM and Met Analyses**

Freeze-dried muscle (approximately 50 mg) and liver samples (liver sample was used in its entirety) were weighed and added to a lysis buffer consisting of 100 mM perchloric acid for muscle (700 µL) and liver (200 µL) and soaked at 4°C for 20 min. A tissue homogenizer pestle (Biomasher II® Closed System Disposable Tissue Homogenizer, NIPPI Inc., Tokyo, Japan) was used to crush the tissue samples and then the suspension was centrifuged at 13,000 × g for 15 min. The supernatant was collected and pH adjusted to 4.5 with KOH and filtered. Flow-through was collected and analyzed for NALM and
Met concentrations using a UPLC (Shimadzu Nexera) system as described by Hyde et al., (2004). Freeze-dried milk samples were dissolved in water (25 w%, 37°C) and then filtered using an Amicon Ultra-0.5 Centrifugal Filter Unit (3KDa cutoff; Cat # UFC5003BK, Millipore). Flow-through samples were analyzed for Met and NALM using UPLC (Shimadzu Nexera).

Statistical Analysis

Data were analyzed as a replicated 3 × 3 Latin square using the Proc Mixed procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC). Data for intake, milk production, and AUC were analyzed with a model that included fixed effects of period and treatment. Cow nested within square and square were random effects. Data for AA were analyzed using the model described above except that time was included as a repeated option. For each variable analyzed, 3 covariance structures (autoregressive order 1, compound symmetry, and unstructured covariance) were evaluated. The covariance structure that resulted in the lowest values for the Akaike information criterion and the Schwartz Bayesian criterion was used (Littell et al., 1998). Kenward-Roger's option was used to calculate the denominator degrees of freedom. Means were separated by use of orthogonal polynomial contrasts: 1) control vs. NALM treatments, 2) linear effect of increasing NALM, and 3) quadratic effect of increasing NALM. Least square means are reported throughout. Differences were considered significant at $P \leq 0.05$, and trends were discussed at $0.05 < P \leq 0.10$. 


RESULTS AND DISCUSSION

Characteristics of Diet Composition and Treatment

Ingredients and chemical composition of the basal diet are presented in Table 3-1. Energy and protein supply and AA balance of the basal diet supplemented with NALM are presented in Table 3-2. The basal diet was formulated to contain 15.7% CP and provide adequate MP supply (60 g/d). All diets provided MP supply in excess of requirements. Additionally, all diets were isoenergetic and supplied NE\textsubscript{L} and ME in excess of requirement (NRC, 2001). Lysine and Met requirements were met for the basal diet (2.0 g/d and 0 g/d balance) by dietary addition of canola meal, dried distillers grains with solubles, and blood meal, respectively. The Met balance for NALM supplemented diets were 15.6 and 31.2 g/d for the 30NALM and 60NALM, respectively.

The NRC (2001) model recommends that dLys and dMet concentrations in MP for maintenance and optimal milk protein production approximate to 7.2 and 2.4%, respectively. Common dietary strategies to achieve ideal Lys and Met concentrations include feeding diets with greater CP concentration or supplementing RPLys and RPMet products to diets with lower CP concentration to balance dLys and dMet in MP. Feeding higher CP diets can result in excess ruminal AA supply, greater N excretion in the environment, reduced N efficiency, and increase feed costs, which is economically unfavorable to dairy producers.
An alternative approach to optimize milk protein production is to maintain dLys and dMet in MP closer to 6.6 and 2.2%, respectively, for a targeted dLys:dMet in MP (efficiency for AA use) close to 3.0:1 (Schwab et al., 2003). Van Amburgh et al. (2015) updated the CNCPS model (version 6.5) and reported optimal ratios of dLys:dMet in MP closer to 2.69:1. The authors stated that to maximize milk protein yield, the calculated estimates from breakpoint analysis in the updated CNCPS model for Lys and Met (% of MP) were 7.00 and 2.60%, respectively, and to maximize milk protein content, 6.77 and 2.85 (% of MP), respectively (Van Amburgh et al., 2015). In the current study, supplementing 30 g/d (30NALM; manufacturer’s recommended dose) and 60 g/d of NALM decreased the dLys:dMet in MP to 2.46:1 and 2.05:1 (Table 3-2), respectively. In addition, the dLys and dMet in MP were 6.53 and 2.66%, respectively, for the 30NALM diet. Furthermore, the dLys and dMet in MP were 6.49 and 3.17%, respectively, for the 60NALM diet. Supplementation of 30 g/d NALM improved the dMet in MP, which suggests a more favorable Met concentration in the diet for animal and production utilization. However, increasing the supplemental NALM dose to 60 g/d reduced the dLys and increased the dMet in MP, leading to suboptimal dLys:dMet in MP.

**Feed Intake, Productive Performance, Feed Efficiency, and Milk Urea Nitrogen**

Intake, milk production, feed efficiency, and MUN data are presented in Table 3-3. Although primary production parameters were measured in the present study, caution needs to be taken when interpreting the data due to the short trial length. Overall, feed
intake, productive performance, and feed efficiency were not affected by NALM supplementation, however MUN linearly decreased with NALM supplementation. The basal diet was formulated to provide adequate MP and the dLys and dMet in MP were not limiting, which may partly explain the lack of response to treatments. A common in vivo technique to measure BA of AA in lactating dairy cows is the production response approach (Schwab et al. 2001; Fleming et al., 2019). Whitehouse et al. (2017) suggested the major limitation for the production response approach is to maintain dietary AA (Met or Lys) deficiency, in all animals, over all treatment dosages administered, so that linearity in milk protein concentration or yield is detected. Although the production response approach was not used in the current study, the absence of positive milk protein response to increasing NALM doses further supports that the basal diet was adequate in dMet, which was expected. The lack of negative production parameter effects serves to indicate that supplementation at 60NALM was still a safe concentration for the animals.

Liang et al. (2019) reported that NALM supplemented at rates of 0, 15, 30 and 60 g/d to mid lactation dairy cows improved milk yield in a quadratic manner, with 30 g/d of NALM seeming to be the optimal supplementation rate for milk yield under the experimental conditions. Amaro et al. (2019) found that milk yield of early lactating Holstein dairy cows tended to be greater \( P = 0.07 \) with intermediate concentrations (15 and 30 g/d) of NALM compared to control animals. The authors noted that NALM supplementation at 30 g/d resulted in the highest numerical milk yield (Amaro et al., 2019), which is in agreement with Liang et al. (2019). Based on limited data in the literature, NALM supplementation to either early or mid-lactation dairy cows has failed
to exhibit beneficial effects on milk protein concentrations (Amaro et al., 2019; Liang et al., 2019). However, Fagundes et al. (2018) reported that NALM supplementation at 30 g/d increased milk fat concentration and yield and 3.5% FCM yield in MP-deficient (-190 g/d) and MP-adequate diets (50 g/d). The basal diet fed in the current study was similar in MP (60 g/d) to that fed by Fagundes et al. (2018); however, each square within the Latin square design consisted of 21-d periods versus 13-d periods for the current study, respectively. Therefore, the short trial length in the present study, along with a 7-d washout period may not have allowed animals to adapt to NALM supplementation to exhibit changes in production parameters.

Liang et al. (2019) noted that MUN concentrations were not affected by oral NALM supplemented at rates of 0, 15, 30 and 60 g/d to mid lactation dairy cows. Milk urea nitrogen can be used as an indicator of N utilization efficiency in dairy cows. Variation in MUN is related to the protein:energy ratio of the diet consumed. With adequate energy in the diet, MUN can be indicative of protein status. All diets in the current study were balanced to be isoenergetic and supplied NEL and ME in excess of requirement (Table 3-2). The linear decrease in MUN concentrations due to NALM dosing was unexpected, however once NALM reaches the small intestine it is deacetylated into the physiological isomer L-Met that is systemically available for use by the body. Although it appears that NALM dosing improved N utilization efficiency through reduced MUN content, milk true protein was similar among NALM dosage rates. Lack of milk true protein change suggests the free useable Met from NALM dosing may have been utilized for other biological processes within the body. Additional research is needed to determine if
NALM supplementation alters N utilization of early to mid-lactation dairy cows by oral supplementation, as abomasal infusion of RPAAs is not a practical means of supplying RPAAs to lactating dairy cows.

**Plasma AA Profiles and NALM**

Supplementation of NALM led to a quadratic trend ($P = 0.06$) toward increases in plasma Arg, Gly, and Met and quadratic increases in plasma His and Ser concentrations (Table 3-4). Alternatively, NALM supplementation linearly decreased plasma AA concentrations of Leu, Phe, and Val and trends were observed for linear decreases in Ile and Ala concentrations. Although plasma Met increased linearly, there was an overall linear trend ($P = 0.08$) toward lower plasma EAA concentration. Total NEAA concentration was not affected by supplementation of NALM. Recently, Liang et al. (2019) described that NALM supplemented at rates of 0, 15, 30, and 60 g/d to mid-lactation dairy cows displayed quadratic increases in plasma Met concentration, with 30 g/d NALM resulting in the highest plasma Met concentration compared to the control treatment (36.7 vs. 31.1 µM). In contrast, Fagundes et al. (2018) found that early to mid-lactation dairy cows supplemented with 30 g/d NALM to MP-adequate diets had no effect on plasma Met concentration, which was an unexpected finding to the authors.

Plasma Met concentrations provide a qualitative measure of post-ruminal delivery and absorption of Met from RPMet products (Blum et al., 1999). Furthermore, plasma AA concentrations can also indicate the balance between AA supply in the diet and
utilization (Meijer et al., 1995). However, the ability of NALM to be absorbed as L-Met from the small intestine is dependent on the enzyme acylase I, which hydrolyzes NALM to L-Met (Giardina, 1997; Baxter et al., 2002). The acylase I enzyme is found in the intestine, liver, and kidney of mammalian tissues and has a high affinity for sulfur and neutral AA derivates (Giardina et al., 1997). No NALM could be detected in either portal or vena caval plasma in young pigs dosed with NALM by a gastrostomy tube (382 mg/kg BW), suggesting deacylation of NALM in the intestinal lumen or mucosal cells (Daabees et al., 1984). In addition, no NALM was detected in the plasma of adult subjects or 1-year-old infants after oral loading (0.0605 mmoles/kg BW) with NALM (Stegink et al., 1980; Stegink et al., 1982). The absence of plasma NALM (Table 3-5) and subsequent increase in plasma Met concentration with NALM dosing in the current study suggests that NALM supplemented by either rumen placement or abomasal infusion to lactating dairy cows is deacylated before entering the central circulation.

In this study, NALM supplementation linearly decreased plasma branched-chain AA (BCAA; Ile, Leu and Val) and EAA concentrations. Appuhamy et al. (2011) reported that jugular infusion of a combination of L-Lys and L-Met led to a 17% reduction in plasma Ile concentration compared to control cows. Blum et al. (1999) noted that plasma concentrations of Val, Ile, and total BCAA were reduced, but only in cows fed a polymer coated RPMet product. Similarly, Fagundes et al. (2018) observed decreased plasma BCAA and total EAA concentrations, but only in a diet supplemented with NALM that was adequate in MP, which was similar to the diet and findings of the current study. In addition, Varvikko et al. (1999) abomasally infused DL-Met to early, mid and late
lactation cows. The authors found that the concentration of total BCAA and each individual BCAA (Ile, Leu, and Val) in plasma decreased linearly with abomasal infusion of DL-Met (Varvikko et al., 1999).

The NALM supplementation in the current study most likely increased BCAA catabolism or tissue uptake, since EAAs that are not removed by the liver or peripheral tissues return to general circulation (Arriola Apelo et al., 2014). Branched-chain amino acids make up roughly 50% of the EAA in milk proteins (Mackle et al., 1999). Feeding RPMet products may result in increased milk protein synthesis or yield, because Met is a limiting EAA in dairy cows. Likewise, increased milk protein yield can enhance mammary gland BCAA uptake, leading to decreased plasma BCAA concentrations. Milk protein yield was not altered by NALM supplementation in our trial, thus suggesting that NALM dosing did not alter BCAA metabolism in the mammary gland. Branched-chain AAs and Met are important in portal drained viscera energy generation in ruminants (Lobley et al., 2003), and dietary energy deficiencies can alter AA partitioning towards greater oxidation and energy generation (Lapierre et al., 2006). All diets in the present study supplied ME and NE_L in excess of requirements according to NRC (2001). Therefore, it is unlikely that the BCAAs were used for additional energy generation in tissues after NALM dosing. It appears that NALM supplementation to lactating dairy cows increases plasma Met concentration and alters individual plasma BCAA profiles; however, more research is needed to understand the mechanisms involved in BCAA metabolism and potential nutrient interactions with NALM dosing.
Bioavailability and kinetic parameter results of NALM dosing are presented in Table 3-5. The mean basal plasma Met concentration was $25.4 \, \mu M$ for the control diet, which is similar to observed values in the literature for lactating dairy cows (Appuhamy et al, 2011; Arriola Apelo et al, 2014). Rumen NALM dosing led to a quadratic increase in the mean plasma Met concentration on d 11 of sampling after 10 d of rumen dosing. The mean plasma Met was $39.7 \, \mu M$ and $39.5 \, \mu M$ for the 30NALM and the 60NALM supplemented cows, respectively. The values reported in the current study were greater than literature values reported for diets supplemented with other types of RPMet products (Lee et al., 2015; Giallongo et al., 2016) and jugular infusion of L-Met to lactating dairy cows (Appuhamy et al, 2011), but similar to values seen with diets supplemented with NALM (Liang et al., 2019). Increased mean plasma Met after rumen NALM dosing suggests that NALM is deacetylated and free Met is absorbed into the central circulation for utilization by the animals.

Peak time and Met height after abomasal infusion of NALM did not differ among treatments (Table 3-5). Plasma Met concentrations increased over the first 3 h after abomasal infusion of NALM and returned to basal concentrations by 8 h post dosing (Figure 3-1). Daabees et al. (1984) noted when equimolar amounts of L-Met and NALM were supplemented to young pigs, the portal and venal caval peak plasma Met concentrations were lower for NALM compared to L-Met. However, peak time after dosing for both portal and vena caval plasma Met concentrations was greater (90 min) for
NALM versus portal (45 min) and vena caval (60 min) plasma Met concentrations after L-Met dosing (Daabees et al., 1984). In humans, peak time after oral administration was delayed for NALM compared to L-Met (Stegink et al., 1980). Peak time after intraruminal supplementation (50 g/d Met equivalent) with either the isopropyl ester of 2-hydroxy-4-(methylthio)-butanoic acid or polymer coated RPMet product in non-lactating dairy cows, averaged 4 h and 22 h 25 min, respectively (Graulet et al., 2005).

The mean plasma Met concentrations after abomasal infusion of each cow on d 12 and 13 for each period were used to determine the AUC (Table 3-5). Abomasal infusion with NALM led to an increased AUC versus control and both linear and quadratic increases in AUC were observed with NALM dosing. The resulting AUC values were expressed as the AUC per Met unit (i.e., AUC divided by the total dMet concentration in each diet) in order to adjust for varying Met doses in the diets. The mean AUC per Met unit (µM × h/L ÷ Met (g)) for the control, 30NALM, and 60NALM diets were 4.68, 5.89, and 5.07, which corresponded to a Met BA (AUC per Met unit ÷ AUC per Met unit_{Control}) of 100, 139, and 115%, respectively. N-acetyl-L-Met has been shown to be BA and capable of replacing the dietary requirement for Met in humans, rats, and swine, but had not been studied in dairy cattle (Boggs et al. 1975; Rotruck and Boggs 1975; Zezulka and Calloway, 1976; Stegink et al. 1980, 1982; Daabees et al. 1984). In the current study, BA estimation by plasma Met appearance was higher for NALM doses, indicating NALM improved plasma Met concentrations relative to the control. These results indicate that NALM is also capable of replacing dietary Met in lactating dairy cattle. However, further studies are warranted to evaluate the BA and kinetic parameters of NALM in lactating
dairy cows relative to unprotected forms of Met (L-Met) and RPMet products with known BA values in the literature. To our knowledge, this is the first time the Met derivative, NALM has been shown to increase plasma Met concentrations and serve as a RPMet source for lactating dairy cows.

**Residue Potential**

Residue potential in lactating Holstein dairy cows supplemented with NALM is summarized in Table 3-6. No NALM was detected in milk, liver, or muscle samples after rumen placement or abomasal infusion. In humans, existing authorizations for NALM have been approved in Germany for use in dietetic foods (BGBI, 1988) and in 1978, the United States approved NALM as a source of L-Met food additive (Federal Register, 1978). Smith et al. (2011) described that the extraction efficiency of N-acetylated AAs is known to be poor which can lead to a lack of detection of NALM. Therefore, tissue Met concentrations were quantified to verify the NALM analysis method by intracellular disruption and tissue Met release. Milk Met concentration was not altered by NALM supplementation. Methionine extraction by the mammary gland, unlike Lys, closely matches the amounts required for milk protein synthesis (Bequette et al., 1998). Therefore, no improvement in milk protein yield due to NALM supplementation would explain the lack of change in milk Met after NALM dosing. Liver and muscle Met concentrations increased with NALM supplementation, resulting in both linear and quadratic effects for increased liver Met concentration and a linear trend ($P = 0.10$) for
increased muscle Met concentration. The liver is responsible for Met metabolism (Brosnan and Brosnan, 2006) and liver Met removal in dairy cows is related to total liver inflow and blood flow, rather than net portal absorption (Lapierre et al, 2005; Arriola Apelo et al, 2014). Additionally, hepatic Met removal varies with the source of RPMet supplemented to lactating dairy cows. Common RPMet products contain equimolar mixtures of D- and L- isomers; however, D-Met is not physiologically functional in the dairy cow and must be converted to L-Met before it can be incorporated into mammalian proteins (Lapierre et al., 2005; Lapierre et al., 2012). Since, NALM only contains the L-isomer of Met, it can be utilized directly for protein synthesis after deacetylation, without conversion to any other isoforms. Liver and muscle Met increased after NALM dosing, demonstrating that free Met from the NALM source was absorbed, delivered to the liver, and was able to be utilized by peripheral tissues (i.e, muscle). Furthermore, in lactating dairy cows, the primary net user of post-liver Met supply is the mammary gland for milk protein synthesis (Lapierre et al., 2005). Elevated Met concentrations in liver and muscle after NALM supplementation, could be explained by the lack of response to milk protein yield seen in the current study. The absence of NALM residue in plasma (Table 3-5), liver, milk, and muscle reveal that the N-acetyl group is cleaved from NALM prior to reaching central circulation and can be considered as a safe and effective RPMet source in lactating dairy cows.

CONCLUSIONS
The current study evaluated the BA and lactational performance of dairy cows supplemented with NALM at 2 different doses. Overall, production parameters were not influenced by NALM supplementation, which was not surprising given the short study periods. Plasma Met increased with NALM dosing, while individual BCAA concentrations decreased. Bioavailability by plasma appearance of Met for NALM treatments was higher for the 30NALM dose compared with the 60NALM and the control, suggesting that optimal supplementation rate for NALM would be 30 g/cow per day. Residue of NALM was not detected in plasma, milk, liver, or muscle samples. This is the first attempt to measure the BA of the Met derivative in lactating dairy cattle and the findings suggest that NALM is deacetylated before reaching central circulation, increases plasma Met concentrations, and supplies lactating dairy cows with a source of RPMet. However, further studies are needed to evaluate the comparative BA of NALM in lactating dairy cows relative to unprotected forms of Met (L-Met) and RPMet products with known BA values in the literature.

ACKNOWLEDGMENTS

The study was supported by CJ CheilJedang (Seoul, South Korea). The authors thank Jed Oyler at Utah State University (Logan) for sampling assistance and the staff of the Caine Dairy Research Center (Wellsville, UT) for their care and handling of the experimental cows.
REFERENCES


Table 3-1. Ingredients and chemical composition of the basal diet

<table>
<thead>
<tr>
<th>Item</th>
<th>% of DM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ingredient</strong></td>
<td></td>
</tr>
<tr>
<td>Alfalfa hay</td>
<td>30.0</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>1.77</td>
</tr>
<tr>
<td>Corn silage</td>
<td>26.5</td>
</tr>
<tr>
<td>Corn grain (steam-flaked)</td>
<td>15.9</td>
</tr>
<tr>
<td>Distillers dried grains with solubles</td>
<td>3.54</td>
</tr>
<tr>
<td>Cottonseed, whole</td>
<td>3.54</td>
</tr>
<tr>
<td>Canola meal</td>
<td>7.07</td>
</tr>
<tr>
<td>Blood meal</td>
<td>1.06</td>
</tr>
<tr>
<td>Beet pulp, shreds</td>
<td>6.19</td>
</tr>
<tr>
<td>Fat supplement(^1)</td>
<td>1.77</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>0.53</td>
</tr>
<tr>
<td>Yeast supplement(^2)</td>
<td>0.18</td>
</tr>
<tr>
<td>Vitamin and mineral mix(^3)</td>
<td>1.94</td>
</tr>
<tr>
<td><strong>Chemical composition</strong></td>
<td></td>
</tr>
<tr>
<td>DM, %</td>
<td>60.5</td>
</tr>
<tr>
<td>CP</td>
<td>15.7</td>
</tr>
<tr>
<td>RDP(^4)</td>
<td>9.33</td>
</tr>
<tr>
<td>RUP(^4)</td>
<td>6.37</td>
</tr>
<tr>
<td>Component</td>
<td>Value</td>
</tr>
<tr>
<td>---------------</td>
<td>--------</td>
</tr>
<tr>
<td>NDF</td>
<td>32.9</td>
</tr>
<tr>
<td>ADF</td>
<td>22.9</td>
</tr>
<tr>
<td>Starch</td>
<td>17.7</td>
</tr>
<tr>
<td>Sugar</td>
<td>6.00</td>
</tr>
<tr>
<td>Ether extract</td>
<td>3.98</td>
</tr>
<tr>
<td>NFC&lt;sup&gt;5&lt;/sup&gt;</td>
<td>37.3</td>
</tr>
<tr>
<td>Ash</td>
<td>10.0</td>
</tr>
</tbody>
</table>

<sup>1</sup>Rumen-protected dry fat supplement (Nurisol®, WLT Distributors Inc., Winnipeg, MB, Canada).

<sup>2</sup>Diamond V XP® (Cargill Inc., Cedar Rapids, IA).

<sup>3</sup>Formulated to contain (per kg DM): 226.7 mg of Se (from sodium selenite), 9278.7 mg of Cu (from copper amino acid complex), 40,537.4 mg of Zn (from zinc amino acid complex), 38,653.4 mg of Mn (from manganese amino acid complex), 552.6 mg of Co (from cobalt carbonate), 1,234,585.2 IU of vitamin A, 152,808.1 IU of vitamin D, 3,815.1 IU of vitamin E, and 295 mg of Rumensin (Elanco Animal Health, Greenfield, IN).

<sup>4</sup>Based on tabular value (NRC, 2001).

<sup>5</sup>NFC = 100 – CP – NDF – ether extract – ash.
Table 3-2. Energy and protein supply and AA balance of the basal diet supplemented with N-acetyl-L-Met (NALM) at 2 different doses

<table>
<thead>
<tr>
<th>Item</th>
<th>Diet¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Energy supply²</td>
<td></td>
</tr>
<tr>
<td>NE₆, Mcal/kg</td>
<td>1.65</td>
</tr>
<tr>
<td>ME, Mcal/d</td>
<td>66.2</td>
</tr>
<tr>
<td>ME, Mcal/kg</td>
<td>2.58</td>
</tr>
<tr>
<td>Protein supply,² g/d</td>
<td></td>
</tr>
<tr>
<td>MP supply</td>
<td>2,940</td>
</tr>
<tr>
<td>MP requirements</td>
<td>2,880</td>
</tr>
<tr>
<td>MP balance</td>
<td>60</td>
</tr>
<tr>
<td>Lys and Met balance,³ g/d</td>
<td></td>
</tr>
<tr>
<td>dLys requirement</td>
<td>191</td>
</tr>
<tr>
<td>dLys supplied by the diet</td>
<td>193</td>
</tr>
<tr>
<td>Lys balance</td>
<td>2.00</td>
</tr>
<tr>
<td>dMet requirement</td>
<td>63.0</td>
</tr>
<tr>
<td>dMet supplied by the diet</td>
<td>63.0</td>
</tr>
<tr>
<td>dMet from Met derivative</td>
<td>0.00</td>
</tr>
<tr>
<td>Total dMet in the diet</td>
<td>63.0</td>
</tr>
<tr>
<td>dMet balance</td>
<td>0.00</td>
</tr>
<tr>
<td>dLys:dMet&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3.06</td>
</tr>
<tr>
<td>----------------------</td>
<td>------</td>
</tr>
</tbody>
</table>

<sup>1</sup>Control = basal diet without NALM supplementation; 30NALM = control diet with 30 g/d of supplemented NALM; and 60NALM = control diet with 60 g/d of supplemented NALM.

<sup>2</sup>All values were estimated using NRC (2001) based on actual DMI, milk yield, milk composition, and BW of the cows throughout the trial.

<sup>3</sup>Digestible Lys (dLys) and digestible Met (dMet) supply from the diets were estimated using NRC (2001); supply of dMet from Met derivative was estimated with Met concentration (78%) and absorption (67%) data provided by the manufacturer. Requirements of dLys and dMet were calculated as 6.6 and 2.2% of MP requirements, respectively.

<sup>4</sup>Lys from diet-to-digestible Met from diet and dMet from NALM.
Table 3-3. Intake, milk production, feed efficiency, and milk urea nitrogen of lactating Holstein dairy cows supplemented with N-acetyl-L-Met (NALM) at 2 different doses

<table>
<thead>
<tr>
<th>Item</th>
<th>Diet^1</th>
<th>Contrast^2</th>
<th>SEM</th>
<th>NALM</th>
<th>L</th>
<th>Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMI, kg/d</td>
<td>20.4</td>
<td>20.7</td>
<td>20.3</td>
<td>1.55</td>
<td>0.96</td>
<td>0.83</td>
</tr>
<tr>
<td>Yield, kg/d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk</td>
<td>30.9</td>
<td>31.3</td>
<td>30.9</td>
<td>1.79</td>
<td>0.84</td>
<td>0.99</td>
</tr>
<tr>
<td>3.5% FCM^3</td>
<td>33.2</td>
<td>33.5</td>
<td>32.6</td>
<td>2.27</td>
<td>0.94</td>
<td>0.85</td>
</tr>
<tr>
<td>ECM^4</td>
<td>32.7</td>
<td>33.0</td>
<td>32.0</td>
<td>2.35</td>
<td>0.91</td>
<td>0.69</td>
</tr>
<tr>
<td>Milk composition, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat</td>
<td>3.98</td>
<td>3.96</td>
<td>3.87</td>
<td>0.157</td>
<td>0.77</td>
<td>0.69</td>
</tr>
<tr>
<td>True protein</td>
<td>2.85</td>
<td>2.87</td>
<td>2.77</td>
<td>0.086</td>
<td>0.75</td>
<td>0.47</td>
</tr>
<tr>
<td>Lactose</td>
<td>4.74</td>
<td>4.73</td>
<td>4.73</td>
<td>0.136</td>
<td>0.77</td>
<td>0.75</td>
</tr>
<tr>
<td>Milk component yield, kg/d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat</td>
<td>1.23</td>
<td>1.23</td>
<td>1.19</td>
<td>0.088</td>
<td>0.89</td>
<td>0.80</td>
</tr>
<tr>
<td>True protein</td>
<td>0.87</td>
<td>0.90</td>
<td>0.85</td>
<td>0.076</td>
<td>0.98</td>
<td>0.65</td>
</tr>
<tr>
<td>Lactose</td>
<td>1.47</td>
<td>1.47</td>
<td>1.46</td>
<td>0.103</td>
<td>0.99</td>
<td>0.87</td>
</tr>
<tr>
<td>Feed efficiency</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk yield/DMI</td>
<td>1.53</td>
<td>1.52</td>
<td>1.54</td>
<td>0.144</td>
<td>0.87</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>3.5% FCM</td>
<td>3% FCM</td>
<td>1.63</td>
<td>1.63</td>
<td>0.096</td>
<td>0.81</td>
</tr>
<tr>
<td>------------------</td>
<td>----------</td>
<td>--------</td>
<td>------</td>
<td>------</td>
<td>-------</td>
<td>------</td>
</tr>
<tr>
<td>yield/DMI</td>
<td>1.65</td>
<td>1.63</td>
<td>1.63</td>
<td>1.63</td>
<td>0.096</td>
<td>0.81</td>
</tr>
<tr>
<td>ECM yield/DMI</td>
<td>1.62</td>
<td>1.60</td>
<td>1.60</td>
<td>1.60</td>
<td>0.086</td>
<td>0.83</td>
</tr>
<tr>
<td>MUN, mg/100 mL</td>
<td>12.3</td>
<td>11.2</td>
<td>10.7</td>
<td>1.19</td>
<td>0.05</td>
<td>0.02</td>
</tr>
</tbody>
</table>

1Control = basal diet without NALM supplementation; 30NALM = control diet with 30 g/d of supplemented NALM; and 60NALM = control diet with 60 g/d of supplemented NALM.

2NALM = control vs. NALM treatments; L = linear effect of increasing NALM supplementation; Q = quadratic effect of increasing NALM supplementation.

33.5% FCM = [0.4324 × milk yield (kg/d)] + [16.216 × fat yield (kg/d)].

4ECM = 0.327 × milk yield (kg/d) + 12.95 × fat yield (kg/d) + 7.2 × protein yield (kg/d).
Table 3-4. Plasma AA concentrations (µmol/L) of lactating Holstein dairy cows supplemented with N-acetyl-L-Met (NALM) at 2 different doses

<table>
<thead>
<tr>
<th>Item</th>
<th>Diet¹</th>
<th>Contrast²</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>30NALM</td>
<td>60NALM</td>
<td>SEM</td>
<td>NALM</td>
</tr>
<tr>
<td>Arg</td>
<td>42.9</td>
<td>46.9</td>
<td>42.0</td>
<td>2.1</td>
<td>0.55</td>
</tr>
<tr>
<td>His</td>
<td>38.9</td>
<td>42.5</td>
<td>37.4</td>
<td>1.66</td>
<td>0.61</td>
</tr>
<tr>
<td>Ile</td>
<td>74.8</td>
<td>74.8</td>
<td>67.2</td>
<td>3.16</td>
<td>0.33</td>
</tr>
<tr>
<td>Leu</td>
<td>136</td>
<td>128</td>
<td>113</td>
<td>5.2</td>
<td>0.02</td>
</tr>
<tr>
<td>Lys</td>
<td>58.2</td>
<td>59.2</td>
<td>54.7</td>
<td>2.51</td>
<td>0.68</td>
</tr>
<tr>
<td>Met</td>
<td>16.0</td>
<td>21.6</td>
<td>22.1</td>
<td>1.07</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Phe</td>
<td>42.2</td>
<td>40.3</td>
<td>36.4</td>
<td>1.56</td>
<td>0.05</td>
</tr>
<tr>
<td>Val</td>
<td>209</td>
<td>202</td>
<td>183</td>
<td>7.8</td>
<td>0.10</td>
</tr>
<tr>
<td>EAA³</td>
<td>617</td>
<td>616</td>
<td>556</td>
<td>24.3</td>
<td>0.30</td>
</tr>
<tr>
<td>Ala</td>
<td>190</td>
<td>179</td>
<td>170</td>
<td>7.78</td>
<td>0.11</td>
</tr>
<tr>
<td>Gly</td>
<td>194</td>
<td>224</td>
<td>211</td>
<td>9.48</td>
<td>0.05</td>
</tr>
<tr>
<td>Ser</td>
<td>54.4</td>
<td>65.6</td>
<td>57.6</td>
<td>2.52</td>
<td>0.02</td>
</tr>
<tr>
<td>Tyr</td>
<td>40.5</td>
<td>41.4</td>
<td>37.3</td>
<td>1.51</td>
<td>0.54</td>
</tr>
<tr>
<td>NEAA⁴</td>
<td>479</td>
<td>510</td>
<td>475</td>
<td>20.6</td>
<td>0.59</td>
</tr>
</tbody>
</table>

¹Control = basal diet without NALM supplementation; 30NALM = control diet with 30 g/d of supplemented NALM; and 60NALM = control diet with 60 g/d of supplemented NALM.
NALM = control vs. NALM treatments; L = linear effect of increasing NALM supplementation; Q = quadratic effect of increasing NALM supplementation.

Essential AA analyzed.

Nonessential AA analyzed.
**Table 3-5.** Determination of bioavailability and kinetic parameters in lactating Holstein dairy cows supplemented with N-acetyl-L-Met (NALM) at 2 different doses

<table>
<thead>
<tr>
<th>Item</th>
<th>Diet¹</th>
<th>Contrast²</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>30NALM</td>
<td>60NALM</td>
<td>SEM</td>
<td>NALM</td>
<td>L</td>
</tr>
<tr>
<td>Basal plasma Met concentration (BPC), µM</td>
<td>25.4</td>
<td>39.7</td>
<td>39.5</td>
<td>2.55</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Peak time after abomasal infusion, h</td>
<td>2:35</td>
<td>2:45</td>
<td>3:15</td>
<td>0.50</td>
<td>0.52</td>
<td>0.38</td>
</tr>
<tr>
<td>Peak Met height after abomasal infusion, µM</td>
<td>45.4</td>
<td>65.4</td>
<td>84.1</td>
<td>13.0</td>
<td>0.16</td>
<td>0.12</td>
</tr>
<tr>
<td>Area under the curve (AUC), µM x h/L</td>
<td>295</td>
<td>463</td>
<td>479</td>
<td>20.5</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>AUC per Met unit,³ (µM x h/L) / Met (g)</td>
<td>4.68</td>
<td>5.89</td>
<td>5.07</td>
<td>0.24</td>
<td>0.01</td>
<td>0.14</td>
</tr>
<tr>
<td>Met bioavailability of NALM,⁴ %</td>
<td>100</td>
<td>139</td>
<td>115</td>
<td>0.07</td>
<td>&lt; 0.01</td>
<td>0.09</td>
</tr>
<tr>
<td>Plasma NALM concentration</td>
<td>ND⁵</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

¹Control = basal diet without NALM supplementation; 30NALM = control diet with 30 g/d of supplemented NALM; and 60NALM = control diet with 60 g/d of supplemented NALM.
2NALM = control vs. NALM treatments; L = linear effect of increasing NALM supplementation; Q = quadratic effect of increasing NALM supplementation.

3AUC per Met unit = area under the curve ÷ total dMet concentration in the diet (g).

4Bioavailability = (AUC per Met unit ÷ AUC per Met unit\text{Control}) \times 100.

5ND = Not detected.
**Table 3-6.** Residue potential in lactating Holstein dairy cows supplemented with N-acetyl-L-Met (NALM) at 2 different doses

<table>
<thead>
<tr>
<th>Item</th>
<th>Diet¹</th>
<th>Contrast²</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>30NALM</td>
<td>60NALM</td>
<td>SEM</td>
<td>NALM</td>
<td>L</td>
</tr>
<tr>
<td>Milk NALM, (µM)</td>
<td>ND²</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Milk Met, (µM)</td>
<td>8.17</td>
<td>6.42</td>
<td>10.3</td>
<td>2.67</td>
<td>0.59</td>
<td>0.58</td>
</tr>
<tr>
<td>Tissue NALM, (µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Muscle</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tissue Met, (µg/g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>15.6</td>
<td>25.4</td>
<td>22.9</td>
<td>1.16</td>
<td>&lt; 0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Muscle</td>
<td>9.18</td>
<td>13.1</td>
<td>13.7</td>
<td>1.27</td>
<td>0.08</td>
<td>0.10</td>
</tr>
</tbody>
</table>

¹Control = basal diet without NALM supplementation; 30NALM = control diet with 30 g/d of supplemented NALM; and 60NALM = control diet with 60 g/d of supplemented NALM.

²ND = Not detected.
Figure 3-1. Plasma Met concentration (µM) versus time plot for lactating Holstein dairy cows abomasally infused with N-acetyl-L-Met (NALM) at 2 different doses
CONCLUSIONS

Amino acid and protein nutrition in lactating dairy cows remain a topic of great interest to researchers and the dairy industry. Substantial progress has been made over the years in the development of rumen-protected forms of lysine and methionine. Lysine and methionine have traditionally been identified as the most limiting essential amino acids for lactating dairy cows. As more complex feeding models are developed, the incorporation of a multiple-limiting amino acid model that includes branched-chain amino acids (i.e., isoleucine, leucine, and valine) and additional essential amino acids, (i.e, histidine, lysine, and methionine) if not all 10 essential amino acids could help to reduce protein overfeeding, improve post-ruminal amino acid supply, decrease nitrogen excretion, and improve lactational performance.

This dissertation investigated the use of acetylated methionine and lysine derivatives as a source of rumen-protected amino acids for lactating dairy cows. Acetylated amino acid derivatives are a free amino acid molecule with a chemical blocking group (acetyl group) added to the either the ε-amino or α-amino group. Chapter 1 focused on investigating production responses and ruminal fermentation characteristics of lactating dairy cows when supplemented with N-acetyl-L-Methionine as a source of rumen-protected methionine in metabolizable protein-deficient and -adequate diets. Improving the total balance of amino acids in metabolizable protein has been one strategy used to optimize lactational performance, while reducing the amount of dietary crude protein fed in lactating dairy cow rations. Previous studies have evaluated the use of rumen-protected
methionine to improve lactational performance in dairy cows, but have obtained inconsistent findings due to various feeding, physiological and complex inter-organ metabolism of methionine by the rumen, liver, and mammary gland. Our objective was to investigate lactational performance and nutrient utilization of early to mid-lactating dairy cows supplemented without or with a developmental N-acetyl-L-methionine in metabolizable protein-deficient or -adequate diets. Overall, the results demonstrated that N-acetyl-L-methionine exerted minor influence on ruminal metabolism, but increased milk fat concentration, which was an unexpected finding, possibly due to accelerated fatty acid delivery from the liver to the mammary gland, resulting in sizable increases in milk fat yield and feed efficiency.

In chapter 2, the objective was to investigate lactational performance and nutrient utilization by mid to late lactating dairy cows supplemented with three different forms of rumen-protected lysine (2 developmental lysine derivatives and 1 commercially available lysine supplement). Results of the study demonstrate that feeding the Nα, ε-acetyl-L-lysine supplemented diet in mid to late lactation dairy cows decreased dry matter intake and maintained milk yield, leading to greater feed efficiency compared to the control diet. These findings suggest that the developmental N-acetyl-L-lysine products (Nα, ε-acetyl-L-Lysine and Nε-acetyl-L-Lysine) may be biologically active in lactating dairy cows, with Nα, ε-acetyl-L-lysine showing the most benefit to improve feed efficiency and increase milk yield. However, further research is needed to discover the role of lysine derivatives on productive performance, ruminal metabolism, and bioavailability in
lactating dairy cows as this was the first attempt to evaluate the use of lysine derivatives in lactating dairy cows.

In chapter 3, the objective of the study was to evaluate the bioavailability and lactational performance of lactating dairy cows supplemented with N-acetyl-L-methionine at 2 different doses (30 and 60 g/d). To date, there had been no research reported on the effects of supplementing N-acetyl-L-methionine on bioavailability and safety to lactating dairy cows. Overall, production parameters were not influenced by N-acetyl-L-methionine supplementation, which was not surprising given the short study periods. Plasma methionine increased with N-acetyl-L-methionine dosing, while individual branched chain amino acid concentrations decreased, which was an unexpected finding. Bioavailability by plasma appearance of methionine for N-acetyl-L-methionine treatments was higher for the 30 g/d N-acetyl-L-methionine dose compared with the 60 g/d N-acetyl-L-methionine and control dose, suggesting that the optimal supplementation rate for N-acetyl-L-methionine is 30 g/cow/d. N-acetyl-L-methionine was not detected in plasma, milk, liver or muscle samples. This again was the first attempt to measure the bioavailability of the methionine derivative in lactating dairy cattle and the findings suggest that N-acetyl-L-methionine is deacetylated before reaching central circulation, increases plasma methionine concentrations, and supplies lactating dairy cows with a safe bioavailable source of rumen-protected methionine. Although there were differences observed between studies in lactational performance (chapter 1-3), the overall major findings of these studies indicate that acetylated methionine is safe for
use in lactating dairy cows and both methionine and lysine derivatives should be considered as a source of rumen-protected amino acids for lactating dairy cows.

The daily profit per lactating dairy cow being fed with rumen protected methionine has been estimated to be around 30 cents (Cho et al., 2007; Chen et al., 2011). Dairy nutritionists commonly report a return on investment of 2.5:1 or higher when rations are balanced for rumen protected lysine and methionine in metabolizable protein (Schwab, 2012). Additionally, increases in income over feed cost approaching 40 to 50 cents per cow per day from precision balancing for rumen degradable protein and rumen undegradable protein, while balancing for lysine and methionine have been described by dairy nutritionists (Schwab, 2012). The concept of the multiple-limiting amino acid model may be profitable based on the values above through precision feeding, however the environmental impacts of reducing nitrogen excretion by lysine and methionine derivative supplementation should not be overlooked.

Consumers of dairy products are aware of the environmental impacts of feeding and raising livestock animals. Animal agriculture has been identified as a major source of nitrogen pollution to water resources. Animal wastes can contribute to nitrogen pollution of the environment as ammonia volatilized to the air, nitrate leached into ground water, and nitrogen that runs off to surface water. Improvement of nitrogen utilization efficiency by lactating dairy cows fed with amino acid derivatives could decrease nitrogen losses from farms and help to reduce the negative environmental impacts of livestock animals.
Although considerable advancements have been made in amino acid nutrition, future works need to focus on the development of products and technologies that promote animal health (i.e., improved immunity) through balanced feeding allowing for lower protein diets and increased microbial protein synthesis. Further works could include the use of acetylated amino acid derivative mixtures supplemented to lactating dairy cows. Additionally, future works should focus on the “real-life” estimates of potential reductions in nitrogen excretion by supplementing amino acid derivatives through improving the amino acid profile reaching the small intestine. Future research needs to focus on longer term feeding periods during various stages of lactation (i.e., early, mid or late). The study durations should be extended to measure responses with larger sample sizes and explore the biological and commercial significance of the dietary treatments. This could also allow researchers to pinpoint when acetylated amino acid derivatives exert the greatest effects to dairy cows during lactation. It will also aid dairy farmers on deciding the optimal time to feed acetylated amino acid derivatives to lactating dairy cows to maximize animal health, reduce environmental nitrogen excretion, and increase profits.

REFERENCES


CURRICULUM VITAE

CONTACT INFORMATION
Mark A. Fagundes, DVM
Ph.D. Candidate, Utah State University Logan, UT
13702 Avenue 18 1/2
Chowchilla, CA 93610
Cell: (559) 999-6928
mafagund@gmail.com

CURRENT POSITION
2017-present Herd Veterinarian and Nutritionist, Avila Family Dairy, LLC, 13644 Avenue 18 ½ Chowchilla, CA 93610

2019-present Dairy Records Analyst, The HEALTHSUM Syndicate, LLC, 445 Barnard Blvd Sunnyside, WA 98944

2020-present Associate Veterinarian, Swinging Udders Veterinarian Services, 8418 Liberty Rd Galt, CA 95632

EDUCATION
Anticipated Ph.D. Utah State University, Logan, UT*
July 2020 Major: Ruminant Nutrition
Major Professor: Jeffery Hall, DVM, Ph.D., Diplomat A.B.V.T.
*First participant through a concurrent DVM/Ph.D.
program at Utah State University School of Veterinary Medicine

2017 DVM Utah State University: School of Veterinary Medicine, Logan, UT/Washington State University: College of Veterinary Medicine, Pullman, WA
WIMU Regional Program in Veterinary Medicine

2012 B.S. California Polytechnic State University, San Luis Obispo, CA
Major: Dairy Science
Minor: Agribusiness
ACADEMIC EXPERIENCE
2017-2019  Clinical Veterinary Internship
Utah State University School of Veterinary Medicine
Clinical Veterinary Services Team
4815 Old Main Hill
Logan, Utah 84322-4815
Clinical Advisor: Rusty Stott, DVM

2019  Senior Paper Discussant
Washington State University: College of Veterinary Medicine, Pullman, WA
Student Presenter: Jessica Thomas
Presentation Date: September 19th, 2019
Topic: Unusual presentations and early recognition of canine hypoadrenocorticism

Student Presenter: Allani Delis
Presentation Date: October 31st, 2019
Topic: Precursor-targeted immune mediated anemia in the dog.

RECENT/CURRENT RESEARCH
2017  Title: Effects of supplementing three different forms of rumen-protected lysine on lactational performance of mid to late lactation dairy cows.

2019  Title: Bioavailability, lactational performance and residue potential of lactating dairy cows supplemented with N-acetyl-L-methionine at different doses.

CURRENT FIELDS OF RESEARCH INTEREST
• Development of feeding strategies to improve nutrient management and environmental performance of dairy cattle
• Improving metabolic and feed efficiency and animal health by enhancing nutrients (protein, carbohydrate, lipid, and mineral) and energy utilization by livestock animals
• Manipulation and characterization of ruminal fermentation and physiology to enhance ruminant production
• Exploring gut physiology and immunology of livestock animals
• Immunonutrients in ruminants
• Strategic use of feed additives (amino acids) in livestock

VETERINARY LICENSURE
Utah  Inactive
Idaho  Active  
California  Pending

ACREDITATION
California  Pending  
Accreditation allows participation in all accreditation work following State and Federal laws and regulations under the direction of the United States Department of Agriculture’s Animal and Plant Health Inspection Service (USDA-APHIS).

ACADEMIC ACHIEVEMENT  
2014-2016  The Research Scholars Program  
Utah State University School of Veterinary Medicine  
Program Goal: Attract to the veterinary student population, and hence to the veterinary profession, individuals of exceptional aptitude who are oriented toward a career of basic or applied research.

2015  1st Prize- Oral Veterinary Student Presentation, ADVS Graduate Student Research Symposium, Logan, UT  

2014  3rd place, poster presentation competition, ADVS Graduate Student Research Symposium, Logan, UT  

2013-2015  Appointed Large Animal Club President  
Utah State University School of Veterinary Medicine  

PROFESSIONAL TRAINING  
2017  Liver Biopsy Wet Lab  
Course Instructor: Jeffery Hall, DVM, Ph.D., Diplomat ABVT  

2013  Milk Quality/LactoCorder Training  
Veterinarian’s Outlet Dublin, Texas  
Course Instructor: Ynte Schukken DVM, Ph.D., MS  
Cornell University College of Veterinary Medicine: Quality Milk Production Services  

2013  Ovum Pickup (OPU) in the bovine  
California Polytechnic State University, San Luis Obispo, CA  
Course Instructors: Joy Altermatt DVM, MS, DACT  
Fernando Campos-Chillon DVM, MS, Ph.D., DACT  

PROFESSIONAL EXPERIENCE  
2017-2019  Clinical Veterinary Internship  
Utah State University School of Veterinary Medicine
Clinical Veterinary Services Team
4815 Old Main Hill
Logan, Utah 84322-4815
Duties: On-call responsibilities of one weeknight each week and one weekend a month. Oversight of research animals. Participation in small ruminant surgeries and routine visits to livestock operations (Dairy, Beef, Small Ruminant, Swine). Participation in classroom/laboratory teaching with undergraduate, graduate and veterinary students.

2011-2013
Veterinary Assistant
Westside Veterinary Services, Inc.
1531 E. Pacheco Blvd.
Los Banos, CA 93635
Duties: Accompanied veterinarians to on-farm calls, administered injections, pregnancy palpation via portable ultrasound, milk quality investigation, routine timing, teat scoring, parlor audits, lameness evaluations, nutrition diagnostics and dairy records analysis.

2012-2013
Associate
Ani-Tech Production Solutions
Duties: Assisted with graphing pulsators, vacuum measurements, CIP evaluations, NMC milking system evaluations and troubleshooting increased mastitis incidence on dairy farms.

2012-2013
Hospital Manager
Red Top Jerseys
21519 Rd 4, Chowchilla, CA 93610
Duties: Diagnosing and treatment of sick animals. Developed treatment protocols and trained employees in the use of an on-farm milk culture system. Employee on-farm training of ultrasonography to determine herd pregnancy status.

PUBLICATIONS
• Journal Articles *indicates corresponding author

• Abstracts in Peer-Refereed Conference Proceedings


- **Oral Conference Presentations** *indicates presenting author


**PROFESSIONAL AFFILIATIONS**

American Veterinary Medical Association
American Association of Bovine Practitioners
Academy of Dairy Veterinary Consultants
American Dairy Science Association
American Society of Animal Science
Dairy Cattle Reproductive Council
National Mastitis Council