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Lactational Performance and Energy Partitioning of Dairy Cows Supplemented with N-Acetyl-L-Methionine During Mid to Late Lactation

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LACTATIONAL PERFORMANCE AND ENERGY PARTITIONING OF DAIRY COWS SUPPLEMENTED WITH N-ACETYL-L-METHIONINE DURING MID TO LATE LACTATION

by

Tyson George Grisenti

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ABSTRACT

Lactational Performance and Energy Partitioning of Dairy Cows

Supplemented with N-Acetyl-L-Methionine during Mid to Late lactation

by

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Utah State University, 2017

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The N-acetyl-L-methionine (**NALM**) molecule is a methionine (**Met**) derivative produced via acetylation of the L-Met α-amino group with an N-acetyl group. This molecule has been shown to be bioavailable and capable of fulfilling the dietary requirement for Met in animals and humans. The current experiment was conducted to test a hypothesis that lactating dairy cows fed with NALM would increase milk production by increasing N and energy utilization efficiencies in a dose dependent manner. Eight multiparous Holstein cows that were mid lactation (124 ± 13 days-in-milk) with similar milk production were used in a 4 × 4 Latin square design for 84 d. A developmental NALM product from CJ CheilJedang (Seoul, South Korea) was used as the supplemental source of rumen-protected Met in the present study. Four dietary treatments included 0 g (control), 15 g, 30 g, and 45 g/d/cow of NALM supplementation. Supplementing NALM significantly increased dry matter intake (linear effect; $P < 0.01$), while milk yield tended to increase quadratically ($P = 0.07$). A linear decrease in milk fat

concentration was seen due to supplementation of NALM in relation to the control ration (P = 0.02). However, milk fat yield was similar across treatments. A trend toward an increase in milk protein yield was observed between the control ration and the ration supplemented with 45 g of NALM (1.18 vs. 1.21 kg/d; P = 0.10). There were no differences in energy-corrected or 3.5% fat-corrected milk yields in response to treatments. It is likely that the supplementation of NALM to mid to late lactating dairy cows may have shifted nutrient and energy utilization toward tissue gain and lactation, which resulted in a decrease in feed efficiency for lactation (P = 0.02). Overall results from the present study suggest that supplementing NALM to mid to late lactating cows can increase milk yield in a dose dependent manner with a shift of net energy partitioning toward milk production and body weight gain. In addition, supplementing NALM increased milk nitrogen (**N**) output without affecting urinary N excretion.

(111 pages)

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LIST OF ABBREVIATIONS

AA = amino acid

ADF = acid detergent fiber

AH = alfalfa hay

BCAA = branched chain amino acids

BHB = beta-hydroxybutyrate

BUN = blood urea nitrogen

BW = body weight

CP = crude protein

CS = corn silage

CWT = hundredweight

DIM = days in milk

DM = dry matter

DMI = dry matter intake

EAA = essential amino acid

ECM = energy corrected milk

FCM = fat corrected milk

HMB = 2-hydroxy-4-(methylthio)-butanoic acid

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

Lys = lysine

MCP = microbial crude protein

ME = metabolizable energy

Met = methionine

MP = metabolizable protein

MUN = milk urea nitrogen

N = nitrogen

NALM = N-acetyl-L-Methionine

NAQ = N-acetyl-L-glutamine

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

PDV = portal drained viscera

RDP = ruminally degradable protein

RPMet = rumen protected methionine

RUP = ruminally undegradable protein

SAM = S-adenosyl-methionine

TMR = total mixed rations

TP = true protein

VFA = volatile fatty acids

VLDL = very low density lipoproteins

INTRODUCTION

Multiple strategies have been employed in the dairy industry to decrease the cost of production while maintaining or increasing milk and milk component production. One method of improving profitability that has received considerable attention is to balance the amino acid (**AA**) profile of the diet. Depending on the dietary composition, both methionine (**Met**) and lysine (**Lys**) have been shown to be most limiting for milk production (NRC, 2001), and Met has been identified as the most limiting AA for milk protein synthesis (Schwab et al., 1976). Therefore, balancing for Met can potentially have a significant impact on dairy production. Various commercial Met products, collectively classified as rumen-protected Met (**RPMet**) products, have been developed using different technologies to deliver bioavailable Met to the small intestine by protecting it from degradation in the rumen. Physical protection of a Met molecule with coating materials allows RPMet products to be effectively resistant to ruminal degradation. Methionine analogues, a different type of RPMet product, are also widely used in the dairy industry, and they utilize a hydroxyl group to protect them from ruminal degradation (Schwab and Ordway, 2003). Despite extensive advancements with these technologies, multiple studies have yielded mixed results on milk and milk component production due to a variety of reasons, such as the method of rumen protection utilized, dietary factors such as crude protein (**CP**) and metabolizable protein (**MP**) concentrations (Lee et al., 2011), animal factors such as breed or stage of lactation (Patton, 2010), and even organoleptic factors (Benefield et al., 2009). The use of Met for other physiological functions by various tissues is also a plausible explanation for the inconsistent milk and

milk component production responses to RPMet supplementation that has commonly been overlooked.

Similar to milk production, Met is limiting for growth and body weight (**BW**) gain (NRC, 2001), and thus the partitioning of Met and its influence on nutrient and energy utilization for milk production and BW gain merits attention. It is commonly understood that a shift occurs in the nutrient and energy partitioning of lactating dairy cows around mid-lactation from milk production to BW gain (NRC, 2001). The components that affect the partition of energy and nutrients have long been of interest to the industry, but are not well understood (Friggens et al., 2013). Studies focusing on the influence of RPMet supplementation on nutrient and energy partitioning are severely lacking and warrants further investigation.

N-acetyl-L-Met (**NALM**) is a Met derivative, meaning it is a free Met molecule with a chemical blocking group (an acetyl group) added to the α-amino group. The acetyl group acts as a barrier that blocks the hydrolysis of the N-terminal of Met which protects it from ruminal degradation (Wallace, 1992). A preliminary study done by our group (Fagundes et al., 2016) reported that cows supplemented with NALM increased milk fat concentration and yield, but not milk protein during early lactation. However, the effects of NALM supplementation and the optimum rate of supplementation in mid to late lactating cows may be different from the initial study due to the distinctive physiological changes for cows in mid to late lactation compared to those in early lactation. Therefore, the present study was performed to explore the effects of NALM supplementation on production parameters and energy partitioning by mid to late lactating dairy cows. We

hypothesized that an increase in metabolizable Met through NALM supplementation would increase body tissue anabolism in a dose dependent manner rather than increasing milk protein synthesis in mid to late lactation Holstein cows.

REVIEW OF LITERATURE

With the advent of the multiple component pricing system in the U.S. dairy industry, dairymen have sought out new and innovative ways to increase the production of both milk protein and milk fat. Methionine has long been recognized as the first limiting AA in milk protein synthesis (Schwab et al., 1976) and one of two limiting AA in milk production (NRC, 2001). Met has also been shown to increase milk fat yield in mid lactation (Overton et al., 1996; Schmidt et al., 1999). For this reason, feeding RPMet, or a similar Met product, has gained popularity with dairyman. This literature review will examine the many different methods that are available for providing Met to ruminants, as well as their effects on feed intake, milk production, BW gain, and N utilization. Special emphasis will be placed on acetylated AA, specifically NALM and the metabolic journey it takes through the rumen, the small intestine, and the liver.

Methionine

Methionine and Ruminant Nutrition

The overall knowledge pertaining to applied ruminant nutrition in the dairy industry has evolved immensely over the past few decades. It is now well known that ruminants do not have a requirement for CP, per se, but rather a requirement for each of the ten essential AA (**EAA**). Thus, when a ration is balanced for CP alone, protein may potentially be underfed, limiting production, or protein may be overfed, decreasing efficiency and increasing the output of nitrogenous manure waste. Balancing rations according to the AA requirements of dairy cows, in theory, should overcome the

uncertainty of over or underfeeding protein in traditionally balanced rations. Of all the AA required by the cow, the most is known about Met.

Methionine is one of 4 AA that contain sulfur and only one of 2 sulfur-containing AA that are proteinogenic (Brosnan, 2006). In ruminants, Met is an EAA, meaning Met cannot be synthesized by the ruminant body in quantities sufficient to meet demands for Met, so Met itself or a precursor for the synthesis of Met must be supplemented in the diet. In mammals, including ruminants, Met is mainly used for the synthesis of new proteins and the synthesis of S-adenosyl-methionine (**SAM**) (Preynat et al., 2009). Synthesis of new proteins includes the synthesis of milk protein and, as mentioned previously, Met is the first limiting AA in milk protein production. This means that milk protein production is normally limited by Met, because it is in the shortest supply in view of the AA requirements for milk protein production. SAM is a primary methyl donor for multiple important biomolecules in all living organisms and is second only to ATP in its use by cells as an enzyme substrate (Atta et al., 2004). Some metabolic health events, such as fatty liver disease, can be combated with proper levels of SAM through sufficient supplementation of dietary Met (Anstee and Day, 2012). In addition to aiding in the metabolic health of the cow, Met aids in the methylation of DNA and is a precursor of taurine and glutathione, both very important antioxidants (Osorio et al., 2014). Also, in typical North American diets Met limits milk production in high-producing dairy cows, especially when soybean meal is used as a protein source (Chen et al., 2011). It is very apparent that Met is essential in milk and milk component production and is also very

beneficial for the metabolic health of the ruminant, but not all forms of Met are equally available and beneficial.

Methionine Isomers

There are 2 isomers of Met: the D and the L isomer. It is important to note that both forms of Met are not equally available to the ruminant. In fact, the D isomer of any AA has to be converted into an L isomer before they can be utilized. The L isomer of Met can be utilized directly for protein synthesis without having to be converted to any other form first; however, it is not known how much of the available D isomer of Met can be converted to the L isomer to be utilized in ruminants. According to Lapierre et al. (2012), the bioavailability of the D isomer of Met depends on the rate of transformation into the L isomer. In monogastrics, this rate of transformation and subsequently the bioavailability of D isomers is well studied (Baker and Boebel, 1980; Arentson and Zimmerman, 1985), but there is a lack of data and information pertaining to this topic in ruminant nutrition (Lapierre et al., 2012). Since RPMet products contain a racemic mixture of both isomers, it stands to reason that it would be beneficial to investigate the efficiency of the transformation of the D isomer in ruminants.

One of the only, and certainly the most current study on the bioavailability of D-Met to dairy cows, was performed by Lapierre et al. (2012) using 4 multiparous cows in 3 different trials. In the first trial, all four of the cows received different treatments of DL-Met in addition to an AA mixture. Trial 2 involved injections of L-Met and D-Met at different time points. Finally, in trial 3 cows received injections of L-Met or DL-Met, again at different time points and in addition to injections of an AA mixture. The results

of these trials indicated that the removal rate for D-Met is slower than that of L-Met, as little as one-sixth as fast in some instances. Also observed was the fact that most of the D-Met is eventually transformed into the L-isomer at some unknown location in the body (Lapierre et al., 2012). This is significant, because D-isomers cannot be extracted by the mammary gland for milk or milk component production. So, D-Met can eventually be utilized by the body after the slow uptake and slow conversion to L-methionine. This then begs the question; what is the best way to deliver a steady supply of postruminal bioavailable Met to the cow?

RPMet

Product Overview of RPMet

Many approaches have been taken in order to deliver the most amount of bioavailable Met to the dairy cow. Many companies have employed various methods to efficiently accomplish this goal. These methods typically include either physically coating Met with different compounds or using compounds that can eventually be converted into Met. Most rumen-protected AA products on the market today fall into one of three categories: physically protected Met, Met analogues, or Met derivatives. Methionine analogues technically are not classified as true rumen protected AA products, but they do offer some level of rumen protection, so for the sake of this literature review I will classify them as Met analogues. Each of these three types of products will be discussed in this review.

By far the most popular method of delivering postruminal Met has been to physically protect the AA from ruminal degradation. Lipids were initially used to protect Met from ruminal degradation by coating the Met with tristearin, a triglyceride. The efficacy of Met supplements is directly related to their ability to bypass the rumen and then be bioavailable in the small intestine. This is where lipid coated Met products face their biggest challenge. It is very difficult to find an effective lipid combination that allows for both a high rumen escape rate and intestinal release rate of Met when using lipids as the primary coating compound (Schwab and Ordway, 2003). Met-Plus (Nisso America, Inc., New York, NY), is one example of a lipid coated RPMet product that is available on the market today, and it contains about 65% Met. Met-Plus appears to be the least studied physically protected Met product because of the inefficiencies related to current lipid coating technology.

The coating of the surface of Met molecules with carbohydrates came along after the advancement of lipid coating technology, along with the hopes of improving the delivery of bioavailable Met to the small intestine. Ethyl cellulose is used to coat a core of DL-Met. According to Schwab and Ordway (2003), enzymatic digestion of ethyl cellulose is minimal and, therefore, it is well protected from ruminal degradation. The problem that arises with using ethyl cellulose is degradation of the product must come from physical action and abrasion. This can reduce the amount of bioavailable Met delivered to the small intestine. Mepron M85 (Evonik Industries, Hanau, Germany) is a popular example of a carbohydrate coated methionine product. Mepron M85, as the name suggests, contains a minimum of 85% DL-Met, which is 20% more than the previously mentioned

Met-Plus. More important than the amount of Met in the product, is the amount of Met that is bioavailable to the cow.

Smartamine M (Adisseo, Inc., Antony, France) provides the most bioavailable Met to the cow when compared with other physically protected Met products, due in part to its coating technology. Smartamine M, similar to Met-Plus, is a lipid coated product with the difference being an additional coating of a pH-sensitive copolymer added to Smartamine M (Schwab and Ordway, 2003). The addition of the copolymer allows for increased protection in the rumen, which permits more Smartamine M to bypass the rumen. The copolymer is degraded in low pH environments. After safe passage through the rumen, Smartamine M will eventually reach the acidic environment of the abomasum where its coating will dissolve allowing the DL-Met to travel on for absorption in the small intestine. Smartamine M contains 75% DL-Met. This product is perhaps the most popular physically protected Met product currently on the market; however, there are more rumen-protected products available.

Another unique approach that has been taken to increase the amount of bioavailable Met is to utilize Met analogues and derivatives. A commonly used Met analogue is 2-hydroxy-4-(methylthio)-butanoic acid (**HMB**), also known as Met hydroxy analogue (Phillips et al., 2003). HMB is technically not a true AA, but it can be converted into Met. Methionine analogues are synthetically produced from the substitution of the α-amino group of Met with a non-nitrogenous group, such as a hydroxyl group, as is the case of HMB (Schwab and Ordway, 2003). The hydroxyl group is, in theory, supposed to partially protect the Met analogue from ruminal degradation. This, however, does not

appear to be an effective form of rumen protection with proportions of ingested HMB that escapes the rumen being reported as low as 5% (Noftsger et al., 2005). This suggests a ruminal mode of action, rather than an abomasal and small intestinal mode of action, or in other words HMB is not technically a true form of RPMet (Noftsger et al., 2003). Esterification of HMB to isopropanol (**HMBi**) does, however, appear to be an effective form of rumen protection, according to St-Pierre and Sylvester (2005), with as much as 50% of HMBi escaping the rumen and being converted to Met (Ordway et al., 2009). Alimet (Novus International, Inc. St. Louis, MO) and Rhodimet AT88 (Adisseo, Inc., Antony, France) are both products that contain HMB and are widely used in the poultry and swine industries, with Alimet being approved for use in the dairy industry (Schwab and Ordway, 2003). A more widely used product in the dairy industry is MetaSmart (Adisseo, Inc., Antony, France), which is an HMBi product. MetaSmart consists of no less than 57% HMBi, which is a 78% Met equivalent and of that percentage, 50% of it would be absorbed and converted to metabolizable Met (Adisseo, Inc., Antony, France). The use of Met analogues is a relatively new concept when compared with physically protected Met, and the use of Met derivatives is an even newer concept.

Methionine derivatives are understudied and currently are not being utilized in the dairy industry. A Met derivative is a free Met molecule that has had 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 differ from Met analogues in the fact that it is adding to the α-amino group, rather than replacing the α-amino group. The focus of this thesis is N-acety-L-Met (**NALM**), which is a Met derivative. Rumen

protection, bioavailability, absorption, metabolism, and impacts on production of this product will be discussed in detail later in this review. For now, I will focus on the impacts of physically protected Met products and Met analogue products on all aspects of production.

Factors Influencing RPMet Efficacy

Plenty of research has been conducted on the effects of Met supplementation on feed intake, milk production, BW gain, and N utilization. The problem with the results of this research, however, is that the overall results can be variable and inconsistent (Patton, 2010). One reason for the variability in these results can be attributed to the different forms of Met being fed. Smartamine M, for example, has a core of DL-Met, and MetaSmart is a Met analogue. Both products have different bioavailabilities, different routes of absorption, and thus, different effects on production parameters. This needs to be considered when comparing the effects of different Met supplement products on the previously mentioned factors. Bioavailability and routes of absorption for these different products will be discussed in further detail at another section in this review, so focus will now be placed on other factors that can influence the efficacy of RPMet products.

One major factor influencing RPMet efficacy is the nutritional composition of the experimental rations that are fed. When studying rumen-protected AA, total CP and MP in the diet can have a major impact on the efficacy of RPMet products. Broderick et al. (2008) observed that milk production increased when total CP in the diet was reduced from 18.3% to 17.3% and then again to 16.1%, but when CP concentration was further reduced to 14.8%, milk production was depressed, even with supplementation of RPMet.

Broderick et al. (2008) also found that higher dietary CP concentrations led to an increase in dry matter intake (**DMI**), milk fat and true protein (**TP**) concentrations, 3.5% fat-corrected milk yield , milk fat yield, TP yield, lactose yield, and solids-not-fat yield. Similar to CP, decreasing MP also decreased milk production (Lee et al., 2011), whereas it has been suggested that overfeeding MP can decrease milk production as well (Lee et al., 2015a).

It has been suggested that some nutritional factors can have different effects on different breeds of dairy cows. In an RPMet meta-analysis, Patton (2010) explained that differences in production according to breed, can perhaps be explained by the nutritional composition of the rations fed. For example, milk production was moderated in Holsteins by neutral detergent fiber (**NDF**) and CP, whereas non-Holstein breeds were moderated by energy balance. In the same meta-analysis on RPMet products, Patton (2010) noticed that production responses were also influenced by the ingredient composition of the rations fed. Alfalfa hay (**AH**)-based rations had greater milk production responses, whereas rations that contained other forages besides AH, had reduced milk production when Met was supplemented (Patton, 2010).

One major factor that could easily vary from trial to trial is days-in-milk (**DIM**), or the location of the cow in her lactation cycle. Schwab et al. (1992) suggested that milk yield did not readily respond to Met supplementation when cows were in mid to late lactation. The findings in Patton's meta-analysis (Patton, 2010) confirm Schwab's thinking; Patton (2010) found most cows in the studies had no response to Met supplementation, were post-peak lactation and, they could have lacked the hormonal drive needed for increased

milk production. Many other factors could come into play as well, such as parity and breed of cow. Benefield et al. (2009) even suggested that the smell of certain products could influence intake and thus production responses. The point proven here is there are many factors that come into play when evaluating the efficacy of an RPMet product, especially when comparing one product to another.

Effects on Feed Intake

Feed intake, also known as DMI, is arguably one of the more important animal responses that should be considered on dairy studies. In general, DMI is directly proportional to positive responses in many aspects of dairy trials, including the health and production of the cow. For example, every extra kg of DMI above the requirements of maintenance provides enough energy to potentially support 2 kg of milk production (Amaral-Phillips et al., 1997). Multiple factors can affect DMI, including the nutritional composition of the diet fed. For instance, keeping focused on AA and protein, supplemental histidine in the diet has been shown to increase DMI (Lee et al., 2012; Giallongo et al., 2016) and deficient MP supplies have been proven to decrease DMI (Lee et al., 2011). In some cases, feeding supplemental Met, in any of the above mentioned forms, can have an impact on DMI. In a meta-analysis study conducted by Zanton et al. (2014) that investigated the effects of Mepron M85, Smartamine M, and HMB on lactational performance, it was reported that DMI was affected by the source of supplemental Met. In fact, it has been suggested that Met, if fed at high concentrations, can have a negative effect on DMI and is the AA that has the potential to impact DMI the most (Benevenga, 1974).

Response in DMI to Met supplementation has been inconsistent and variable. Smartamine M seems to be the most studied RPMet product on the market today and, therefore, there are multiple recorded DMI responses to supplementation of Smartamine M. Osorio et al. (2014) supplemented Smartamine to transition dairy cows, from 21 d pre-partum to 30 d post-partum. An overall post-partum increase in DMI was noted, with an even more pronounced increase in DMI seen from 7 d to 30 d post-partum. Conversely, Socha et al. (2005) also in post-partum cows, found a decrease in DMI when supplemented with Smartamine M. Studies have also shown that supplementation of Smartamine M can have no effect on DMI, as is the case with Chen et al. (2011). When compared with other types of Met products, Smartamine M seems to have a more pronounced effect on DMI in some cases. Cermakova et al. (2012) demonstrated that dairy cows supplemented with Smartamine M had significantly higher DMI than those supplemented with MetaSmart. Responses in DMI with other products have also been seen.

Mepron M85, another physically protected Met product, has been shown in multiple studies to decrease DMI. Zanton et al. (2014) reported that Mepron M85 decreased DMI when compared with their respective control cows. Similarly, Benefield et al. (2009) observed a decrease in DMI after supplementation of Mepron M85. MetaSmart, a Met analogue, has been shown to both increase and decrease DMI. Osorio et al. (2014) demonstrated that MetaSmart fed to post-partum cows, increased DMI. An increase in DMI upon supplementation of Met analogues is understandable, because it has been reported that Met analogues have a stimulatory effect on certain rumen bacteria, which

could increase passage rate, thus increasing DMI (Lee et al., 2015b). In contrast, Cermakova et al. (2012) reported a decrease in DMI in MetaSmart supplemented cows when compared to those fed a control diet. There are multiple other examples of both increases and decreases in DMI being reported with supplementation of different Met products. It seems the current data on DMI related to Met supplementation is very variable and may not be reliable. More research needs to be conducted on the variables that are related to Met supplementation and DMI under different physiological conditions.

Effects on Milk and Milk Component Production

Beginning in 2000, the Federal Milk Marketing Administration implemented the multiple component pricing system (AMS-USDA, 1999). This means there is a monetary value on the pounds of fat, protein, and other solids in the milk that are produced rather than just total volume of milk produced. To maximize producers' milk check, dairy farmers focus on fat and protein concentrations in the milk and total milk volume. Other solids and solids-not-fat are hard to manipulate, unlike milk fat and milk protein, and also do not yield nearly as good of an economic return as do milk fat and milk protein. For that reason, other solids is usually not a main focus of dairy producers today. Therefore, this literature review will only focus on the effects of Met supplementation on milk, milk fat, and milk protein production and how different RPMet products affect production in their own separate ways.

Total volume of milk produced per cow per day, or the herd average milk production, is the glory number on any dairy. It is often used as a benchmark number, or a way to

compare how well a dairy is performing. More practically, this number directly correlates with how many pounds of milk fat and milk protein the dairyman will get paid for, and for that reason it is of great importance to focus on increasing milk production. The best and most recorded responses in milk production have come through the use of physically protected Met products, especially Smartamine (Rulquin et al., 2006). After compiling data from 18 different studies that used Smartamine, Patton (2010) reported that on average across all 18 studies, an increase in milk production was observed. Mepron has also shown multiple positive responses in milk production (Lara et al., 2006; Broderick et al., 2008). Some studies have also shown no response in milk production with supplementation of Smartamine (Rulquin et al., 2006) and Mepron (Leonardi et al. 2003). In spite of the fact that some studies show no response, physically protected Met products still prove to be effective in increasing milk production (Chen et al., 2011).

Some Met analogue products, such as MetaSmart, can also have a positive impact on milk production, but not all Met analogue products show such positive results. In review, there are two types of Met analogues, 2-hydroxy-4-(methylthio)-butanoic acid or the isopropyl ester of HMB; both have different effects on milk production. Generally, HMB does not affect milk production, but HMBi, because of its significant metabolizable Met supply to dairy cows, has the potential to increase milk production (Rulquin et al., 2006). Many studies have compared the two Met analogues and their effects on milk production. St-Pierre and Sylvester (2005) compared two popular Met analogue products-Alimet (HMB) and MetaSmart (HMBi). They found milk production in cows supplemented with HMB was not affected by HMB, but those supplemented with HMBi produced an

additional 2.9 kg of milk when compared with the control cows. Noftsger et al. (2003 and 2005) has suggested the reason HMB does not help increase milk production is because it is not a major source of metabolizable Met, but rather it acts in the rumen and influences the microbial populations of the rumen. Lee et al. (2015b) had similar findings by supplementing HMB, as they observed a 1 kg numerical decrease in milk production for every 0.05% of dry matter (**DM**) increase in HMB. In periparturient cows, Piepenbrink et al. (2004) actually found a quadratic increase in milk production with HMB supplementation with a decrease occurring at a supplementation rate of 45 g/d of HMB. The rumen protected isopropyl ester of HMB (HMBi) has proven to have a somewhat better response with increasing milk production. Confirming the findings of St-Pierre and Sylvester (2005), Xia et al. (2012) reported an increase of up to 3.9 kg/d milk yield with supplementation of HMBi both pre and postpartum. Methionine analogues have been proven to be more effective in increasing milk fat, rather than total milk yield.

Unlike milk production, milk fat production shows a more positive response to HMB and HMBi supplementation. The use of HMB has been studied for a longer period of time than HMBi and, therefore, there are earlier studies that show the effect of HMB on milk fat production. In fact, there are multiple early studies that show a positive response in milk fat production from supplementation of HMB (Holter et al., 1972; Lundquist et al., 1983; Huber et al., 1984). Interestingly enough though, the results of current studies pertaining to milk fat production and HMB are mixed and not conclusive (Lee et al., 2015b). In contrast, HMBi has recently received a lot of attention and has shown an ability to increase milk fat production. Xia et al. (2012), when comparing milk fat

production in cows supplemented with HMBi post-partum and pre/post-partum to control cows, reported an increase in milk fat yield (1,120 and 1,160 vs. 980 g/d, respectively). St-Pierre and Sylvester (2005) also noticed an increase of 166 g/d of milk fat yield with HMBi supplementation. Traditional RPMet products, such as Smartamine and Mepron, are not usually associated with increases in milk fat production, but rather with increases in milk protein production.

True rumen-protected forms of Met, normally do not contribute to an increase in milk fat because of their lack of a ruminal mode of action, unlike HMB and HMBi. Many responses in both milk protein concentration and yield have been recorded for RPMet products such as Smartamine and Mepron. Patton (2010) who summarized the findings of 35 studies (17 evaluating Mepron and 18 evaluating Smartamine) showed, on average, supplementing these products increased both milk protein yield and concentration. Many studies have also been conducted comparing true RPMet products to Met analogue products and their effects on milk protein yield. Ordway et al. (2009) showed an increase in milk protein concentration in cows fed MetaSmart as well as Smartamine compared to those fed a control diet. Osorio et al. (2013) observed an increase in milk protein yield, but they reported a larger increase in Smartamine supplemented cows when compared with those supplemented with MetaSmart (1.24 vs 1.23 kg/d). The findings of Rulquin et al. (2006) also confirm the results of Osorio et al. (2013) in that Smartamine supplemented cows produced more grams per day of milk protein than MetaSmart supplemented cows, and both groups produced more than the control group. The important message here is when Met is made available post-ruminally, whether it be

through RPMet products or Met analogue products, milk protein is increased. Methionine, if not utilized by the cow for milk and milk component production, can also potentially play a pivotal role in BW gain.

Effects on BW Gain

Effects of supplemental Met on BW gain in dairy cattle seems to be sparse and lacking. However, in other species information concerning this topic is abundant; for example, in the poultry industry supplementation of dietary Met is a common practice and is well researched. Lemme et al. (2002) found a positive response in BW gain, feed conversion, carcass yield, and breast meat yield with supplementation of both DL-Met and HMB, with better responses coming from DL-Met supplementation. Similarly, Meirelles et al. (2002) concluded that DL-Met increased BW gain and feed conversion more than HMB, although HMB also increased BW gain and feed conversion when compared with a control diet. In both studies HMB was found to have an efficacy on weight gain of about 65% when compared with DL-Met as a source of dietary Met (Lemme et al., 2002; Meirelles et al., 2002). Growing pigs also respond positively in weight gain and feed conversion to Met supplementation. Zimmermann et al. (2005) reported that pigs supplemented with DL-Met or HMB had higher weight gains than those consuming a control diet (501 and 488 g vs. 432 g). Very little information concerning Met supplementation and BW gain in dairy cows is available, but there is information on other ruminants.

Although there is data concerning weight gain in steers and lambs supplemented with Met, this data has been inconsistent and quite disappointing. Some studies showed

positive responses in BW gain and feed efficiency with supplementation of RPMet (Deetz et al., 1985; Oke et al., 1986). Others, such as Strasia et al. (1986) reported no response in BW gain. More recently, Hussein and Berger (2014) observed no response in BW gain with as much as 50 g of RPMet supplementation to Holstein steers. A lack of response to RPMet supplementation suggests that Met was not limiting in these experimental diets. Data on the effects of supplementation of Met analogues and derivatives are currently even more sparse and lacking than that of true RPMet supplementation. Even though BW gain results have been somewhat disappointing, Met has the potential to aid in BW gain in diets where Met is the most-limiting AA.

Similar to milk protein synthesis in dairy cattle, Met is the first-limiting AA in growing cattle (Richardson and Hatfield, 1978). This is significant, because Met functions as a precursor for protein synthesis and, therefore, a deficiency of Met will lead to a decrease in the turnover of dietary protein into protein deposition in the body, thus limiting potential BW gain (Loest et al., 2002). In competition with its use for protein synthesis, Met can also be converted to SAM in the liver, which acts as a methyl group donor for many transmethylation reactions (Finkelstein, 1990). SAM aids in the formation of phospholipids (Obeid and Herrmann, 2009), which in turn aids in the formation of the phospholipid bilayer of very low density lipoproteins (**VLDL**). Then, VLDL carries triglycerides, which, especially in finishing cattle, aids in fat deposition and intramuscular marbling, leading to an increase in BW gain (Dodson et al., 2010). Methionine has the potential to positively affect BW gain, but more research needs to be performed to clearly understand its mechanism.

Effects on N Utilization

Unlike the effects of Met supplementation on BW gain, the effects of Met supplementation on N utilization are well known and reported. As discussed earlier, supplementation of RPMet can increase milk yield (Patton, 2010) and milk protein yield (Ordway et al., 2009). Therefore, the use of RPMet may allow for the feeding of less CP in diets without adversely affecting production (Broderick et al., 2008). Reducing dietary CP concentration, while maintaining production, could potentially have a huge impact on N utilization and excretion, because when dairy cows are fed to meet MP requirements, they consume excessive N, resulting in 75% of the dietary N consumed being lost to the environment in urine and feces (Arriola Apelo et al., 2014). This potential decrease in N utilization is important today because of the need to continue to feed the increasing world population while focusing on the ever-present environmental concerns (OECD/FAO, 2015).

Many studies show a positive response in production and a decrease in N excretion in relation to a decrease in dietary CP accompanied by Met supplementation. Broderick et al. (2008) conducted a trial in which dietary CP was reduced from 18.6% to 14.8% with an increase in RPMet supplementation from 0 to 15 g/d in four different diets. As expected, significant increases in milk production and N efficiency were observed: 39.7 to 41.6 kg/d milk yield and 26.2 to 31.7% N utilization. Supplementation of HMB has also proven to increase both milk production and N efficiency. Wang et al. (2010) observed that upon HMB supplementation in a MP deficient diet, milk production was increased from 26.5 to 28.5 kg/d coupled with N efficiency improvement from 26 to

28.3%. In both studies, excretion of N was reduced in milk, urine, and manure. The ability to reduce dietary CP while maintaining or increasing production is of great value when focusing on the potential environmental impact of dairying.

Economic Analysis

The worth of Met supplementation to a dairyman hinges on a positive return on investment. Dairymen routinely measure their return on investments in terms of the cost invested to increase milk and milk component production compared with how much milk and milk component production actually increases. Currently, as this review is being written, the market price of class III milk is $16.74 per hundredweight (**CWT**) (www.cmegroup.com; May 2017), $2.05 per lb. of milk fat, and $2.30 per lb. of milk protein (www.milkpay.com; May 2017). Smartamine, a popular RPMet product, is currently priced at $6.72 per lb., and the recommended feeding rate is 12 g/cow/d, depending on dietary deficiency of Met. Metasmart, a popular HMBi Met analogue product, is currently priced at $2.73 per lb. and the average feeding rate is 40 g/cow/d. At the average feeding rate the cost of feeding Smartamine would be $0.18/head/d, whereas the cost of feeding Metasmart would be $0.24/head/d. At the current cost of milk, a dairy farmer would need to produce 1.08 lbs. of extra milk to cover the cost of investment in Smartamine and an extra 1.43 lbs. of milk to cover the cost of investment in Metasmart. Both of those numbers are attainable. It is important to note, and has been discussed previously in this review, that Smartamine and Metasmart can have different impacts on milk component production. Dependent on the targeted milk component, either Smartamine or Metasmart may have the biggest financial impact for the dairy producer.

Acetylated AA

Although the aforementioned methods of supplying useable Met to dairy cows have been successful, the ever-evolving dairy industry is always looking for new and improved methods of increasing production and herd health, while decreasing the environmental impact of dairying. As another way of delivering bioavailable AA to the dairy cow, acetylation of AA has garnered attention. Acetylation of AA occurs when the NH_2-terminals of AA are acetylated with an acetyl group (Gade and Brown, 1981). The beginning products of this reaction are L-AA and acetic anhydride (EFSA, 2003). The purpose of acetylating AA is to protect the N-terminal amino group from hydrolysis by rumen microbes (Wallace, 1992). The acetylation process is not new; however, the use of acetylated AA in ruminant nutrition is new and as a result of this, research pertaining to this topic appears to be sparse.

Bioavailability

As a result of the chemical makeup of acetylated AA (L-AA and an acetyl group), the bioavailability of the AA in question depends on the separation of the AA and the attached acetyl group. The acetyl group acts as a barrier that blocks the hydrolysis of the NH_2-terminal of the amino acid which protects it from ruminal degradation (Wallace, 1992). The protection provided by the acetyl group also prevents absorption of the attached AA in the small intestine. Thus, acetylated AA themselves are not bioavailable, per se, until the acetyl group and attached AA are separated. The hydrolytic reaction that separates these 2 compounds is catalyzed by an enzyme called acylase 1 or aminoacylase

1 (Baxter et al., 2001). This enzyme is found in multiple mammalian tissues, including the small intestine, which is the most important site in the study of the bioavailability of acetylated AA (Giardina et al., 1997).

The hydrolytic reaction separating the acetyl group and the AA is not possible without the aminoacylase 1 enzyme, thus the bioavailability of acetylated AA is dependent on the presence of the aminoacylase 1 enzyme, and the bioavailability can be predicted based on the ability of aminoacylase 1 to catalyze this hydrolytic reaction (Baxter et al., 2001). Furthermore, Gade and Brown (1981) found that the aminoacylase enzyme is the major and the only enzyme that catalyzes the hydrolysis of acetylated AA, especially acetylated Met. Therefore, if the aminoacylase enzyme is the only enzyme capable of hydrolyzing acetylated AA, then the theory of Baxter et al. (2001) that states the bioavailability of acetylated AA is directly related to the amount of enzyme present and the ability of this enzyme to catalyze hydrolytic reactions, holds true. Baxter et al. (2001) reinforced their hypothesis by studying the hydrolytic effects of aminoacylase on N-acetyl-L-glutamine (**NAQ**) and NALM. They concluded that the hydrolyzation of both NAQ and NALM was linearly dependent on the concentration of aminoacylase and time (Baxter et al., 2001). Another way to investigate the efficacy of aminoacylase is to measure the K_m and V_{max} of the enzyme.

An easy and simple way to define K_m and V_{max}, in the context of the aminoacylase enzyme, is that K_m is a measure of how easily and quickly aminoacylase binds to acetylated AA, and V_{max} defines how fast aminoacylase can catalyze the hydrolytic reaction to yield the L-AA and an acetyl group (Dixon et al., 1979). Galaev and Svedas

(1982) found the K_m constant of the hydrolysis of acetyl-L-Met by aminoacylase to be 0.14 ± 0.03 M while Baxter et al. (2001) found a K_m constant of 0.00136 ± 0.00015 M. The low K_m constant values presented in the two studies above suggest that aminoacylase binds to acetylated AA quickly and easily, or in other words aminoacylase has a high affinity for acetyl-L-Met and only a small amount of substrate is needed to saturate the enzyme. The V_{max} of this same reaction is not quite as fast, when compared with the hydrolysis of other acetylated AA. The V_{max}, as found by Baxter et al. (2001) is 7.48 ± 0.28 nM Met/min/µg acylase 1. When comparing the K_m and the V_{max} of the hydrolytic reaction of acetyl-L-Met, one can conclude there is a high affinity for the substrate, but once it is saturated the reactions proceeds to completion at a moderate pace. One can also conclude in the presence of aminoacylase and upon completion of the hydrolytic process, much of the free AA released by this process will be bioavailable to the dairy cow. There are, however, other factors which may influence the efficacy of this hydrolytic process.

One major factor that influences the efficacy of hydrolyzing acetylated AA is the type of AA isomer being utilized. Birnbaum et al. (1952) concluded acetylated D-isomers of AA were hydrolyzed much more slowly than their corresponding L-isomers. Similarly, while studying the bioavailability of different acetylated AA, Boggs (1978) found comparable results to Birnbaum et al (1952). Boggs (1978) fed N-acetyl-D-Met and NALM in diets deficient in Met to rats under similar conditions. Growth of rats fed NALM was 62.7 ± 9.5 g/d, while the growth of rats fed N-acetyl-D-Met was 23.9 ± 0.6 g/d, which clearly supports the assumption that acetylated D-isomers of Met are not as

bioavailable as the L-isomer. The pH at the location of enzymatic activity can also greatly influence the hydrolytic efficacy of the aminoacylase enzyme. Gade and Brown (1981) found the optimum pH for which aminoacylase can most effectively hydrolyze acetylated AA was approximately 8.5. In the pH range of 6.0 to 10.3, enzymatic activity was still 90% effective, but when the pH dropped down to 3.8, no enzymatic activity could be detected. Similarly, Galaev and Svedas (1982) concluded the optimum pH range of aminoacylase activity was somewhere around a pH of 7.0 – 8.0. The pH in the small intestine of ruminants falls in the range of 7.0 – 8.0, which creates the perfect pH environment for the hydrolyzation of acetylated AA. The concentration of aminoacylase in the small intestine is very high (Boggs, 1978). Thus, because of the optimal pH conditions in the small intestine and the high concentration of aminoacylase in the small intestine, the potential bioavailability of the free AA released from the hydrolytic process will be high.

Bioavailability of Other Met Supplements

Unlike acetylated Met, the bioavailability of RPMet and Met analogues is more well-known and studied. Many different methods of delivering useable Met to the dairy cow have been described above and although they all have the same objective in mind of protecting the Met in the rumen, the bioavailability of these different methods and products differs. Understanding and studying the bioavailability of these different products is important in determining their efficacy in the dairy industry, because although all of these products provide useable Met to some degree to the dairy cow, dairy

producers should choose a product based on its ability to provide the most bioavailable Met to the cow. The bioavailability of RPMet products is ultimately determined by the Met concentration of the product, the ruminal stability of the product, and the intestinal release of the product (Berthiaume et al., 2000). The bioavailability of different rumen-protected products and different Met analogue products will be discussed in detail.

Bioavailability of RPMet

As stated above, it stands to reason the Met concentration of RPMet products directly influences the degree of bioavailable Met delivered to the small intestine. As discussed in the RPMet Product Overview section of this literature review, different RPMet products have differing concentrations of DL-Met. As far as true RPMet products are concerned, potentially the most popular commercial product, Smartamine (Adisseo, Inc., Antony, France) contains 75% DL-Met, while another popular product, Mepron (Degussa Corporation, Germany) contains 85% DL-Met, and Met-Plus (Nisso America, Inc., New York, NY) has 65% DL-Met. In review, Met analogues do not contain DL-Met, but rather they contain the compound 2-hydroxy-4-(methylthio)-butanoic acid, which eventually can be converted to Met. Metasmart (Adisseo, Inc., Antony, France), the isopropyl ester form of HMB, is potentially the most popular Met analogue available on the market. MetaSmart consists of no less than 57% HMBi, which is a 78% Met equivalent, and of that 50% is reported to be absorbed and converted to metabolizable Met. Therefore, the actual concentration of Met in each of the products varies, with Mepron containing the most Met (85%). Although the concentration of Met in each of the

products is important, it does not independently dictate the amount of bioavailable Met in the product itself, ruminal degradation also influences this.

Ruminal degradation rate, or ruminal outflow rate, of any RPMet product is influenced by the particle size and density of the product (Koenig and Rode, 2001). Adisseo Inc., reports a particle size of 1.4 to 2.5 mm and a density of 0.7 g/cm^3 for Smartamine and a particle size of approximately 0.3 mm and a density of 0.75 g/cm^3 to 0.80 g/cm^3 for MetaSmart, while Degussa Corporation reports a pellet particle size of 1.8 × 3 mm and a density of 0.71 g/cm^3 for Mepron. To compare how particle size affects ruminal outflow rate, Smartamine has a mean rumen retention time of 31.9 h in lactating dairy cows (Mambrini and Peyraud, 1997). Also influencing ruminal outflow rate is the form of protection used to help Met bypass the rumen. Smartamine uses a pH-sensitive polymer coating (Adisseo, Inc., Antony, France), and Mepron uses a coating of ethyl cellulose (Degussa Corporation, Germany) to protect a core of DL-Met, while MetaSmart utilizes the analogue HMBi (Adisseo, Inc., Antony, France), which will be converted to Met after being absorbed. Differing particle sizes, densities, and forms of rumen-protection result in differing bioavailabilities. Schwab and Ordway (2003) reported average ruminal outflow rates of 90% for Smartamine and 80% for Mepron. In other words, 90% and 80% of ingested Smartamine and Mepron, respectively will pass undegraded through the rumen to the acidic abomasum where the encapsulated Met will be released. MetaSmart has a rumen bypass rate of 50%, although technically MetaSmart does not bypass the rumen in the traditional manner (Adisseo, Inc., Antony, France). MetaSmart is absorbed across the rumen wall where it then becomes bioavailable. After

assessing the Met content and the rumen bypass rate of an RPMet product, the abomasal release and intestinal absorption of the product needs to be determined in order to quantify the potential efficacy of the product in question.

Once RPMet products, with the exception of Met analogues, bypass the rumen and enter the abomasum, the encapsulated Met is released in the acidic abomasal environment where it then passes onto the small intestine. Similar to Met concentration and ruminal bypass rate, the rate of release of Met from the abomasum and the subsequent absorption rate in the small intestine, differ among all products. One way to measure intestinal absorption is to measure the intestinal disappearance of Met expressed as a percentage of total Met entering the small intestine from the abomasum. Koenig and Rode (2001) stated that the intestinal disappearance rate of Mepron averaged 31.9%, while Sudekum et al. (2004) similarly found an average Mepron intestinal disappearance rate of 28%. Schwab and Ordway (2003) reported an average intestinal disappearance rate of 90% for Smartamine. This large difference in intestinal disappearance between Mepron and Smartamine can most likely be attributed to the difference in the form of rumen protection employed in the products, and the efficacy of the eventual release of Met. The polymer coating of Smartamine is degraded in low pH environments, while the coating of Mepron is degraded through abrasion and physical breakdown, which may not be as reliable as the pH-sensitive Smartamine coating. After assessing the Met concentration, the ruminal bypass rate, and the intestinal disappearance rate, the bioavailability of the RPM product can be calculated.

Berthiaume et al. (2000) stated that RPMet bioavailability is based on the products' AA concentration, their ruminal stability, and intestinal digestibility. Similarly, Koenig and Rode (2001) defined RPMet bioavailability as a combination of effective ruminal degradability and intestinal disappearance with the actual calculation being (100 – effective ruminal degradability) × intestinal digestibility. Based on these calculations, Koenig and Rode (2001) found the bioavailability of Mepron to be a low 23.6%. In comparison, only 25 to 35% of feed protein in normal dairy rations reaches the small intestine. By this comparison, Mepron does not seem to be an effective source of bioavailable Met. Contrary to the findings of Koenig and Rode (2001), the Degussa Corporation reports a bioavailability of 80 to 85% for Mepron. Abdi-Benemar (2016) reported a bioavailability of 61% for Mepron, which is closer to the bioavailability reported by Degussa Corporation. These differences in the bioavailability of Mepron may arise from the ethyl cellulose matrix coating of this product. The coating of this product is designed to be thinner at both ends of the pellet so that both ends can be opened, and the DL-Met core can be solubilized and flushed out of the pellet (Berthiaume et al., 2000). The problem that potentially may arise with this mode of action is that Mepron relies on abrasion and physical forces for the degradation of the ethyl cellulose coating, rather than relying on enzymatic or pH activity, which may lead to a decrease of available methionine (Schwab and Ordway, 2003). Unlike Mepron, Smartamine relies on a pH-sensitive polymer coating to protect the DL-Met core, which makes estimating bioavailability more constant and easier. According to Robert and Williams (1997), the bioavailability of Smartamine falls somewhere between 75 to 97%. Similarly, Graulet et

al. (2005) determined the bioavailability of Smartamine to be 74%. Providing more evidence to the consistency of the bioavailability of Smartamine, Schwab (1995) found the bioavailability to be 80%. Although Met analogues are different in both composition and their mode of action from traditional RPMet products, the bioavailability of Met analogues can still be assessed and compared to the bioavailability of RPMet products. Graulet et al. (2005) compared the Met bioavailability of HMBi with that of Smartamine and found the Met bioavailability of HMBi and Smartamine to be 48 and 71%, respectively. This is in accordance with estimations from Robert et al. (2001, 2002) of a bioavailability of 40 to 58% for HMBi. According to the studies cited, Smartamine provides the most bioavailable Met to the dairy cow, which creates a standard for the future bioavailability studies of Met derivatives.

Absorption of NALM in the Small Intestine

The use of acetylated AA in ruminant nutrition is a fairly new concept, and consequently information concerning its bioavailability is sparse. In review, acetylation of AA occurs when the NH_2-terminal of an AA is protected with an acetyl group (Gade and Brown, 1981). The purpose of acetylating AA is to protect the N-terminal amino group from hydrolysis by rumen microbes (Wallace, 1992). Once NALM bypasses the rumen and arrives at the small intestine, the aminoacylase enzyme hydrolytically separates the acetyl group from the L-Met molecule, thus making both the acetate and the L-Met molecule available for absorption in the small intestine. Providing both an acetyl group and an L-Met molecule is a very unique aspect of NALM that distinguishes it from

other RPMet or Met analogue products. Without the separation of these 2 molecules, however, L-Met would not be available for absorption in the small intestine.

Intestinal Absorption of AA

Once AA reach the small intestine, they can be incorporated into protein (constitutive or secretions), converted into other AA for biosynthetic processes, oxidized to CO_2, or transported through enterocytes into the mesenteric portal vein (Stoll and Burrin, 2006). The small intestine is divided into three distinct regions consisting of duodenum, jejunum, and ileum. Absorption rates differ in each of the different sections of the small intestine and the site where the majority of AA absorption occurs differs according to species. In ruminants, it is believed that the majority of AA absorption occurs in the ileum (Webb and Matthews, 1994). Williams (1969) also proposed that the site of AA absorption can change according to the AA being absorbed. For example, Williams (1969), in agreement with Webb and Matthews (1994), found that the majority of AA absorption occurred in the ileum of sheep with the exception of Met which was absorbed in equal amounts in the jejunum and ileum. Similar to the influence of AA on the site of absorption, they can also have an impact on the absorption rate, or in other words, different AA are absorbed at differing rates. Armstrong et al. (1977) and Christiansen and Webb (1990) observed that Met was absorbed at the greatest relative quantity when compared to all other AA. Likewise, the mode of transportation and the mechanism used to transport AA through the enterocytes can differ according to the individual AA.

Amino acids can be transported from the intestinal lumen to the enterocytes through either simple diffusion, facilitated diffusion, or active transport (Wilson and Webb,

1990). Facilitated diffusion mechanisms are also known as sodium-independent systems, and active transport is referred to as sodium-dependent transport. Sodium-dependent systems require energy to pump AA into the cell. This process is regulated by Na^+/K^+ ATPase, which pumps Na and AA into the cell and K out of the cell (Webb and Matthews, 1994). Sodium-independent systems do not require energy to pump AA into the cell. Amino acid concentration in the small intestine dictates which transportation method is utilized. Only when concentrations of AA are low does transportation through facilitated diffusion, and active transport exceed absorption through simple diffusion (Wilson and Webb, 1990). Amino acid transport systems can also be classified according to substrate preference (Wu, 2013). Many transport systems have an affinity for more than one substrate.

It is important to recognize the AA profile that reaches the small intestine will not be the same AA profile that will be absorbed through the enterocytes and enter the portal circulation. As stated earlier, AA can be oxidized for energy or utilized for other purposes, such as cellular protein synthesis or mucin production, in the small intestine. As these AA are irreversibly utilized, they are then nutritionally unavailable to the cow (Stoll and Burrin, 2006). Depending on the AA in question, small intestinal metabolism could drastically change the amount available for absorption. As much as 20-90% of dietary AA can potentially be catabolized in and by the small intestine, depending on the AA (Wu, 2013). Measurements of AA disappearance in the ruminant small intestine is lacking, but there are studies concerning AA disappearance in the monogastric small intestine. In similar swine studies, Wu et al. (2010) and Stoll and Burrin (2006) found

some AA, such as glutamate and aspartate, are almost completely catabolized by the small intestine and nearly 30% of Met is metabolized. In contrast, Seal and Parker (2000) suggested only 20% of the ruminants' small intestinal AA requirement comes from lumen-derived dietary AA, with the rest being derived from the arterial supply of AA. Stoll and Burrin (2006) suggested the reason oxidation of AA in the small intestine can potentially be high compared to any other substrate is because it is the body's way of meeting the high metabolic demands of the small intestine while preserving and ensuring the delivery of glucose, the bodies' most important energy substrate, to the peripheral tissues. Once AA are transported into the enterocytes, catabolization appears to be negligible. Chen et al. (2009) reported that catabolism of Met in piglet enterocytes was negligible. Amino acids travel through the enterocytes where they pass through the basolateral membrane and enter mesenteric circulation on their way to the liver.

Liver Metabolism

The liver is a very large organ that is essential for nutrient metabolism and nutrient distribution in the body. The liver has a variety of functions including detoxification of certain substrates, decomposition of red blood cells, protein synthesis, hormone production and removal, triglyceride production, control of feed intake, and bile production, just to name a few. However, one of the main functions of the liver is to filter the hepatic portal vein blood before allowing it to pass to the rest of the body. In ruminants, blood accounts for 25% of the livers mass, and the liver receives 25% of the cardiac output (Lobley et al., 2000). Blood supply to the liver comes from both the

hepatic portal vein and the hepatic artery. The hepatic artery only contributes 8-12% of total hepatic blood flow in cattle and sheep, with the rest of blood flow coming from the hepatic portal vein (Lobley et al., 2000). Blood flow to the liver through the hepatic portal vein is not controlled by the liver itself and is actually inversely related to arterial supply of blood to the liver (Lautt, 1996). Lobley et al. (2000) suggested that adenosine released in the liver and the subsequent removal of adenosine through hepatic and arterial flows may be responsible for regulating hepatic blood flow. In the hepatic portal vein, AA are carried to the liver in the plasma and not in the red blood cells (Houlier et al., 1991).

Liver Removal of AA

Similar to the small intestine, when AA reaches the liver they can either pass directly through the liver to be utilized either in peripheral tissues, or in the liver for synthesis of proteins, urea, or glucose (anabolism), or they may also be oxidized (catabolism) (Bequette et al., 2003). The liver has the difficult function of ensuring a sufficient supply of AA to peripheral tissues while maintaining non-toxic levels of AA in the bloodstream. Removal of AA from hepatic circulation is usually expressed relative to the amounts absorbed from the portal drained viscera (**PDV**), and it is important to note that the PDV includes the whole gastrointestinal tract, pancreas, and spleen. This is critical to note, because only a small portion of the AA in the portal vein actually comes from first pass absorption from the small intestine (Lobley and Lapierre, 2003). Similar to studies on the metabolism of AA in the small intestine, results on the uptake of AA by the liver are mixed. Variation in the uptake of AA by the liver is influenced by many factors,

including the nutrient profile of the ration being fed and the physiological state of the animal (Lobley and Lapierre, 2003). Some of this variation can also be attributed to the long-standing question of whether the liver acts as a “controller” and regulates the amount of AA that reaches peripheral tissues or whether the liver act as a “responder” and removes the AA that are not used by the peripheral tissues (Lobley and Lapierre, 2003). Another big factor influencing AA uptake by the liver is the type of AA being absorbed (EAA vs. non-essential AA (**NEAA**), for example).

Essential AA tend to have different removal rates in the liver when compared with NEAA, and even some EAA, such as branched chain amino acids (**BCAA**), have different removal rates when compared with other EAA. Bequette et al. (2003) reported in dry dairy cows 0 to 30% of absorbed BCAA are removed across the liver, and in lactating dairy cows there is actually a net release of BCAA from the liver. This coincides with the findings of Blouin et al. (2002) who also recorded a net positive release of BCAA from the liver. Lobley (1992) proposed BCAA catabolism in the liver is very limited and once this threshold is reached, the removal of BCAA by the liver will stop, and catabolism of BCAA will occur in non-hepatic tissues. Concerning all other EAA, the amounts removed by the liver varies. According to Bequette et al. (2003), 43% of Met, 50% of phenylalanine, 11% of threonine, and 28% of histidine were removed in the liver from the net portal supply of AA. Lapierre et al. (2000) reported liver removal rates of 29% for Met, 64% for phenylalanine, 23% for threonine, and 39% for histidine. Liver extraction of NEAA tends to be higher than the extraction of EAA (Bequette et al., 2003). It appears the extraction of NEAA by the liver exceeds that of the extraction of EAA

because of their role in metabolic functions, such as gluconeogenesis (Hanigan et al., 2004). No matter the removal rate, AA are removed for a specific purpose, including the use of Met for multiple functions in the liver.

Liver Utilization of Met

In the liver, Met that is removed from the hepatic blood flow is converted to SAM, homocysteine, and cysteine via the methylation cycle. Then, SAM is primarily used for transmethylation, trans-sulfuration, and polyamine synthesis, SAM appears to be second only to ATP in the number of reactions in which it is a cofactor (Lu, 2000). According to Mudd and Poole (1975), Met prefers the catabolic pathway of SAM synthesis in the liver, where up to half of the daily intake of Met is converted to SAM. In the liver, Met and ATP are converted to SAM with the help of the enzyme Met adenosyltransferase (Mato et al., 1997). Once the methyl group of SAM is removed in the transmethylation processes, SAM will then be converted to S-adenosylhomocysteine. The adenosine is then removed from S-adenosylhomocysteine, resulting in the formation of homocysteine. From this point, homocysteine can either be transsulfurated to make cysteine or remethylated back to Met (Loest et al., 2002). Remethylated Met can either enter the Met cycle again, or it can be exported out of the liver to be used elsewhere.

Physiological Impacts of Met

Methionine is most well known for its potential impact on milk and milk component production, but Met can also have a large impact on cow health through its role in liver lipoprotein synthesis, as a substrate for antioxidant reactions, and for its role in immune

function (Osorio et al., 2013). The effects on health because of Met supplementation appear to be most pronounced and beneficial in transition cows and is an essential nutrient for transition cows (Sun et al., 2016). The term transition refers to the 3 wks. before calving and the 3 wks. after calving. During this time, cows are much more susceptible to fatty liver disease, ketosis, retained placenta, hypocalcemia, clinical mastitis, and displaced abomasum (Sun et al., 2016). Cows are highly susceptible to these metabolic disorders at this time for a variety of reasons, one of which being the rapid growth of the fetus prior to parturition and the rapid increase in milk production postpartum. This rapid increase in milk production creates a high demand for energy which usually cannot be met with the limited DMI of fresh cows, and then results in a negative energy balance. This unique problem demands innovative ways of supplementing and feeding cows in such a way as to increase their profitability and viability during this stressful transition period. Methionine supplementation may alleviate some of the problems associated with the transition period.

The synthesis of SAM from Met plays a central role in the health of transition cows; SAM is a very important methyl donor in the formation of phosphatidylethanolamine to phosphatidylcholine, which is important in the packaging of VLDL (Osorio et al., 2014). This is significant in transition cow health, because when transition cows are in a negative energy balance state, they mobilize their body fat reserves to meet their high energy demand. As body fat is mobilized, non-esterified fatty acids (**NEFA**) are formed and sent to the liver where they can be re-esterified to form triglycerides (Gross et al., 2013). Choline, which is synthesized from Met, facilitates the movement of NEFA to the

liver (Goselink et al., 2013). Triglycerides can be carried out of the liver through VLDL, or they can be stored in the liver, which is a cause of fatty liver disease. Sun et al. (2016) observed an increase in NEFA concentrations in postpartum transition cows supplemented with RPMet. The same study reported an increase in plasma VLDL concentrations, suggesting that Met influences liver lipid metabolism and exportation (Sun et al., 2016). Methionine can also have a variety of other effects on the health of transition cows as well.

It has been proposed that Met supplementation can have an impact on the proliferation of T lymphocyte production. For example, Soder and Holden (1999) reported an increase in T lymphocyte concentrations when 15 and 30 g/d of RPMet was supplemented to mid-lactation dairy cows. In comparison, Osorio et al. (2013) reported a tendency for an increase in phagocytosis in cows supplemented with RPMet. The mechanism behind the increase in T lymphocyte concentrations and phagocytosis is not well understood, but it underlines the fact that Met supplementation potentially can have an impact on the immune function of cows. Methionine supplementation has also been shown to counteract the negative inflammatory effects of parturition. Osorio et al. (2013) observed an increase in albumin in Met supplemented cows when compared to control cows. Decreased albumin concentrations in control cows not supplemented with Met is understandable, because the lack of albumin is indicative of inflammatory conditions in transition cows. Methionine supplementation appears to negate some of the effects of inflammation in transition cows. Methionine can also potentially help in the antioxidant capacity of transition cows. Glutathione is a product of the methionine cycle in the liver

and also acts as the primary antioxidant defense in the prevention of oxidative liver injury (Halsted, 2013). Supplementation of RPMet has been shown to increase and expedite the synthesis of glutathione, which in theory would affect the antioxidant status of the transition cow (Osorio et al., 2014). Because of the role of Met in the liver, in lipoprotein synthesis, as a substrate for antioxidant reactions, and for its role in immune function, it can be very beneficial to the health of the transition cow.

Conclusions

Methionine has long been recognized as a limiting AA for milk protein synthesis and milk production (Schwab et al., 1976). In order to meet the Met requirements of the lactating dairy cow, many different methods have been utilized to deliver useable Met to the dairy cow. These methods include protecting Met from ruminal degradation or utilizing Met analogues. A new approach to delivering useable Met to the dairy cow is the utilization of Met derivatives. A Met derivative is a free Met molecule that contains a chemical blocking group added to the α-amino group or a Met molecule in which the acetyl group has been modified (Schwab and Ordway, 2003). The Met derivative that is the focus of this study is NALM. Acetylated AA may be an effective way of delivering bioavailable Met to the small intestine where it is absorbed and utilized. This study will present the impacts of NALM on the lactational and physiological performance of eight mid to late lactation cows.

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 January 25, 2016 to April 18, 2016.

Cows, Experimental Design, and Dietary Treatments

Eight multiparous lactating Holstein cows were used during this trial. At the onset of the trial the cows averaged 124 ± 13.0 DIM with an average BW of 694 ± 39.7 kg and 757 ± 55.5 kg at the end of the trial.

A double 4 × 4 Latin square design was utilized in this experiment. The duration of the trial was 84 d which consisted of 4 periods of 21 d each (14 d of treatment adaption and 7 d of data collection and sampling). Within each square, cows were randomly assigned to a sequence of four diets during each of the periods. The four dietary treatments included 0 (control), 15, 30, and 45 g/d/cow of NALM supplementation. In a previous study done by our group (Fagundes et al., 2016), the same NALM product was added at a rate of 0.13% DM, to provide approximately 15.7 g/d/cow of available Met. In the current study, we included half (low dose) and double the previous dose to test if the NALM supplementation would have a linear and/or a quadratic effect on production as well as ruminal fermentation parameters. Metabolizable Met and Lys concentrations and Lys:Met ratios were estimated using the CPM dairy ration analyzer program. The

experimental NALM product was top-dressed onto each of the diets supplemented with 15, 30, and 45 g/d/cow of NALM.

A 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 its Met concentration was reported to be 78.0%, 67% bioavailable, with 99.5% purity and 20-30 wt % solubility in water.

Diets were formulated based on NRC (2001) guidelines to ensure sufficient net energy for lactation (**NE_L**), MP, vitamins, and minerals for production of 40 kg/d of milk with 3.5% fat and 3.0% true protein. Average dietary forage-to-concentrate ratios of 54:46 (DM basis) utilized in this trial were similar to that of a typical high-producing dairy ration in the Intermountain West (i.e., Utah, Idaho, Wyoming, Montana, and parts of Arizona and Nevada). Experimental diets consisted of good quality AH with a chemical composition of 21.4, 32.8, and 24.2% DM for CP, NDF, and ADF, respectively, whereas CS contained 8.51, 44.4, and 24.0% DM for CP, NDF, and ADF, respectively. AH and CS accounted for 47 and 46%, respectively, of total forage in the diet.

Cows were housed individually in tie stalls fitted with rubber mattresses covered with straw and allowed free access to water. Cows were fed twice daily for ad libitum intake at a level of 110% of 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. Milk was sampled for 2 consecutive d (d 17 and 18)

during the a.m. and p.m. milkings during each period. Individual milk samples were analyzed by the Rocky Mountain DHIA Laboratory (Wellsville, UT) for fat, TP, lactose, and milk urea nitrogen (**MUN**). Milk composition was expressed on weighted milk yield of a.m. and p.m. samples. Milk fat and TP yields were calculated by multiplying milk yield from the respective day by fat and TP concentration of the milk from an individual cow. To convert milk TP to milk N, a conversion factor of 6.38 was used (DePeters and Cant, 1992), and total milk N (kg/d) was calculated as milk TP/6.38 + MUN, where milk TP and MUN were expressed as kg/d.

All cows were weighed on the first 2 d of the trial (d 1 and 2) and on the last 2 d of each period (d 20 and 21) after the a.m. milking and before the a.m. feeding. These weights were used to calculate the mean BW of cows for each experimental period. Energy partitioning was determined using the data of milk yield, milk composition, and BW of experimental animals. Energy utilization was determined by calculating energy for maintenance 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 × milk fat concentration) + (0.0563 × milk TP 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 Sampling and Analysis

Samples of AH, oat hay, and CS were pulled weekly and composited for the duration of each period to determine DM, and diets were adjusted accordingly to account for any change in DM concentrations. On d 15 to d 21 of each period, samples of total mixed

rations (**TMR**) and orts were collected from individual cows. All samples were frozen directly after they were pulled and remained frozen until processing. At the end of each period, all samples were dried at 60°C for 48 h, ground to pass a 1-mm screen (standard model 4; Arthur H. Thomas Co., Swedesboro, NJ), and stored for chemical analysis. 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 organic matter (**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).

Ruminal Fermentation Characteristics

Ruminal fluid samples were collected on d 15 and d 19 at 4 h after the morning feeding during each period using a Geishauser probe. The fluid was collected with a solid, tube-like probe with rows of small holes on the end (Geishauser, 1993). To avoid any possible contamination from saliva, the first 100 mL of ruminal fluid extracted was discarded, and the next 15 mL was kept for analysis and strained through a polyester

screen (pore size 355 μm; B & S H Thompson, Ville Mont-Royal, QC, Canada). Immediately upon extraction from the rumen, the pH of the ruminal fluid was measured using a portable pH meter (Oakton pH 6; Oakton Instruments, Vernon Hills, IL). Five mL of the filtered ruminal fluid were mixed with 1 mL of 1% sulfuric acid and stored frozen (−40°C) for analysis of ammonia-N (**NH_3-N**). Concentration of NH_3-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 filtered ruminal fluid was added to 1 mL of 25% meta-phosphoric acid, and then stored at −40°C for determination of volatile fatty acid (**VFA**) content. Ruminal VFA were separated and quantified using a gas chromatograph (Model 5890, Hewlett-Packard Lab, Palo Alto, CA) 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).

Urine Sampling and Analyses

On d 15, d 16, and d 18, spot urine samples were collected at 0600 and 1800 h from each cow. Each period, 6 samples of urine were collected from each cow, and after each sample was collected it was acidified to pH < 4.0, stored at −40°C, and composited by cow per period. 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-heptane sulfonic acid and 0.086% ammonium dihydrogen phosphate ($NH_4H_2PO_4$). 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 (1998). 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 and Gomes (1992) and Janicek et al. (2008). In addition to MCP estimations, urinary-urea N was assayed using the Stanbio Urea Nitrogen Kit 580 (Stanbio Laboratory, Inc., San Antonio, TX) according to the instructions provided.

Blood Sampling and Analyses

Blood was drawn from the coccygeal artery or vein into serum and whole blood vacuum collection tubes on d 15, d 16, and d 19 at 0 h prior to a.m. feeding and on d 19 and d 20 at 4 and 8 h after a.m. feeding. Blood was drawn for analyzing the concentrations of beta-hydroxybutyrate (**BHB**), glucose, NEFA, blood AA, and blood urea nitrogen (**BUN**). Immediately after drawing blood, BHB concentration was

measured using a handheld electronic BHB meter and test strips (Precision Xtra, Abbott Diabetes Care, Abingdon, UK) according to Iwersen et al. (2009). The BHB values were multiplied by 10.3 to convert from mmol/L to mg/100 mL (Oetzel and McGuirk, 2007). Similar to BHB, blood glucose was measured and recorded immediately after blood was drawn using a handheld electronic glucometer and glucose test strips (Precision Xtra). After sampling, blood samples were immediately transported on ice to the laboratory for further processing. Samples were centrifuged at 2,300 × *g* for 20 min, and serum was then collected and stored at −40°C. At the end of the trial, serum samples were sent off to the Animal Health Diagnostic Center, Cornell University (Ithaca, NY) for NEFA and BUN analysis, and the remaining serum samples were kept for AA analysis.

Serum 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 was 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.

Statistical Analysis

Analysis of variance was conducted using the MIXED procedure (Littell et al., 1998) of SAS (SAS Institute, 2016) for all the statistical analyses. The model included effects of square, dietary treatment, day, and interactions among the fixed effects, with cow within

square and period within square designated as random variables. The effect of day was included as a fixed repeated measurement. Simple, autoregressive one, and compound symmetry covariance structures were used in the analysis depending on low values for the Akaike's information criteria and Schwartz's Bayesian criterion. The relationship between N intake and N excretions into milk, urine, and feces was determined by linear regression using the PROC REG procedure of SAS. For all analyses, degrees of freedom were estimated with the Kenward-Roger specification in the models. 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 were reported throughout. Treatment effects were declared significant if $P \leq 0.05$, and differences were considered to indicate a trend toward significance if $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Characteristics of Experimental Diets

Ingredient and chemical composition of experimental diets are presented in Table 1. All diets supplied NE_L in excess of requirement (NRC, 2001). Mean concentrations of NDF, ADF, starch, ether extract, and non-fiber carbohydrates (**NFC**) were similar for all treatments. As expected, CP concentrations did not increase with NALM supplementation due to the top-dressing application onto the basal TMR. Supplementation of NALM at varying doses tested in the current trial resulted in 7.84, 15.7, and 23.5 g/d/cow of absorbable Met in the diet in addition to the 54.9 g/d of absorbable Met from the basal TMR, which resulted in total absorbable Met amounts of 62.7, 70.6, and 78.4 g/d/cow in the rations supplemented with 15, 30, and 45 g/d of NALM, respectively. The amount of total absorbable Met in the respective rations compared to amounts of MP led to concentrations of 1.95, 2.22, 2.50, and 2.77% of MP. Ideal concentrations of Met that are needed in MP for maximum milk protein yield have been reported to be 2.4% by NRC (2001) and 2.5% by Doepel et al. (2004). Comparing metabolizable Lys to metabolizable Met led to Lys:Met of 3.43:1, 3.00:1, 2.67:1, and 2.40:1 for rations supplemented with 0, 15, 30, and 45 g/d of NALM, respectively. Optimal metabolizable Lys:Met for maximum milk and milk protein yield have traditionally been reported as a 3.00:1 (NRC, 2001, Whitehouse et al., 2009), but very recently optimal ratios as low as 2.69:1 were reported by Van Amburgh et al. (2015) after calculating the optimum efficiency of use for Lys and Met using data derived from a

Table 1. Ingredients and chemical composition of the experimental diets with varying doses of N-acetyl-L-Met (NALM) supplemented to mid to late lactating Holstein dairy cows

	Diet			
Item	0 g/d NALM	15 g/d NALM	30 g/d NALM	45 g/d NALM
Ingredient, % of DM				
Alfalfa hay	25.0	25.0	25.0	25.0
Oat hay	3.76	3.75	3.75	3.75
Corn silage	24.9	24.8	24.8	24.8
Beet pulp	3.02	3.01	3.01	3.01
Cottonseed	4.89	4.88	4.88	4.88
Corn, steam-flaked	19.9	19.9	19.9	19.9
Corn grain, high-moisture	3.67	3.66	3.66	3.66
Canola meal	5.88	5.87	5.87	5.87
Soybean meal	5.88	5.87	5.87	5.87
NALM[1]	0.00	0.06	0.11	0.17
Fat supplement[2]	0.49	0.49	0.49	0.49
Sodium bicarbonate	0.58	0.58	0.58	0.58
Vitamin and mineral mix[3]	2.03	2.03	2.03	2.03
Chemical composition, % of DM				
DM, %	58.3 ± 1.08	58.3 ± 1.42	58.4 ± 1.23	58.7 ± 1.68
OM	89.3 ± 0.79	90.0 ± 0.73	89.8 ± 0.77	90.1 ± 0.59
CP	17.3 ± 0.70	16.7 ± 0.85	17.7 ± 1.12	17.1 ± 1.16
RDP[4]	11.1	11.1	11.2	11.2
RUP[4]	6.19	6.20	6.26	6.28
NDF	30.6 ± 2.40	31.8 ± 2.21	30.3 ± 1.72	30.2 ± 1.16
ADF	18.3 ± 2.00	19.1 ± 1.74	17.8 ± 1.53	18.3 ± 1.20
Starch	20.2 ± 1.25	20.8 ± 1.08	19.3 ± 1.36	20.2 ± 0.95
Ether extract	1.95 ± 0.842	2.18 ± 0.219	1.73 ± 0.563	1.82 ± 0.572
NFC[5]	39.2 ± 1.72	39.3 ± 1.60	40.1 ± 0.87	41.0 ± 1.86
NE_L,[4] Mcal/kg	1.55	1.55	1.55	1.55

table continues

Absorbed Met_{TMR},[6] g/d	54.9	54.9	54.9	54.9
Absorbed Met_{NALM},[7] g/d	0.00	7.84	15.7	23.5
Total absorbed Met,[8] g/d	54.9	62.7	70.6	78.4
Absorbed Lys,[4] g/d	188.3	188.3	188.3	188.4
Metabolizable Lys,[9] % of MP	6.68	6.67	6.67	6.66
Metabolizable Met,[10] % of MP	1.95	2.22	2.50	2.77
Lys:Met[11]	3.43:1	3.00:1	2.67:1	2.40:1

[1]Developmental NALM product made by CJ CheilJedang Co. (Seoul, Korea).
[2]Calcium salts of palm oil (EnerGII®, Virtus Nutrition, LLC, Corcoran, CA).
[3]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).
[4]Based on tabular value (NRC, 2001).
[5]NFC = 100 – CP – NDF – ether extract – ash.
[6]Estimated using NRC (2001) for basal TMR without NALM supplementation.
[7]Estimated supply of absorbed Met from NALM product (assuming 78% Met content and 67% bioavailability).
[8]Total absorbed Met = absorbed Met_{TMR} + absorbed Met_{NALM}.
[9]Metabolizable Lys = absorbed Lys/MP supply.
[10]Metabolizable Met = total absorbed Met/MP supply.
[11]Lys:Met = metabolizable Lys/metabolizable Met.

meta-analysis of 40 published papers (Doepel et al., 2004) and a trial performed by Lapierre et al. (2007).

Feed Intake and Productive Performance

Increasing NALM supplementation linearly increased intakes of DM and CP with a trend toward a linear increase in ADF intake ($P = 0.09$) but no effect on NDF intake (Table 2). The increase in DMI was unexpected, because little evidence exists to suggest that an increase in AA supplementation has any effect on DMI in dairy cows (Allen, 2000). Similarly, Kung and Rode (1996) stated that in general, RPAA supplementation did not improve DMI, which corresponds to the findings of other studies performed by Leonardi et al. (2003) and Chen et al. (2011). In contrast, a limited number of studies (Broderick et al., 2009; Zanton et al., 2014) have shown an increase in DMI with RPMet supplementation, while Ordway et al. (2009) and Zanton et al. (2014) reported decreases in DMI. Intake of DM generally can increase or decrease for a number of factors, including animal factors, management, climatic conditions, and dietary composition (Hayirli et al., 2002). For example, decreases in DMI upon supplementation of RPMet have been correlated with excessive concentrations of Met (Robinson et al., 2000), the presence of co-limiting AA (Patton, 2010), and the sulfur smell of RPMet products (Benefield et al., 2009), while increases in DMI upon RPMet supplementation have been associated with dietary increases in CP (Broderick et al., 2009), parity (Ordway et al., 2009), and the type of RPMet product utilized (Zanton et al., 2014). Supplementation of a Met analogue, HMBi, has also increased DMI (Osorio et al., 2014) because of the

Table 2. Intake of DM and nutrients and productive performance of mid to late lactating Holstein dairy cows supplemented with varying doses of N-acetyl-L-Met (NALM)

	NALM					Contrast[1]		
Item	0 g/d	15 g/d	30 g/d	45 g/d	SEM	NALM	L	Q
Intake, kg/d								
DM	29.0	29.4	30.5	31.7	1.14	0.04	< 0.01	0.57
CP	4.98	4.85	5.44	5.44	0.253	0.02	< 0.01	0.65
NDF	8.89	9.30	9.31	9.54	0.384	0.44	0.14	0.76
ADF	5.32	5.57	5.50	5.79	0.249	0.26	0.09	0.89
Yield, kg/d								
Milk	38.0	37.4	37.7	38.7	2.90	0.18	0.17	0.07
3.5% FCM	38.9	38.2	37.8	38.5	2.87	0.45	0.48	0.72
ECM	38.4	38.8	38.4	39.2	2.82	0.49	0.73	0.16
Milk composition, %								
Fat	3.64	3.60	3.54	3.44	0.167	0.10	0.02	0.54
True protein	3.13	3.13	3.14	3.09	0.084	0.68	0.43	0.36
Lactose	4.76	4.75	4.74	4.78	0.057	0.55	0.76	0.21
Milk component yield, kg/d								
Fat	1.39	1.36	1.32	1.35	0.110	0.27	0.16	0.26
True protein	1.18	1.16	1.19	1.21	0.078	0.28	0.10	0.29
Lactose	1.82	1.78	1.79	1.86	0.152	0.12	0.19	0.03
Dairy efficiency								
Milk yield/DMI	1.31	1.26	1.23	1.22	0.078	0.30	0.07	0.66
3.5% FCM yield/DMI	1.34	1.30	1.25	1.22	0.078	0.23	0.05	0.93
ECM yield/DMI	1.35	1.32	1.26	1.24	0.075	0.12	0.02	0.84

[1]NALM = control (0 g/d) vs. NALM treatments; L = linear effect of increasing NALM; Q = quadratic effect of increasing NALM.

potential stimulatory effect of HMBi on cellulolytic bacteria and the subsequent increase in passage rate and DMI (Lee et al., 2015b). In the current study, it appears that NALM supplementation may have had a similar effect by stimulating MCP synthesis, thus increasing passage rate and subsequently increasing DMI, which will be discussed in more detail later in this paper.

In spite of the linear increase in DMI with increasing NALM supplementation, milk production did not follow the same pattern (Table 2). Supplementation of NALM tended ($P = 0.07$) to have a quadratic effect on milk yield, as the greatest NALM supplementation (45 g/d) increased milk yield, but not at 15 and 30 g/d NALM compared with control. Supplementing NALM did not affect yields of 3.5% FCM and ECM regardless of dose rate. Similar to the present study, multiple studies investigating RPMet have shown similar results of little to no change in milk yield (Leonardi et al. 2003; Rulquin et al., 2006). Patton (2010) found that many different factors affect the efficacy of RPMet on milk yield, including the presence of co-limiting AA, the severity or lack of Met deficiency in the ration, breed, type of forage fed, and stage of lactation. In the current study, stage of lactation appeared to be the main factor that interfered with milk yield, because cows that are in mid to late lactation generally tend to lack the hormonal drive necessary to increase milk yield (Oltenacu et al., 1980; Penasa et al., 2016) and, therefore, supplementation of NALM may be ineffective at increasing milk yield when supplemented in mid to late lactation cows.

Supplementation of NALM had no effect on milk true protein and lactose concentrations, but had a negative linear effect on milk fat concentration (Table 2). No

changes were recorded in milk fat yield, whereas milk protein yield tended to increase linearly ($P = 0.10$) with increasing NALM supplementation. RPMet has long been promoted as an effective way to increase milk protein concentration and yield, and many studies have shown increases in milk protein concentration and yield when RPMet has been supplemented (Ordway et al., 2009; Patton, 2010; Osorio et al., 2013). For example, Rulquin et al. (2006) reported a linear increase in milk protein yield (962 to 1,003 g/d) and Wang et al. (2010) reported a linear increase in milk protein yield (870 to 920 g/d) upon supplementation of RPMet. Feed efficiencies based on yields of milk and 3.5% FCM tended to decrease ($P = 0.07$ and 0.05, respectively), while feed efficiency based on ECM yield decreased linearly, because ECM yield did not increase in relation to the increase in DMI. This is a significant parameter, as it helps define the efficiency and productivity of a dairy herd in converting feed inputs into saleable outputs. The linear decrease in feed efficiency (milk yield/DMI) from 1.31 to 1.22 coupled with increasing NALM supplementation in our study is below the average feed efficiency of 1.36 reported by Britt et al. (2003). Although some production parameters in the current study increased, the decrease in feed efficiency suggests that NALM supplementation in mid to late lactation cows may not be economically beneficial when looking only at production parameters.

Change of BW and Net Energy Utilization

No changes were seen in BW gain with supplementation of NALM (Table 3). However, there was a numerical increase (0.72 to 1.04 kg/d) in BW gain seen when comparing the control diet to the diet supplemented with 45 g/d of NALM, which

Table 3. Change of BW and net energy utilization of mid to late lactating Holstein dairy cows supplemented with varying doses of N-acetyl-L-Met (NALM)

	NALM					Contrast[1]		
Item	0 g/d	15 g/d	30 g/d	45 g/d	SEM	NALM	L	Q
BW								
Initial, kg	724	726	724	714	16.4	0.47	0.21	0.29
Mean, kg	739	732	744	736	16.5	0.18	0.77	0.95
Gain, kg/d	0.72	0.30	0.97	1.04	0.372	0.31	0.21	0.40
Calculated net energy values, Mcal/d								
Maintenance	11.3	11.3	11.4	11.3	0.22	0.11	0.44	0.67
BW gain	2.93	2.50	5.77	4.68	1.826	0.40	0.22	0.82
Milk	26.3	26.0	25.5	26.2	1.63	0.42	0.64	0.17
BW gain + milk	29.3	27.9	32.0	31.6	2.62	0.37	0.19	0.79
Total[2]	40.6	39.2	43.4	42.9	2.67	0.35	0.19	0.80
NE_L,[3] Mcal/kg of DMI	1.40	1.36	1.41	1.36	0.076	0.77	0.73	0.92
Net energy partitioning, % energy intake								
Maintenance	29.0	29.6	26.3	27.1	1.69	0.16	0.09	0.93
BW gain	5.92	5.56	13.1	9.15	3.73	0.29	0.21	0.56
Milk	65.3	66.0	59.2	62.3	3.25	0.18	0.15	0.60
BW gain + milk	71.0	70.4	73.7	72.9	1.69	0.16	0.09	0.93

[1]NALM = control (0 g/d) vs. NALM treatments; L = linear effect of increasing NALM; Q = quadratic effect of increasing NALM.
[2]Net energy used for maintenance, BW change, and milk.
[3]Calculated NE_L = calculated total net energy, Mcal/d ÷ DMI (kg/d).

suggests that supplementation of NALM may have played a role in BW gain. Information concerning the effects of RPMet supplementation on BW gain in ruminants is lacking, but some studies have reported increases in BW gain upon RPMet supplementation (Deetz et al., 1985; Oke et al., 1986). Due to the chemical makeup of NALM (an acetyl group and a Met molecule), it would stand to reason that supplementation of NALM may potentially have an impact on BW gain. It is well understood that Met is a limiting AA for growing cattle (Richardson and Hatfield, 1978) and, therefore, an increase in available Met will aid in 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 aid in fat deposition in growing cattle (Obeid and Herrmann, 2009). In addition, acetate is also recognized for its role as a carbon source for fat synthesis (Bauman and Griinari, 2001). The small number of animals (n = 8) utilized in this study may account for the lack of a significant effect on BW gain witnessed; however, understanding the energy partitioning of lactating dairy cows may help highlight the importance of the numerical increase in BW gain observed in the current study.

Supplementation of NALM did not influence net energy partitioning as a percentage of energy intake of BW gain and milk yield (Table 3). However, there was a numerical increase in the proportion of energy intake that was partitioned to BW gain where supplementation of NALM increased the percentage of energy intake partitioned to BW gain from 5.92% in the control diet to 9.15% in the diet supplemented with 45 g/d of NALM. The numerical increase in the percentage of energy intake that was partitioned to BW gain tended to have an effect on increasing the net energy partitioning toward BW

gain and milk yield combined ($P = 0.09$). The net energy consumed by a dairy cow can be utilized and partitioned to one of three physiological processes, maintenance, growth, and lactation. The efficiency at which each physiological process operates is different, and the partitioning of nutrients to each of these processes changes throughout lactation, as can be seen in a normal lactation cycle where milk production, DMI, and BW gain follow a curvilinear pattern (NRC, 2001). During a normal lactation cycle, DMI will lag behind milk production until maximum milk production is reached, at which point DMI will continue to increase for a period of time and then decrease in the same pattern as milk production (NRC, 2001). When the energy necessary for milk production can be obtained through feed intake alone, the lactating dairy cow will stop mobilizing body fat and protein reserves and begin to partition energy toward BW gain (Kirkland and Gordon, 2001). Kirkland and Gordon (2001) stated that this curvilinearity may be explained by the increased partitioning of nutrients and energy from milk production to BW gain. Therefore, it may be inferred that any influence on DMI in mid to late lactating dairy cows will have an effect on energy partitioning. Vandehaar and St-Pierre (2006) stated that either changes in diet composition or DMI can influence the partitioning of energy and nutrients from the mammary gland to other body tissues. For example, a study using mid lactation cows (70 ± 7 DIM) fed high-grain, low-fiber diets reported an increase in energy intake (10%) with most of that energy being partitioned toward BW gain rather than milk yield (Oba and Allen, 2000). As reported earlier, DMI in the current study linearly increased with increasing NALM supplementation, possibly explaining the effect on net energy partitioning to both BW gain and milk production.

Stage of lactation also plays a major role in energy and nutrient partitioning. For instance, in early lactation, high-producing dairy cows partition 90 to 430 g/d of AA from tissue protein reserves to counteract the deficiency in ingested AA (Bequette et al., 2003). Later in lactation due to tissue sensitivity and substrate supply, an equivalent amount of AA will be partitioned back to the tissue protein reserves to replenish the loss accrued in early lactation (Bequette et al., 2003). Utilizing multiple regression analysis, Burt (1957) reported that only milk yield drove the partitioning of energy and nutrients, whereas Coulon and Remond (1991) and Kirkland and Gordon (2001) found that both DIM and milk yield directed the partitioning of energy and nutrients. Both increasing DIM and diminishing milk yield throughout the current study in mid to late lactation appear to have likely influenced the energy and nutrient partitioning of dairy cows when supplemented with NALM, channeling more energy into BW gain and, therefore, impacting the combined energy proportioned to BW gain and milk production. Regardless of the mechanism behind the partitioning of net energy and nutrients to both BW gain and milk production, an effect such as the one seen in the present study would be beneficial to the modern, high-producing dairy cow. As cows progress later into lactation, there is a competition for nutrients to be utilized for milk production, BW gain, and fetus development. Body weight gain is often overlooked, but is a crucial factor during lactation because it influences the longevity and fertility of high-producing dairy cows in subsequent lactations (Coffey et al., 2002). Thus, NALM supplementation appears to be beneficial in aiding mid to late lactating cows partition more energy to both BW gain and milk production, potentially increasing fertility and longevity. The lack of a

significant effect seen in the current trial, coupled with the numerical increase seen suggests more work needs to be done to assess the potential effects of NALM supplementation.

Utilization of N

As anticipated because of the clear effects seen on DMI, cows fed the rations supplemented with varying doses of NALM consumed more N than those fed the control (linear effect; Table 4). A previous study performed by our group (Fagundes et al., 2016) did not find an increase in N intake upon supplementation of NALM. Traditionally, supplementation of RPMet products have not been shown to increase N intake because of the lowered dietary CP concentration in basal diets normally associated with RPMet supplementation (Leonardi et al., 2003; Broderick et al., 2009). Supplementation of NALM at varying doses had no effect on milk N excretion in the current study. As a result of the linear increase in N intake and the lack of response in milk N excretion after supplementation of NALM, milk N:N intake decreased linearly from 0.25 to 0.23. The N utilization efficiencies reported in the current study are relatively low compared to the average efficiencies (25 to 35%) reported in the literature (Chase, 1994; Hristov et al., 2004) but fall within the normally accepted range (15 to 45%; Dijkstra et al., 2013). There are multiple studies that collaborate with the results of the current study that increased N intake decreases N utilization efficiency (Castillo et al., 2000; Kalscheur et al., 2006; Dijkstra et al., 2013). The lack of response in milk N when NALM was

Table 4. Utilization of N by mid to late lactating Holstein dairy cows supplemented with varying doses of N-acetyl-L-Met (NALM)

	NALM					Contrast[1]		
Item	0 g/d	15 g/d	30 g/d	45 g/d	SEM	NALM	L	Q
N intake, g/d	798	777	871	870	40.4	0.02	< 0.01	0.65
Milk N,[2] g/d	197	199	194	196	10.1	0.27	0.29	0.98
Milk N:N intake[3]	0.25	0.25	0.23	0.23	0.011	0.04	0.02	0.82
MUN, mg/100 mL	16.2	16.4	15.7	15.5	0.61	0.15	0.04	0.58
BUN, mg/100 mL	18.7	20.5	18.7	18.6	1.32	0.10	0.40	0.10
NH_3-N,[4] mg/100 mL	19.4	15.4	17.7	16.1	1.80	0.26	0.28	0.42
Urinary N excretion,[5] g/d	313	311	304	298	15.7	0.23	0.05	0.70
Fecal N excretion,[6] g/d	287	272	370	373	36.8	0.02	< 0.01	0.71
Manure N excretion,[7] g/d	600	583	674	670	33.8	0.03	0.01	0.77

[1]NALM = control (0 g/d) vs. NALM treatments; L = linear effect of increasing NALM; Q = quadratic effect of increasing NALM.
[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).
[6]Predicted using the equation: N intake, g/d – urinary N excretion, g/d – milk N, g/d.
[7]Manure N, g/d = urinary N excretion, g/d + fecal N excretion, g/d.

supplemented was related to the lack of response in milk production seen in the current study, suggesting that NALM was utilized elsewhere in the body tissues.

Supplementing NALM did not affect ruminal NH_3-N concentration in spite of a significant increase in N intake by NALM supplementation (Table 4). Ruminal NH_3-N concentration is a result of the balance between production (proteolysis) and assimilation (De Visser et al., 1997), and thus any efforts to maximize N utilization in the rumen should involve an optimal balance between the 2 metabolic processes. Yet, concentrations and components of dietary CP can influence microbial activity, as RDP supplies peptides, amino acids, and NH_3-N derived from microbial proteolysis for use in microbial protein synthesis (Wallace et al., 1997). Given the fact that greater N input into the rumen associated with increased N intake as a result of NALM supplementation did not have an effect on NH_3-N concentration, signifies that dietary N utilization in the rumen may have been manipulated due possibly to a degraded fraction of NALM in the rumen.

Concentration of MUN linearly decreased with supplementation of NALM (Table 4), and it ranged from 15.5 to 16.2 mg/100 mL, which is higher than the accepted, optimal range of 10 to 14 mg/100 mL (Wattiaux et al., 2005), but the slight reduction in MUN concentration with NALM supplementation (Appendix A) may have a biologically minor impact. However, it is noteworthy to indicate that the linear increase in N intake with increasing NALM supplementation did not correspond to MUN concentration. Supplementation of RPMet has been shown to decrease MUN concentration in multiple studies (Broderick et al., 2008; Wang et al., 2010; Arriola Apelo et al., 2014), but this

decrease in MUN concentration was associated with decreased N intake as a result of decreased CP in the diets supplemented with RPMet. For example, Broderick et al., (2008) tested 4 experimental diets with decreasing concentrations of CP (18.6 to 14.8%) and corresponding increases of supplemental RPMet (0 to 15 g/d). As CP decreased and RPMet supplementation increased, milk production and N utilization efficiency increased linearly, N intake decreased linearly, and concentrations of MUN decreased linearly (14.5 to 7.9 mg/100 mL; Broderick et al., 2008). In a different trial in the same study conducted by Broderick et al. (2008), two levels of CP (17.3 and 16.1%) were tested with either 0 or 10 g/d of RPMet. No effect of RPMet supplementation on MUN was reported (Broderick et al., 2008). This study collectively suggests that the effectiveness of RPMet would be minimal on decreasing MUN concentration. Rather, the changes in MUN concentration was a direct consequence of decreasing concentrations of CP in the diets while maintaining potential production.

Concentration of BUN showed a tendency to decrease ($P = 0.10$) in response to NALM supplementation, and urinary N excretion linearly decreased. Urea N found in blood, urine, and milk is derived from NH_3-N in the rumen and, therefore, the amount of urea in urine is directly proportional to that of urea in blood which is proportional to MUN (Jonker et al., 1998). The decrease in urinary N excretion upon increasing supplementation of NALM in the present study suggests that NALM supplementation may have improved the AA balance in MP, and thus decreased deamination of absorbed AA, leading to a decrease in urinary N output (Wang et al., 2010). The linear decrease in urinary N excretion (Appendix A) with increasing NALM supplementation is impactful,

because urinary N is the most environmentally volatile form of N excreted (Varel et al., 1999). Unlike the many factors that affect MUN, BUN, and urinary N, fecal N is directly correlated with DMI and N intake (Huhtanen et al., 2008). Therefore, because of the substantial increase in N intake, NALM supplementation linearly increased fecal N (Appendix A) as well as manure N excretions in our study. Hence, in view of overall consideration on environmental N management, a partial benefit by decreasing urinary N output by supplementing NALM would be discounted because of the increased N excretion in manure.

Ruminal Fermentation Characteristics

Supplementation of NALM had no effect on ruminal pH, which ranged from 6.15 to 6.23 (Table 5). Total VFA concentration was similar across treatments. Except a trend ($P = 0.06$) toward a quadratic response on valerate proportion, VFA composition did not differ among treatments. These results are consistent with other studies; Davidson (2006) reported minimal effects on ruminal fermentation in continuous cultures receiving RPMet supplementation because of limited impact on energy metabolism in the rumen from traditional RPMet products. The current study, along with previous studies, suggest that the effects of RPMet including NALM on ruminal VFA profiles is minimal.

In spite of the minimal impact on VFA profiles in the rumen, NALM supplementation linearly increased MCP yield (Table 5). Supplementation of RPMet traditionally has not had an impact on microbial production, with the exception of Met analogues such as HMBi because of their ruminal degradability. Several studies have shown positive effects

Table 5. Ruminal fermentation characteristics of mid to late lactating Holstein dairy cows supplemented with varying doses of N-acetyl-L-Met (NALM)

	NALM					Contrast[1]		
Item	0 g/d	15 g/d	30 g/d	45 g/d	SEM	NALM	L	Q
Ruminal pH	6.23	6.15	6.21	6.22	0.066	0.63	0.82	0.31
Total VFA, mM	104	110	102	108	4.1	0.14	0.80	0.93
Individual VFA,[2] mM								
Acetate (A)	63.3	66.9	63.6	66.7	3.01	0.37	0.43	0.90
Propionate (P)	22.6	23.8	21.3	23.9	1.50	0.33	0.80	0.54
Butyrate	13.0	14.2	13.4	12.9	0.77	0.35	0.66	0.14
Valerate	1.90	2.04	2.01	1.91	0.910	0.27	0.99	0.06
Isobutyrate	1.31	1.27	1.22	1.29	0.125	0.54	0.54	0.25
Isovalerate	1.84	1.75	1.74	1.80	0.167	0.90	0.76	0.50
A:P	2.83	2.71	2.64	2.60	0.185	0.54	0.16	0.77
MCP,[3] g/d	1835	1758	1923	1891	78.6	0.02	0.05	0.50

[1]NALM = control (0 g/d) vs. NALM treatments; L = linear effect of increasing NALM; Q = quadratic effect of increasing NALM.
[2]Expressed as mol/100 mol.
[3]Microbial protein production, g/d = ({[purine derivatives production – (0.385 × $BW^{0.75}$)]/0.85} × 70 × 6.25)/(0.13 × 0.83 × 1,000) (Janicek et al., 2008).

of HMB and HMBi supplementation on aspects of ruminal fermentation, including increases in total concentrations of VFA (Martin et al., 2013), NDF digestion (Noftsger et al., 2005), and MCP yield (Lee et al., 2015b). It is unclear how NALM supplementation increased MCP yield in the current study. It has been reported the efficiency of MCP yield increases when AA or peptides are used as a source of N rather than ammonia (Maeng et al., 1976). Multiple in vitro studies have shown a positive effect of AA supplementation on microbial growth (Maeng et al., 1976; Argyle and Baldwin, 1989; Russell and Strobel, 1993). In vivo studies have also shown similar results (Lundquist et al., 1985; Rooke and Armstrong, 1989). Studies concerning the effects of Met specifically on microbial growth are sparse, but it has been reported that rumen microbes utilize Met for different physiological aspects such as incorporation into cellular material (Patterson and Kung, 1988) and lipid biosynthesis (Patton et al., 1970). Based on the aforementioned data, it may be concluded if any amount of NALM was degraded in the rumen and converted to Met, then the available Met may stimulate MCP production.

A continuous culture study estimated the ruminal protection of NALM to be 67% (Windschitl and Stern, 1988), which was the same rate we found for the NALM product used in the current study after a 24-h incubation using an in vitro batch culture incubation (data not reported). In order for NALM to be degraded or hydrolyzed, the aminoacylase enzyme needs to be present (Baxter et al., 2001). This enzyme is found in multiple mammalian tissues (Giardina et al., 1997), but studies involving its presence or lack thereof in the rumen were not found. It has been reported, however, that certain microorganisms can produce this enzyme (Tripathi et al., 2000), including bacteria that

reside in the rumen. Aminoacylase has been purified from *Bacillus* spp. and *Pseudomonas* spp. (Story et al., 2001), which both reside in the rumen. Other rumen dwelling bacteria such as *Aspergillus* spp. and *Alcaligenes* spp., can also produce the aminoacylase enzyme (Gentzen et al., 1980; Wakayama et al., 1996). There is no in vivo evidence currently in the literature to validate the in vitro findings. Therefore, more research needs to be done on the prevalence of the aminoacylase enzyme in the rumen and the possible effect of its presence on NALM degradation and partial conversion to Met in the rumen.

Plasma Metabolite and AA Profiles

Supplementation of NALM did not have an effect on plasma concentrations of NEFA, BHB, and glucose (Table 6), but it led to a trend toward a linear increase in Met (P = 0.08), a linear increase in Gly, and a quadratic increase in Ala concentration. As plasma Met concentration provides a qualitative measure of the postruminal delivery of Met from RPMet products (Blum et al., 1999), it is important to note the relationship between plasma Met concentration and RPMet supplementation. In a study done by Koenig and Rode (2001), RPMet was supplemented at 20 and 63 g/d, and as expected a 32.5 and 65.5% increase in plasma Met concentration above the control diet (0 g of RPMet) was observed for the 20 and the 63 g/d RPMet supplemented diet, respectively. The ranges reported by Koenig and Rode (2001) were similar to the increase in plasma Met concentration (51 to 71%) the authors reported in the same study when Met was infused into the duodenum at similar rates. Blum et al. (1999) also reported a linear increase in plasma Met concentration (16.6 to 144.8 µmol/L) 5 d after supplementation with 60 and

67 g/d of RPMet products, when compared with plasma Met concentration 3 d before supplementation. The relatively small increase in plasma Met concentration with NALM supplementation recorded in the present trial may have occurred because Met requirements for other physiological functions, such as BW gain, were not met. It is reported in the literature when available EAA are below their requirement, the plasma EAA concentrations will either increase marginally or not increase at all (Broderick et al., 1974; Bergen, 1979; Koenig and Rode, 2001). Very little information exists concerning the relationship between duodenal AA supply and plasma AA concentrations and the factors affecting that relationship and the control of the removal of plasma AA (Patton et al., 2015). Interestingly, Patton et al. (2015) hypothesized that Met supplementation may stimulate MCP production which in turn would demand more removal of plasma Met, which supports the increase in MCP production discussed earlier in this paper and may account for the modest increase in plasma Met concentrations seen in the current study.

Table 6. Blood chemistry parameters and AA concentrations in plasma of mid to late lactating Holstein dairy cows supplemented with varying doses of N-acetyl-L-Met (NALM)

	NALM					Contrast[1]		
Item, μ*M*	0 g/d	15 g/d	30 g/d	45 g/d	SEM	NALM	L	Q
NEFA,[2] mEq/L	0.08	0.09	0.08	0.09	0.008	0.10	0.43	0.79
BHB, mg/100 mL	5.03	4.47	4.71	4.73	0.384	0.25	0.46	0.12
Glucose, mg/100 mL	40.8	41.8	42.2	41.7	1.04	0.34	0.22	0.17
EAA[3]								
His	63.4	61.6	65.1	65.2	3.09	0.26	0.17	0.53
Ile	104	96.0	102	98.8	5.08	0.13	0.44	0.36
Leu	177	162	177	171	10.5	0.06	0.86	0.35
Lys	87.6	88.7	89.0	86.7	5.68	0.97	0.89	0.65
Met	20.8	22.1	23.0	22.5	0.93	0.09	0.08	0.22
Phe	87.9	78.6	81.0	78.6	3.76	0.20	0.11	0.33
Thr	59.6	57.4	58.5	57.4	3.71	0.78	0.48	0.74
Val	242	231	339	234	53.4	0.38	0.72	0.35
Total EAA	840	795	934	809	57.9	0.26	0.84	0.46
NEAA[4]								
Ala	172	162	170	177	7.5	0.09	0.22	0.04
Asp	21.9	22.1	22.5	17.7	5.05	0.76	0.46	0.49
Glu	29.9	29.0	33.4	32.4	3.59	0.48	0.24	0.99
Gly	171	180	184	188	10.0	0.11	0.02	0.64
Pro	82.3	79.6	86.1	85.0	4.56	0.20	0.16	0.72
Tyr	105	99.9	102	105	5.0	0.81	0.94	0.37
Total NEAA	579	566	591	601	20.9	0.26	0.12	0.38

[1]NALM = control (0 g/d) vs. NALM treatments; L = linear effect of increasing NALM; Q = quadratic effect of increasing NALM.

table continues

[2]NEFA = nonesterified fatty acids.
[3]EAA = essential AA.
[4]NEAA = non-essential AA

CONCLUSIONS

The developmental NALM product was supplemented in a typical lactation diet in the current study with the expectation of improving the conversion of feed inputs into production outputs through more efficient partitioning of nutrients and energy in mid to late lactating Holstein dairy cows. A linear increase in DMI was observed with increasing supplementation of NALM, which may potentially be related to a linear increase in MCP yield. However, we have yet to investigate the potential stimulatory effects of NALM supplementation on the rumen microbiota and also the presence and concentration of the aminoacylase enzyme in the rumen, which may contribute to identifying additional evidence for the positive effect of supplementing NALM in lactation diets. The increase in DMI allowed for more nutrients and energy to be partitioned to different physiological processes, but because of the stage of lactation of cows tested in the present study, the extra energy ingested was partitioned to both milk production and BW gain which resulted in a quadratic increase in milk yield with an increase in milk protein yield. Caution should be exerted to extrapolate overall data of BW and net energy utilization in the current study due to the small number of animals (n = 8) and the short length of data collection and, therefore, further investigation is needed to confirm our data with a relatively longer period of experimentation and with a larger number of experimental animals. From an environmental standpoint, the decrease in urinary N excretion due to NALM supplementation highlights an additional benefit because of the decrease in the most volatile form of ammonia excreted by cows, but with the linear increase in DMI, fecal N excretion also linearly increased, possibly counteracting the beneficial effect of

decreasing urinary N excretion. The increase in DMI without a subsequent increase in milk yield decreased the N utilization efficiency in the current study, which opposes the general goal of the dairy industry today. Overall, the current study suggests that NALM supplementation has a positive impact on increasing milk protein yield and energy partitioning of dairy cows in mid to late lactation by aiding in BW gain, potentially affecting the fertility and longevity of high-producing dairy cows.

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APPENDICES

APPENDICES

Appendix. The relationship between N intake and excretion of N into milk, feces, or urine by mid to late lactating Holstein dairy cows supplemented with N-acetyl-L-Met (NALM) with varying doses (n = 32 on individual data set).