Evaluating Ruminally Protected Methionine Products to Improve the Efficiency of Dairy Cattle Protein Utilization Using the Cornell Net Carbohydrate and Protein System

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ABSTRACT

Reducing protein fed to cattle while maintaining milk production increases environmental and economic sustainability. Rumen-protected methionine (RP Met) improves precision of methionine supply to cattle, reducing dietary protein and nitrogen excretion. This experiment compared a newly manufactured RP Met (RP Met 2) to a commercialized RP Met that is known to adequately supply metabolizable methionine (RP Met 1). *In vitro* analyses were conducted to estimate ruminal and intestinal digestibility; results were used to calculate the amount of metabolizable methionine for the Cornell Net Carbohydrate and Protein System v6.5.5 for diet formulation. The *in vivo* trial involved 39 early lactation cattle, ranging in parity. After a 10-day covariate, three diets were assigned for nine weeks, each to 13 cows: control diet, control diet with RP Met 1 (16g/d), and control diet with RP Met 2 (23g/d). Different inclusion rates were used to supply equal amounts of metabolizable methionine. At comparable feeding rates of metabolizable methionine, based on *in vitro* ruminal and intestinal degradation, it was hypothesized that both RP Mets would equally increase energy corrected milk when compared to the control. Using SASv9.4, analysis of variance analyzed fixed effects (diet, time, and their interaction) and random effects (cows). *In vitro* results indicated better ruminal protection for RP Met 1 than RP Met 2. Energy corrected milk was not statistically modified by either RP Met. Overall, the *in vivo* and *in vitro* results did not align, and a larger sample size could determine whether the RP Mets statistically differ from the control.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AA	Amino Acids
ADF	Acid Detergent Fiber
ADICP	Acid Detergent Insoluble Crude Protein
ANOVA	Analysis of Variance
AMTS	Agricultural Modeling and Training Systems, L.L.C.
BCS	Body Condition Score
BW	Body Weight
CNCPSv6.55	Cornell Net Carbohydrate and Protein System version 6.5.5
CS	Corn Silage
CURC	Cornell University Ruminant Center
DM	Dry Matter
DMI	Dry Matter Intake
EAA	Essential Amino Acids
ECM	Energy Corrected Milk
MMG	Mixed Mainly Grass
Met	Methionine
MP	Metabolizable Protein
MUN	Milk Urea Nitrogen
N	Nitrogen
NDF	Neutral Detergent Fiber
NDICP	Neutral Detergent Insoluble Crude Protein
NPN	Non-Protein Nitrogen
RP	Rumen-protected
RDP	Rumen Degradable Protein
RUP	Rumen Undegradable Protein
SAS	Statistical Analysis System
TMR	Total Mixed Ration
uN	Undigested Nitrogen

INTRODUCTION

Reducing nitrogen (N) fed to dairy cows while maintaining milk production is a critical strategy in providing economic and environmental sustainability to the dairy industry. Overfeeding N to a dairy herd leads to excretion of feed N, negatively impacting income over feed costs and the efficiency of nutrient utilization in the cow and for the farm (Ipharraguerre 2005; Chase and Van Amburgh 2009). In addition, an accumulation of urinary and fecal N in the environment increases the potential for volatilization, release of nitrous oxide into the atmosphere, and leaching of nitrate into groundwater (Schwab et al., 2005; Broderick et al., 2008; Broderick et al., 2003). Underfeeding N also has detrimental consequences, increasing the susceptibility of metabolic disease and limiting the lactational performance of the herd. Thus, a balance between overfeeding and underfeeding N must be found.

Measurement of N supply in feed has historically been accomplished through crude protein (CP) measurements, using the conversion factor of CP/16 to approximate N. The conversion factor originates from the concept that average N content of all amino acids (AA) is 16%. However, this strategy oversimplifies the conversion, as proteins differ in their AA composition, and the nitrogen content of AA varies considerably due to molecular weight of and number of nitrogen atoms in AA (Mariotti et al., 2008). Thus, the use of CP does not allow for adequate description of the N requirements of the cow and creates errors in the prediction of AA requirements. Bias in this conversion factor can result in the oversupply of some AA, leaving others potentially undersupplied. A more effective strategy for determining N supply is through measuring the amount and profile of AA. Adequate supply of N to a ruminant is important for proper rumen function, while AA that are absorbed by the small intestine contribute to milk protein, tissue growth, and maintenance of homeostasis (Ipharraguerre, 2005).

Formulating diets for AA requirements can be accomplished through several formulation programs and models. The model used in the study detailed in this thesis is the Cornell Net Carbohydrate and Protein Supply version 6.5.5 (CNCPS v6.5.5). The CNCPSv6.5.5 uses animal and feed descriptions and characteristics to formulate diets that satisfy the model's calculated animal requirements (Higgs et al., 2015; Van Amburgh et al., 2015). Examples of animal descriptions include current and target body weight, milk production, and milk components. Feed characteristics include any ingredient in the intended diet and their respective nutrient composition values, such as dry matter and crude protein content. The CNCPS v6.5.5 accounts for N from ammonia, and the true protein in both rumen soluble and insoluble forms, allowing for better prediction of metabolizable protein (MP)

from AA and small peptides that can escape rumen fermentation. In addition, the CNCPS v6.5.5 utilizes AA profiles on a whole-feed basis, as opposed to insoluble protein, which does not measure rumen undegradable AA (Van Amburgh et al., 2013). Overall, the CNCPS v6.5.5 is a useful tool in providing the cow with the nutrients needed for growth, maintenance, reproduction, and lactation while avoiding unnecessary waste (Van Amburgh et al., 2019).

Nutritionally, AA are categorized into non-essential AA (NEAA) and essential AA (EAA). Non-essential AA are produced by animal cells and are therefore not required in the diet (Wu et al., 2012). Essential AA cannot be, or are insufficiently, synthesized by the animal; thus, these must be provided to meet requirements for maintenance, growth, and milk production (Wu et al., 2012). Methionine (Met) is considered to be the first limiting EAA in milk production for high producing lactating dairy cattle, and its supplementation typically increases milk protein synthesis and yield (Schwab 1996, Armentano et al., 1997).

Methionine limitation is exacerbated by low dietary CP (Sinclair et al., 2014); CP can get as low as 14-16% of the diet and still provide for high milk production with proper feeding strategies and management (Chase and Van Amburgh 2009). Supplementing Met allows for lower levels of CP to be fed by improving the profile of essential AA in metabolizable protein (MP). Metabolizable protein is defined as the amount of AA and peptides absorbed by the small intestine, from microbial protein, endogenous protein, and feed protein sources that escape ruminal degradation. Altering feed rations to contain less CP comes with challenges beyond proper diet formulation, including the need for sufficient dry matter intake (Broderick et al., 2008; Sinclair et al., 2014), proper on-farm feeding management, and general comfort of the farmer to significantly reduce unnecessary CP (Chase and Van Amburgh, 2009).

Feeding crystalline methionine leads to its digestion in the rumen, where the microbes can alter the dietary AA profile absorbed in the small intestine. Creating a coating around a Met core can protect the AA from ruminal action while allowing Met to be absorbed by the small intestine. The coating of a rumen-protected Met (RP Met) could contain lipids, ruminally-inert pH sensitive polymers, or ethyl cellulose and stearic acid that is digested via abomasal action, exposing the Met core in the small intestine (Schwab, 1995; Chen et al., 2011). Escaping rumen fermentation and being absorbed in the small intestine increases N use efficiency of Met (Schwab, 1995).

To assess the efficacy of a newly developed rumen-protected (RP) Met product, two *in vitro* methods are used in the Van Amburgh lab. One method, known as the Ross Assay, was developed in

the laboratory and mimics the digestive tract of the cow to test rumen protection and intestinal digestibility (Ross et al., 2013; Ross, 2013). Another method looks at the stability of the RP Met in the rumen over time by analyzing samples in 4 or 6 hour increments out to 30 hours of in vitro incubation and then mathematically analyzing the degradation curve to develop a rate of degradation, which coupled with the intestinal digestibility, allows for the prediction of intestinally available AA. *In vitro* ruminal and intestinal data are used to determine the pool size of available AA as well as the rate of digestion in the rumen. Altogether, the *in vitro* data allows for the calculation of the amount of metabolizable AA supplied to the intestine, which is subsequently inputted into the CNCPS to formulate dietary treatments.

To confirm the two *in vitro* assessments, a feeding trial is conducted with lactating dairy cattle to understand how animals respond to RP Met supplements. All three methods are important for determining the extent of under-protection or overprotection of the AA from ruminal digestion. An under-protected RP product degrades due to microbial action in the rumen, indicating that the coating surrounding the Met is insufficient. An over-protected RP product escapes both the rumen and intestine and is excreted in the feces. In this case, the coating does not break down from the enzymes or acidity in the abomasum or in the small intestine.

Although several RP Met products exist in the dairy industry, few have been directly compared within one study (Schwab, 1995; Koenig and Rode, 2001; Zang et al., 2017). The primary objective of this research trial was to measure the performance of a newly manufactured RP Met (RP Met 2) against a long-standing commercially available RP Met (RP Met 1). Each product contained a different coating, designed for rumen protection, surrounding the amino acid. Rumen-protected Met 1 had a complex polymer coating, and RP Met 2 had a fat coating. Frequently fed in the dairy industry, RP Met 1 has a known and repeatable capacity to deliver methionine to the small intestine and circulation of the cow (Blum et al., 1992; Armentano et al., 1997; Chen et al., 2011; Osorio et al., 2014). In the market, RP Met 1 is considered the standard to which all other products should compare; and what the manufacturer of RP Met 2 was attempting to match or exceed. *In vitro* and *in vivo* methods were used to develop the digestion characteristics of the two RP Met supplements necessary to predict the amount of methionine available at the small intestine. Based on the *in vitro* analysis of both RP Mets and the amount of metabolizable methionine that was limiting milk yield and components, the hypothesis was that at comparable feeding rates of metabolizable Met, energy corrected milk yield for RP Met 1 and RP Met 2 would equally increase when compared to the control.

LITERATURE REVIEW

Nitrogen in the Environment

Loss of N from dairy production systems is an environmental and public concern that receives attention from regulatory standards throughout the world. When N is fed beyond an animal's requirement, N is excreted in feces and urine, with 70% of excess N excreted in the urine (Castillo et al., 2001). The N is present in the urine as urea, which is converted to ammonia by urease enzymes; this ammonia is in equilibrium with ammonium (Powell et al., 2008). Ammonium converts back to ammonia through volatilization, influenced by pH of the soil and presence of evaporation. Atmospheric ammonia combines with other chemicals to form acid rain, nitrate deposits, and fine particles that lodge in lungs, all of which are harmful to natural ecosystems and public health (Powell et al., 2008). Several studies have shown that the reduction of dietary CP and precise N feeding is a key strategy in decreasing N excretion and ammonia emissions (Arriaga et al., 2010; Castilo et al., 2001; Powell et al., 2008; Schwab et al., 2005). For example, Arriaga et al. 2010 found that N use efficiency (measured by milk N use efficiency, milk urea nitrogen (MUN), and excreted N per milk yield unit) decreased with increasing CP, leading to an increase in ammonia emissions from the barn floor. However, minimizing dietary CP must be achieved without compromising lactation performance. Therefore, utilization of N in dairy cows must be properly understood and accurately modeled.

Nitrogen Utilization in Dairy Cows

Ruminants can be thought of as having two sets of N requirements: providing N to microbes in the rumen and providing N to the ruminant body through absorption from the small intestine (Ipharraguerre and Clark, 2005; Schwab et al., 2005). Dietary N supply is divided into rumen degradable protein (RDP) N, rumen undegradable protein (RUP) N, and non-protein N (NPN). RDP is subjected to microbial action in the rumen, where bacteria attach to the feed particles, leading to proteolysis and the breakdown of protein and peptides to AA (Bach et al., 2015). From there, AA can follow a few pathways. One fate is to contribute to microbial protein; some of which will remain in the rumen, while the rest flows to the small intestine. Amino acids can also be deaminated into ammonia and a carbon backbone where the carbon can then be used for cellular activity within the microbe, leading to the production of VFAs, including acetate, propionate, and butyrate. The ammonia from the deaminated AA can be converted to urea for recycling throughout the body or be excreted through the urine (Bach et al., 2015).

Unlike RDP, RUP escapes microbial action in the rumen, is subjected to abomasal and intestinal digestion, and can be absorbed through the intestinal wall and directly utilized by the cow. Microbial action on protein can alter the AA originally supplied in the diet; escaping ruminal action allows the original AA profile to be conserved and supplied directly to the cow. Finally, NPN sources, including free AA, peptides, amides, ammonia, and nitrates, have two fates: face microbial action in the rumen to become peptides and AA or flow into the small intestine as soluble N (Ipharraguerre 2005, Ross 2013). Appendix 1 depicts a diagram from Ipharraguerre 2005 detailing the utilization of N within the rumen and into the small intestine. Once AA are absorbed in the small intestine, they are used as building blocks for protein synthesis for milk production, tissue growth, maintenance, and reproductive functions (Ipharraguerre 2005). Thus, milk protein production and efficiency of feed N utilization is dependent on the pattern of AA absorbed from the intestine (Schwab 1996). Enhancing the efficiency of converting N to animal protein depends more on the profile of AA than on total AA supplied (Lapierre et al., 2001).

Essential Amino Acids and Methionine

Methionine and lysine are commonly considered to be the first limiting amino acids in the milk production of dairy cows fed a corn or legume forage diet (Schwab et al., 1976; Schwab, 1996); the recommendation for the diet is approximately 3:1 of Lysine to Met in MP. For the diets discussed in this thesis, the ratio of Lysin to Met is 3.26:1. When Lysine and Met are in proper balance, other EAA are utilized effectively to increase protein synthesis (Appuhamy et al., 2011; Dong et al., 2018). Essential AA are substrates for protein synthesis in the mammary gland and act as signaling molecules to regulate pathways affecting protein output (Arriola Apelo et al., 2013). For example, a signaling pathway for protein translation and elongation occurs through enhancing the activity of mTORC1, a protein kinase that upregulates protein synthesis (Dong et al., 2018).

Supplemented Met is considered a non-protein source of N, so it can be subjected to ruminal degradation. However, to avoid the possibility of facing microbial breakdown in the rumen, Met can be encapsulated by a protective coating (Schwab, 1995). A lipid and/or carbohydrate coating is the safest, cheapest, and most readily available method of ruminally protecting Met in the market. Another option is to coat the Met core with ruminally-inert pH sensitive polymers, which do not break down in the rumen due to neutral pH, but are soluble in the acidic post-ruminal environment (Schwab, 1995). Lastly, ethylcellulose and stearic acid with poly(2-vinylpyridine-co-styrene) can be used to surround the Met core (Schwab, 1995). Also, some analogs of Met have more stability in the rumen

than others, such as HMBi (Chen et al., 2011). In addition to providing protection from the rumen, a coating developed to surround a Met core needs to be broken down in the small intestine and resist stresses from transportation and storage (Schwab, 1995). Rumen protection increases the duodenal flux of Met, reflecting a higher digestibility of Met in the small intestine (Berthiaume et al., 2001; Berthiaume et al., 2006). Once RP Met degrades in the small intestine, Met is absorbed in the hepatic portal vein, where the AA is transported to the liver to contribute to various metabolic functions throughout the animal.

Supplementing with RP Met increases milk protein synthesis and yield (Schwab, 1996; Pisulewski et al., 1996; Armentano et al., 1997), specifically through the increase of casein protein (Donkin et al.,1989). Rumen-protected Met allows for a lower CP to be supplied, increasing N use efficiency (Broderick et al., 2008; Chen et al., 2011) and decreasing urinary N excretion (Broderick et al., 2008). Broderick et al. 2008 conducted a trial with varying dietary CP and RP Met inclusion rates, finding that apparent N efficiency was greatest with the lowest CP diet and the highest amount of RP Met. Results from their trial confirm the strategy of using RP Met to reduce dietary CP and urinary N excretion, without sacrificing milk production.

In addition to milk and milk protein synthesis, Met follows methyl donor pathways to reduce fatty liver disease and produce antioxidants (Jacometo et al., 2017; McFadden et al., 2020). Once Met enters the Met cycle within the body, it is converted to S-Adenosyl methionine (Finkelstein and Martin, 1984), a universal methyl donor throughout the body (McFadden et al., 2020). Via the phosphatidylethanolamine N-methyltransferase pathway, three molecules of S-Adenosyl methionine methylate phosphatidylethanolamine for it to be converted into phosphatidylcholine (Martinez-Una et al., 2013). Phosphatidylcholine is important in early lactation cows as it contributes to milk production, very low-density lipoprotein synthesis, and prevention of fatty liver disease (Osorio et al., 2014, Zhou et al., 2016, McFadden et al., 2020). After donating methyl groups, S-adenosyl methionine is converted to S-adenosylhomocysteine to be hydrolyzed into homocysteine. From here, homocysteine can be reformed into Met via the Met cycle or enter the transsulfuration pathway (Jacometo et al., 2017). In the transsulfuration pathway, homocysteine becomes cystathionine to be catabolized to cysteine (McFadden et al., 2020). Cysteine can then be converted into glutathione and taurine, antioxidants that helps prevent oxidative damage (Osorio et al., 2014; Jacometo et al., 2017). For a complete overview of the Met cycle and transsulfuration pathways, as well as how they fit into

other aspects of methyl donor metabolism, refer to Appendix 2 (Zhou et al., 2016; McFadden et al., 2020).

Commercial Rumen-Protected Product

Throughout the trial discussed in this thesis, a commercialized RP Met (referred to in this paper as RP Met 1) is used as a standard for which a newly manufactured RP Met is compared. Numerous trials have demonstrated the efficacy of the commercial RP Met in ruminal protection and intestinal digestion. Chen et al. 2011 looked at the effect of supplementing rumen-protected methionine products on milk components, and the commercial RP Met was among those products studied. The authors found a response from supplementing a 15.5% CP diet with the commercial RP Met, with significantly higher energy corrected milk over dry matter intake and milk protein content when compared to the negative control (15.5% CP with no Met supplementation). Fat yield and content, as well as protein yield, tended to be greater with the RP Met supplemented diet as opposed to the negative control. In addition, the N use efficiency was greatest for the diet with the commercial RP Met supplemented. Rulquin and Delaby 1997 found that an increase in milk protein content could be accomplished by supplementing the commercialized RP Met, even under low dietary energy levels. The authors found a significant increase in protein yield, protein content, and casein content from the inclusion of the commercial RP Met. Other studies agree that supplementation with the commercial RP Met increases milk protein yield and content (Armentano et al., 1997; Osorio et al., 2014). Benefits beyond milk components have been shown from supplementing diets with the commercial RP Met, including increasing liver gluconeogenesis under low energy balance (Rulquin and Delaby 1997), decreasing inflammation (Osario et al., 2014), and improving oxidative status. The commercialized RP Met used in this thesis has also been shown to have higher bioavailability than other RP Met products on the market, indicated by higher plasma Met concentrations (Blum et al., 1992; Sudekum et al., 2004). The commercial RP Met is considered a reliable product based on the many trials showing its positive effects on milk components and herd health, as well as its overall bioavailability to the cow.

Modeling and Cornell Net Carbohydrate and Protein System

Used throughout agricultural and food system research, modeling helps to understand systems of interactions. Models aggregate and interrelate information to help solve problems or answer a question, whether it is of limited scope or used across disciplines (Sterman, 1991; Tedeschi et al., 2011). Key characteristics of trustworthy models include using robust data, defining any assumptions made, reflecting the complexity of the information, and having the ability to be updated with new and

improved data (White and Hall, 2017; Horton et al., 2017). Animal production models can give insight into the relationship between livestock and climate change and the economic consequences associated with animal production decisions (Tedeschi et al., 2011; White and Hall, 2017). Data obtained from models informs policy makers, stimulating debate and affecting the fate of legislation (Sterman, 1991; Horton et al., 2017). The creation of models also makes information more accessible and usable in non-academic settings, including on-farm applications (Van Amburgh et al., 2015).

Used in both research and in the dairy industry, the Cornell Net Carbohydrate and Protein System (CNCPS) is a dairy nutrition model that determines animal requirements from environmental and nutritional inputs and allows formulation of diets to meet these requirements (Fox et al.,2004; Tylutki et al.,2008; Higgs et al.,2015). Chemical feed characteristics are used to calculate pool sizes of various fractions within feeds and subsequent feed intake, and the rumen degradation and passage rates of carbohydrates and proteins are calculated within the CNCPS to predict ruminal fermentation, microbial protein, and post-ruminal absorption of feed sources (Fox et al., 2004; Van Amburgh et al., 2015).

Over the last twenty years, laboratory analysis, animal sampling techniques, and on-farm trials have been used to test and improve CNCPS (Fox et al., 2004; Tylutki et al., 2008; Van Amburgh et al., 2015), with the version used in this thesis being CNCPSv6.5.5. Recent updates include those made to predicting nutrient requirements and supply, as well updating the feed library (Higgs et al., 2015; Van Amburgh et al., 2015). Feed library updates are important for improving intestinal digestibility estimates for protein sources (Ross et al., 2013). One of the most recent updates is the prediction of milk yield from the most-limiting nutrient, whether that be MP or metabolizable energy (Van Amburgh et al., 2015). This component was utilized in the *in vivo* trial discussed in this thesis, with the goal of MP being the limiting nutrient so the diets could respond to the addition of RP Met.

In addition, the profile of AA is a focus of improvement in CNCPS. Because analyzing AA from insoluble residue does not account for rumen undegradable AA, the analysis of AA was changed to a whole-feed basis (Van Amburgh et al., 2013; Higgs et al., 2015). In addition, Van Amburgh et al. 2015 identified higher Lys and Met AA concentrations of feed that had previously been improperly analyzed. As used in the *in vivo* trial detailed in this thesis, Agricultural Modeling and Training Systems L.L.C. (AMTS.Cattle) uses CNCPS for formulating and evaluating diets (Van Amburgh et al., 2015).

In Vitro Methodology

Assessing the N and undigested N (uN) content of feedstuffs and supplements in an *in vitro* environment involves ruminal and intestinal analyses by mimicking the chemistry of those compartments. In the context of the laboratory analyses described below, uN is the N remaining after exposure to each compartment.

Ross et al. 2013 developed an *in vitro* intestinal digestion assay, known as the Ross Assay. The methods of the Ross Assay are outlined in the Materials and Methods section of the thesis and a detailed flow chart is provided by **Figure 1** (Ross et al., 2013). When assessing ruminal digestion, other assays use bags with feedstuffs, placing them in situ in a fistulated cow for fermentation (Vanzant et al., 1998; Ross et al., 2013). However, bags produce variable results and it is difficult to differentiate N loss from digestion or solubility (Vanzant et al., 1998; Ross, 2013). Instead, the Ross Assay creates an *in vitro* ruminal environment by adding feed to Erlenmeyer flasks, along with rumen fluid for microbial activity and the Van Soest buffer (Goering and Van Soest, 1970) for a pH of 6.8. Rumen fluid should be retrieved at last six hours after cattle are fed to have optimum microbial activity (Ross, 2013).

The Erlenmeyer flasks are placed in a water bath at 39°C and then maintained under constant carbon dioxide to create an anaerobic environment. After 16 hours, a subset of the flasks is filtered with a vacuum through glass microfiber filters (Ross et al., 2013). Any nitrogen remaining on the filter is then determined via combustion assay (Leco FP-928 N Analyzer, Leco Corp., St. Joseph, MI), giving the uN value of the sample at 16 hours in the rumen.

The flasks that are not filtered are subjected to abomasal digestion, where 3M HCl is added to the flask, adjusting its pH to 2 and creating an acidic abomasum environment. Pepsin is then added, and the samples are incubated for one hour (Ross, 2013). After neutralizing the samples with sodium hydroxide to a pH of 6, enzymes are added for the transition from the abomasum to the intestine. As opposed to other assays that use pancreatin, the Ross Assay uses an enzyme mix of trypsin, chymotrypsin, amylase, and lipase in the intestinal digestion phase of the assay for a more defined enzyme profile (Ross et al., 2013). After the addition of intestinal enzymes, the flasks are incubated for 24 hours at 39°C. The contents of the flasks are then filtered through the glass microfiber filters, and the N determination of the residue is determined via combustion assay (Leco FP-928 N Analyzer, Leco Corp., St. Joseph, MI). The uN resulting from the ruminal environment and the intestinal environment are compared to determine the proportion degraded in each compartment. Results from

the Ross Assay have been used to update CNCPS, including the correction of NPN pools and soluble true protein of feedstuffs (Van Amburgh et al., 2010).

In addition to the Ross Assay, a Rumen Stability Time Course provides insight into how the feed or supplement is digested by the cow. A Rumen Stability Time Course determines the stability of the product in the rumen over a 30-hour period using four time points: 8, 16, 24, and 30 hours. Nitrogen remaining from the samples are determined through the same methods as described above. The rate of N digestion in the rumen can then be calculated based on the degradation at each time point. In the context of this thesis, the rate of digestion is used to calculate the amount of metabolizable Met available from the RP Met products.

Both *in vitro* analyses are important for determining the extent of under-protection or overprotection of a RP product. An under-protected RP product faces microbial action in the rumen, so the coating surrounding the Met is insufficient. In the context of the *in vitro* methods, an under-protected product in the rumen will have greater loss, at a greater rate, and less will be found in the intestinal assay. An over-protected RP product escapes the entire digestive tract and is excreted. In this case, the coating does not break down from the enzymes or acidity in the abomasum or in the small intestine. Over-protected RP products in the *in vitro* analyses will show a relatively high and constant level of degradability in the rumen, and the Ross Assay results will show a higher uN in the intestinal compartment.

Conclusion and Study Overview

The supplementation of RP Met to the dairy cow reduces nitrogenous waste while maintaining milk production. Modelling techniques, *in vivo* analyses, and *in vitro* assessments allow for the evaluation of an RP Met to determine the amount metabolizable methionine supplied to the animal. The purpose of this research trial was to measure the performance of a newly manufactured RP Met (RP Met 2) against a long-standing, commercially available RP Met (RP Met 2). Based on the *in vitro* analysis of both RP Mets and the amount of metabolizable Met that was limiting milk yield and components, the hypothesis was that at similar feeding rates of metabolizable Met, the cow performance for energy corrected milk for both RP Mets would equally deviate from the control.

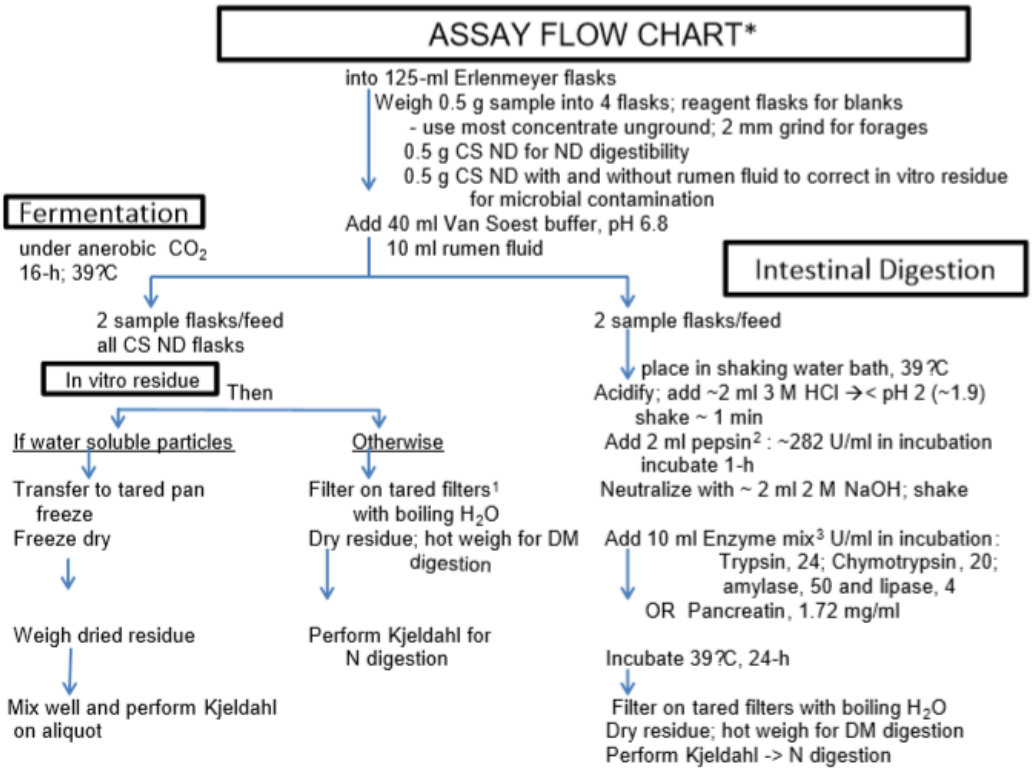

Figure 1: Ross Assay Flow Chart to determine undigestible N of feedstuffs (Ross et al., 2013).

MATERIALS & METHODS

In Vitro Evaluation of Rumen-Protected Products

Two *in vitro* assessments were performed on the RP Met products, both designed to mimic the biology of the cow.

A Rumen Stability Time Course was used to evaluate each RP Met products ability to avoid breakdown and digestion in the rumen over a thirty-hour period. Four timepoints were used to measure the products' integrity throughout the exposure to a rumen environment: 8-hr, 16-hr, 24-hr, 30-hr. First, 40mL of a Van Soest buffer was added to 0.5g of dry matter of RP Met in an Erlenmeyer flask. Obtained from fistulated dairy cattle, rumen fluid was filtered and subjected to carbon dioxide for an anaerobic environment. The filtrate (10mL) was then used to inoculate each flask, and flasks were subjected to anaerobic conditions, 39°C water, and 6.8 pH to mimic a rumen environment. At each timepoint, a subset of the flasks was removed, and the residue was filtered on a glass filtering apparatus. Nitrogen determination of the residue was determined via combustion assay (Leco FP-928 N Analyzer, Leco Corp., St. Joseph, MI). Any N remaining within the residue was assumed to be uN.

Another *in vitro* evaluation was performed on the methionine products to estimate their ruminal stability at 16 hr, as well as their intestinal digestibility after abomasal and intestinal action (**Figure 1**, Ross et al., 2013). All samples were subjected to an *in vitro* rumen environment, using the same techniques as described above. After 16-hr incubation, half of the samples were filtered for N determination via combustion assay, and the other half continued through the *in vitro* abomasal and intestinal compartments. To mimic the abomasum, 3 M HCl was added to lower the pH of each flask to 2. After one minute in a shaker bath set to 39°C, 2 mL of a pepsin enzyme mix was added, with the purpose of breaking proteins into peptides. The flasks were then incubated in the shaker bath for one hour, and the 39°C was maintained. Afterwards, 2 ml of 2 M sodium hydroxide was used to neutralize the samples (pH of 5), reflecting the transition from the abomasum to the small intestine. Lastly, 10 mL of an enzyme mix containing trypsin, chymotrypsin, amylase, and lipase was added and the flasks were incubated in 39°C in the shaker bath for 24-hrs. The solution and residue were then filtered as described previously, and the N content of the filter paper with residue was measured via combustion assay (Leco FP-928 N Analyzer, Leco Corp., St. Joseph, MI). Blood meal and heat-treated blood meal were also put through the Ross Assay as positive and negative controls due to their known N digestion when subjected to both rumen fermentation and intestinal digestion. Blood meal was a positive control, while heat-treated blood meal was a negative control.

By generating the pool size of available AA and the rate of ruminal digestion, the *in vitro* results were used to calculate the amount of metabolizable methionine available from both products. Dietary treatments were formulated with this knowledge to test the validity of these *in vitro* results through an *in vivo* model.

In Vivo Evaluation of Rumen-Protected Products

[1]The *in vivo* assessment of the RP Met products was conducted in tie-stalls at the Cornell University Ruminant Center (CURC) (Harford, New York). Each cow had access to feed and water, and the stalls were bedded with sawdust. Thirty-nine total lactating dairy cattle (27 primiparous and 12 multiparous) (2.46 ± 1.35 lactations), between 21 and 40 DIM, were assigned to this trial. Early lactation animals, as opposed to mid or late lactation, were chosen for their greater milk yield response to post-ruminal amino acid supplementation (Schwab 1996). The trial took place between May 16, 2019 and July 25, 2019, with a 10-day covariate period followed by nine-weeks with three separate diets, each with 13 cows.

Dietary treatments were formulated using Agricultural Modeling and Training Systems (AMTS) ration formulation software. The AMTS platform utilizes CNCPS v.6.5.5 to calculate nutrient requirements for cattle as well as calculate the supply of nutrients to those animals given the nutrient profile and digestibility of the feeds fed. To evaluate the efficacy of each RP Met product, three diets were formulated and fed. First, a control diet (Control) was formulated for adequate metabolizable energy but was slightly deficient in MP (90% MP Requirements). A control was used to allow for any responses from supplementing the RP Met products to be observed. Two subsequent diets were formulated, both using the same formulation as the control diet; however, one supplemented the commercially available RP Met product (Control + RP Met 1) and the other supplemented the newly produced RP Met product (Control + RP Met 2). Each of the RP products were added at a different inclusion rate to ensure the same amount of metabolizable methionine was being supplied in the dietary treatments: 16g/d of RP Met 1 and 23g/d of RP Met 2. The forages were provided by CURC, and the grain mix was purchased by Purina Animal Nutrition (Caledonia, New York). **Table 1** provides the ingredient composition of each diet fed throughout the experiment.

[1] The Cornell University Animal Care and Use Committee approved all procedures involving the use of animals.

Table 1: Ingredient composition of the three diets, %DM[1]

Ingredient, % DM	Control	Control + RP Met 1[2]	Control + RP Met 2[3]
Corn Silage	45.36	45.33	45.32
Grass Silage	17.30	17.29	17.28
RP Met 1[2]	----	**0.06**	----
RP Met 2[3]	----	----	**0.09**
Grain Mix Composition			
Corn Meal	11.53	11.53	11.52
SoyPass	6.15	6.15	6.15
SoyHulls	4.73	4.73	4.72
Canola Meal	3.84	3.84	3.84
Molasses	3.84	3.84	3.84
Wheat Midds	1.85	1.84	1.84
Corn Distillers	1.81	1.81	1.81
Megalac	1.58	1.58	1.57
Salt White	0.56	0.56	0.56
Urea	0.38	0.38	0.38
Limestone	0.31	0.31	0.31
Calcium Phosphate	0.23	0.23	0.23
Vitamin Pack	0.22	0.22	0.22
Rumen-protected Lysine	0.15	0.15	0.15
Min AD	0.09	0.09	0.09
Levucell SC	0.08	0.08	0.08
Total	100.00	100.00	100.00

[1]DM=dry matter. [2]Met 1=commercial rumen-protected methionine. [3]RP Met 2=new rumen-protected methionine

Sampling Procedure and Laboratory Analysis

To analyze the effects of each diet, milk samples, feed samples, animal parameters, and blood samples were taken throughout the experiment.

Milk weights were recorded three times a day during milking using a DelPro system (DeLaval Inc., Kansas City, MO), at 0600 h, 1400 h, and 2200 h. The daily milk weights were averaged across the week to be used with the weekly milk sample data. Milk samples were taken one day each week for three consecutive milking times (Wednesday 0600 h, 1400 h, 2200 h). The milk samples were preserved in 2-bromo-2-nitropane, 3-diol at 4°C until analyzed by Dairy One (Ithaca, NY). The resulting analysis reported protein, fat, lactose, solids, and milk urea nitrogen (MUN). Energy corrected milk (ECM) was calculated using the following equation, adapted from Tyrrell and Reid (1965): ECM kg = (0.327 * milk kg) + (12.95 * fat kg) + (7.65 * protein kg).

Feed was delivered daily at 1400 h using a Data Ranger Feed Cart (American Calan; Northwood, NH). The control TMR was brought to the barn where the RP Met products were hand added, mixed into the TMR, and diets were then fed in individual feed bins in front of each cow. Daily intakes for each animal were recorded as the amount of feed delivered less the amount refused on the following day. Samples of TMR were taken weekly from the individual feed bins from five random cows per dietary treatment and composited based on dietary treatment. Refused TMR samples from five random cows were taken the following day before the feed was replaced; these were also composited based on dietary treatment. Corn silage (CS) and mixed mainly grass (MMG) were sampled weekly from the feed bunks. Grain samples were taken during the covariate week, week 2, and week 5. Whenever sampled, subsamples of the offered TMR, CS, MMG, and grain were sent to Cumberland Valley Analytical Services (CVAS) for feed chemistry analysis. Feed chemistry reported by CVAS included: CP, neutral detergent insoluble CP (NDICP), acid detergent insoluble CP (ADICP), starch; sugar, ash, ether extract, fiber acid detergent fiber (ADF), and neutral detergent fiber (NDF).

Remaining samples were stored in a freezer. The TMR samples were weighed for as-fed kg, dried at 60°C for 48 hours, and weighed again for dry matter (DM) kg. Resulting DM measurements were used to calculate dry matter intake (DMI). After determining the as-fed and DM weights of the samples, the TMR samples from the offered and refused, as well as the CS and MMG were ground in the Wiley Mill to be stored for potential future analysis.

Body weights (BW) were recorded weekly after the 1400 h milking, during which a body condition score (BCS) was determined using a 5-point scale. To reduce bias, BCS was averaged between two researchers.

In addition to weekly and daily samples, blood samples were taken after one milking during the covariate week, week 3, and week 7. Blood was extracted with a vacuum tube from the coccygeal vein underneath the cow's tail after being disinfected with alcohol solution. Immediately following blood drawing, the tubes were centrifuged, and plasma was collected and frozen. Samples were subjected to an enzymatic colorimetric assay to evaluate urea nitrogen content (No.640; Sigma-Aldrich, St. Louis, Mo).

Statistical Analysis

The data was analyzed using Statistical Analysis System version 9.4 (SAS v9.4; Cary, NC). Data normality and homogeneity of variance were assessed for BW, BCS, DMI, milk components, and PUN. Residuals were used to account for differences between the observed values and the predicted

values. These data were then analyzed using a mixed analysis of variance (ANOVA) test to account for fixed effects of diet, time, and their interaction as well as the random effect of cow. Whenever available and applicable, a covariate measurement of the response variable in question was used in the statistical model. Least square means and differences among least square means using a Tukey's multiple comparison adjustment were reported for analysis. A p-value less than 0.05 was considered significant, while a p-value between 0.05 and 0.10 was considered a trend. The means for the feed chemistry were also found to compare with the feed chemistry for the formulated diet.

RESULTS AND DISCUSSION

In Vitro Results and Discussion

Based on the Ross Assay, the rumen-protected (RP) Met 2 had 5.87% dry matter (DM) of total digested N, while RP Met 1 had 6.42% DM of total digested N. These values are the product of the total N % DM before the Ross Assay and the total % N considered digested by the Ross Assay. The extent of rumen protection over multiple timepoints of the RP Met supplements is shown in **Figure 2**. Rumen-protected Met 1 remained relatively stable in the rumen, with 97.3% uN at 30-hrs in the rumen *in vitro* environment. Conversely, RP Met 2 faced rumen action and degraded over time, with 67.6% uN at 30 hrs. Based on the Ross Assay results in **Figure 3**, RP Met 2 lost more N in the rumen than RP Met 1, further showing that RP Met 2 product has less protection from ruminal action. In addition, RP Met 1 had more metabolizable Met available than RP Met 2, with 6.42% DM of total digested N and 5.87% DM of total digested N respectively.

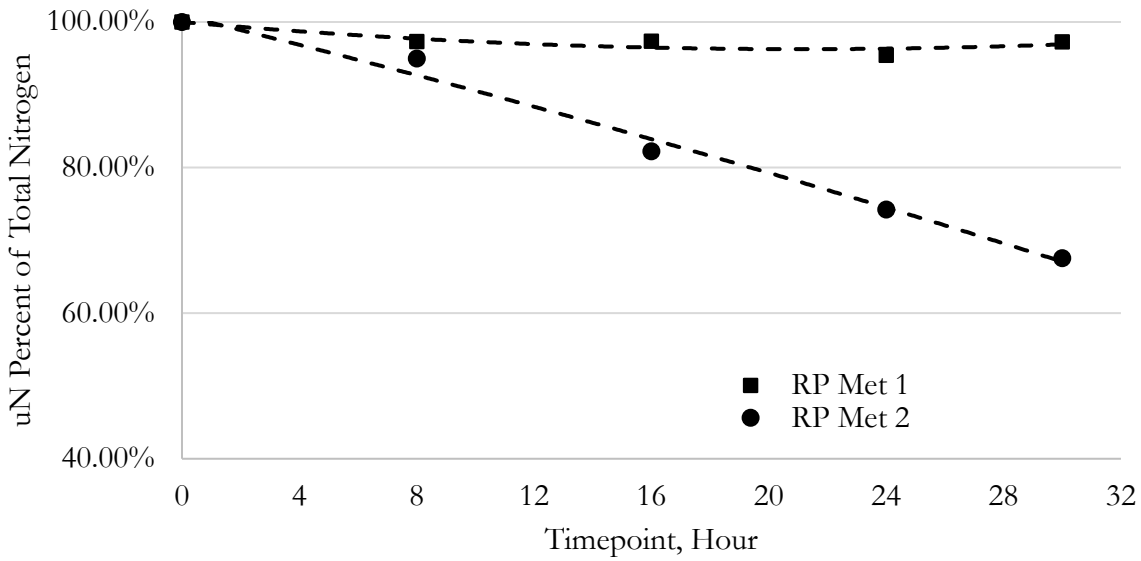

Figure 2: Rumen Stability Time Course results for commercial rumen-protected (RP) Met (RP Met 1) and new RP Met (RP Met 2). Each point represents *in vitro* rumen undigestible N (uN) % of the RP Met product at the indicated time points

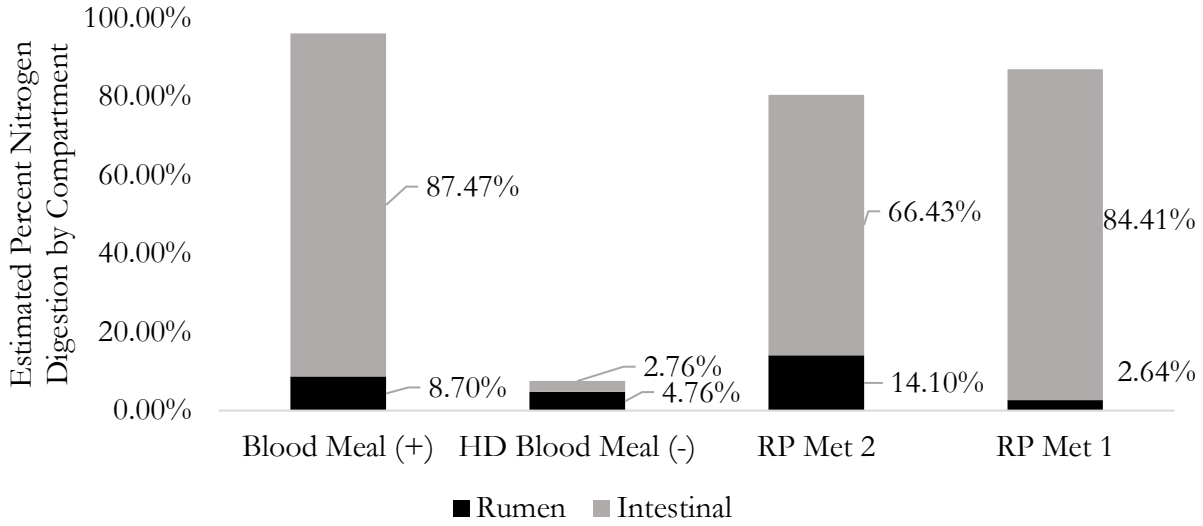

Figure 3: Ross Assay results showing percentage of digestible nitrogen of the rumen-protected (RP) Met products by compartment. Blood meal and heat-damaged (HD) blood meal were used as controls due to their known nitrogen digestibility under the assay conditions.

Feed Chemistry Results and Discussion

Comparing the nutrient composition between the formulated and experimental diets is important to assess how closely the diets fed to the cows matched the expectations. **Table 2** shows the nutrient composition of the formulated and experimental diets (control, RP Met 1, RP Met 2) as percent of the diet in either dry matter (DM) or crude protein (CP) measurements. Starch was lower in the three diets than formulated, with approximately a 4% DM decrease. Despite this difference, the three experimental diets deviated from the formulated diet to a nearly identical extent. The similarity in the nutrient composition for the control, RP Met 1, and RP Met 2 diets supports the concept that the only change in the diet is the presence and type of RP Met.

Table 2: Comparison of nutrient composition for the formulated and actual diets

Nutrient Composition[3]	Formulated[2]	Experimental Diets[1]		
		Control	RP Met 1	RP Met 2
Dry Matter, %	40.10%	37.53%	37.34%	37.36%
Crude Protein, % DM	15.87%	16.55%	16.71%	16.57%
Soluble Protein, % CP	40.75%	46.24%	46.68%	46.90%
NDICP, % CP	15.00%	15.14%	15.13%	14.88%
ADICP, % CP	6.0%	5.96%	5.83%	5.84%
Sugar, % DM	4.65%	3.56%	3.84%	3.81%
Starch, % DM	26.42%	22.73%	22.52%	22.73%
NDF, %DM	31.97%	33.82%	33.48%	33.67%
ADF, %DM	20.35%	21.63%	21.30%	21.57%
Ash, % DM	7.52%	6.82%	6.74%	6.56%
EE, % DM	4.67%	5.15%	5.27%	5.01%

[1]Three experimental diets; Control= base diet, no RP Met supplementation, RP Met 1= base diet with commercial RP Met supplementation, RP Met 2= base diet with new RP Met supplementation. [2]Formulated chemistry as reported by AMTS.Cattle. [3]Nutrient composition of experimental diets as reported by Cumberland Valley Analytical Services

In Vivo Results and Discussion

It was hypothesized that based on the *in vitro* data, at the feeding rates implemented in this study, the RP Met 1 and RP Met 2 will provide similar amounts of metabolizable methionine and allow for similar productivity by the cattle, particularly for energy corrected milk yield. Rumen-protected Met 1 has been shown to increase milk components and yield (Armentano et al., 1997; Rulquin and Delaby 1997; Chen et al., 2011; Osorio et al., 2014), so its supplementation in this trial is expected to result in a biological response from the animal. Because the same amount of metabolizable Met for each RP Met product (16g/d for RP Met 1 and 23g/d for RP Met 2) was added to their respective diets, the RP Met supplements were expected to equally deviating from the control to give responses indicating higher N-use efficiency, such as higher milk components. However, despite numerical differences for RP Met 1, no statistically significant deviations were found between the two RP Met products and the control. Since the diets supplemented with the RP Mets did not deviate from the control diet, the results are inconclusive.

As shown above, the *in vitro* assays indicate some degree of protection from the rumen for both RP Met products. However, the *in vivo* trials showed no significant benefits in milk yield or milk

components from including either product. It should be noted that the planned n value of the *in vivo* experiment was 72, rather than 39; with a higher n value comes a higher statistical power and more reliable data. An insufficient statistical power in the *in vivo* trial resulted in a discrepancy between the *in vivo* and *in vitro* trials.

Gutierrez-Botero et al. 2015 conducted an experiment that compared the Ross Assay to *in vivo* results, with a similar experimental design to the study detailed in this thesis. Comparing two blood meals with different predicted digestible protein levels, Gutierrez-Botero et al. 2015 evaluated the products through the Ross Assay and conducted a feeding study, evaluating milk yield, milk components, BW, BCS, DMI, and feed efficiency. The results from Gutierrez-Botero et al. 2015 indicated consistency between the Ross Assay and the *in vivo* trial, providing confidence in the accuracy of the Ross Assay.

The effect of the three diets (control, RP Met 1, and RP Met 2) on body weight, body condition score, plasma urea nitrogen, and milk urea nitrogen are depicted in **Table 3**. There was no significance between diet or from the interaction of diet and time. However, there was significant change in the parameters over time. For BW and BCS this can be expected, as the trial involved early lactation cows (21-40 DIM), and cows typically gain body condition over their lactation. It can be assumed that the RP Met supplementations were not allocated towards growth because no significant difference for the model of diet or diet*time was found for BW and BCS. This is also consistent with other *in vivo* trials studying RP Met (Broderick et al., 2008; Osorio et al., 2017). Establishing that the RP Met does not contribute towards growth shows the potential for the supplements to be used in other metabolic pathways, including milk production.

The milk urea nitrogen (MUN) and plasma urea nitrogen (PUN) results are displayed together in **Table 3**. On average, the PUN concentration was slightly greater than MUN for the control, the RP Met 1, and the RP Met 2 diets. Plasma urea nitrogen is expected to be higher than MUN due to the laws of passive diffusion. Numerically higher PUN and MUN were seen for RP Met 2 when compared to the control and RP Met 1, which could indicate a lower N use efficiency of RP Met 2.

Table 3: Body weight, body condition score, plasma urea nitrogen, and milk urea nitrogen (LS means) for the experimental diets. Fixed effects of diets, time, and the diets throughout time for the same parameters

	Diet[1]				p-Value[2]		
	Control	RP Met 1	RP Met 2	SEM	Diet	Time	Diet*Time
BW kg	676.3	676.7	671.1	4.072	0.56	< 0.01	0.23
BCS	2.90	2.88	2.90	0.027	0.88	< 0.01	0.48
PUN mg/dL	10.71	11.08	11.16	0.331	0.59	<0.01	0.50
MUN mg/dL	10.50	10.68	10.83	0.237	0.64	<0.01	0.12

[1]Control= base diet, no RP Met supplementation, RP Met 1= base diet with commercial RP Met supplementation, RP Met 2= base diet with new RP Met supplementation. Least squared means are reported for all parameters. [2]p-Value for fixed effects, significance if $p<0.05$, tendency if $p<0.1$#. [3]SEM=Standard Error of the Mean for least squared means.

Production responses to the three experimental diets are depicted in **Table 4**, showing the effects of diet, time, and diet over time for DMI, feed efficiency, milk yield, ECM, protein, fat, and lactose. No significant difference was found in the results for the effect of diet and diet over time for the parameters. However, the effect of time was significant for all indicators.

As depicted in **Table 4**, the commercial RP Met (RP Met 1) used in this experiment did not show a significant response in production performance when compared to the control. However, other *in vivo* trials show improved production performance when diets were supplemented with the commercial RP Met. Energy corrected milk usually increases with the addition of commercial RP Met to a Met-limiting diet (Chen et al., 2011; Osorio et al., 2014). Chen et al. 2011 found that RP Met improved ECM due to an increase in milk protein synthesis. Other studies agree that RP Met supplementation increases milk protein yield (Armentano et al., 1997; Osorio et al., 2014). The lack of production response to RP Met 1 indicates confounding effects may have compromised an effective response to the *in vivo* supplementation.

All three diets had low milk protein yields when compared to other AA experiments. For example, Appuhamy et al. 2011 studied the effects of adding Met and lysine to the diet, finding a milk protein yield of 1.52 kg/d for the diet with Met. When Chen et al. 2011 compared different Met sources, the milk protein yield did not fall below 1.2 kg/d. As depicted in **Table 4**, the control, RP Met 1, and RP Met 2 all had low protein yields compared to other experiments (1.098 kg/d, RP Met: 1.126 kg/d, 1.111 kg/d respectively).

Experimental feed chemistry of the diets depicted in **Table 2** gives insight into the depressed milk protein yield, as the starch content of the diets was approximately 4% less than formulated. It can be

speculated from the low starch levels that adequate energy was not provided to the rumen for substantial microbial protein production (Fessenden 2016), causing the low protein levels in all diets. When energy is limiting, the fate of AA in the rumen is to deaminate into VFAs, rather than contribute to metabolizable protein production (Bach et al., 2015). Broderick 2003 studied the effects of varying levels of CP and dietary energy, finding that an increase in energy allowed for better utilization of CP. Increasing dietary energy results in an increase in milk yield and milk protein components. A rise in dietary energy also increased the ratio of milk N to N intake, as well as a decrease in MUN (Broderick 2003).

Although there was no statistical difference when looking at the model of different dietary treatments for milk components, there were numerical differences. Rumen-protected Met 1 had numerically higher ECM and fat yield than the control and RP Met 2. A possible explanation for the slight increase in fat yield is the higher availability of metabolizable Met of RP Met 1 contributing to the production of phosphatidylcholine, a component of milk fat (Zhou et al., 2016).

Table 4: Dry matter intake, feed efficiency, milk yield, and milk components (LS means) for the experimental diets. Fixed effects of diets, time, and the diets throughout time for the same parameters

	Diet[1]				**p-Value[2]**		
	Control	RP Met 1	RP Met 2	SEM[3]	Diet	Time	Diet*Time
DMI kg/d	24.4	24.8	24.7	0.301	0.71	<0.01	0.73
Feed Efficiency[4]	1.840	1.865	1.837	0.039	0.85	<0.01	0.41
Milk Yield kg/d	43.94	43.86	43.38	0.855	0.88	<0.01	0.13
ECM kg/d	45.28	46.56	45.80	1.300	0.78	<0.01	0.18
Protein kg/d	1.098	1.126	1.111	0.037	0.86	<0.01	0.23
Fat kg/d	1.739	1.823	1.782	0.061	0.61	<0.01	0.29
Lactose kg/d	2.049	1.995	1.990	0.069	0.80	<0.01	0.52

[1] Control= base diet, no RP Met supplementation, RP Met 1= base diet with commercial RP Met supplementation, RP Met 2= base diet with new RP Met supplementation. Least squared means are reported for all parameters. [2]p-Value for fixed effects, significance if $p<0.05$, tendency if $p<0.1$. [3]SEM=Standard Error of the Mean for least squared means. [4]Feed efficiency is the quotient energy corrected milk/dry matter intake

Conclusions

The primary objective of this research trial was to measure the performance of a newly manufactured RP Met (RP Met 2) against a commercially available RP Met frequently fed in the dairy industry (RP Met 1). The *in vitro* trial results indicate lower metabolizable Met available from RP Met

2 as compared to RP Met 1. Based on the *in vitro* analysis of both RP Mets and the amount of metabolizable methionine that was limiting milk yield and components, the hypothesis was that at comparable feeding rates of metabolizable Met, energy corrected milk yield for RP Met 1 and RP Met 2 would equally increase when compared to the control. As shown by the descriptive statistics, the *in vivo* dietary treatments were not statistically different. For BW, BCS, and DMI, having no significant difference between the diets is expected. Although RP Met 1 had a numerically higher production response than the control and RP Met 2, the RP Mets did not significantly deviate from the control. Having a lower n value than expected in the *in vivo* trial indicates the need for higher statistical power to achieve significant results.

Future Directions

With the number of animals at 39 (or 13 per dietary treatment), the study was limited in statistical power as the planned n value was 72. The numerical increases from RP Met 1, particularly in the milk components, might have seen a statistically significant response if more cows were involved in the trial. To receive more robust results, another trial with the same methods and statistical models should be employed with a greater number of cows.

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Appendix 1: Schematic Representation of N transaction in the rumen (Ipharraguerre 2005)

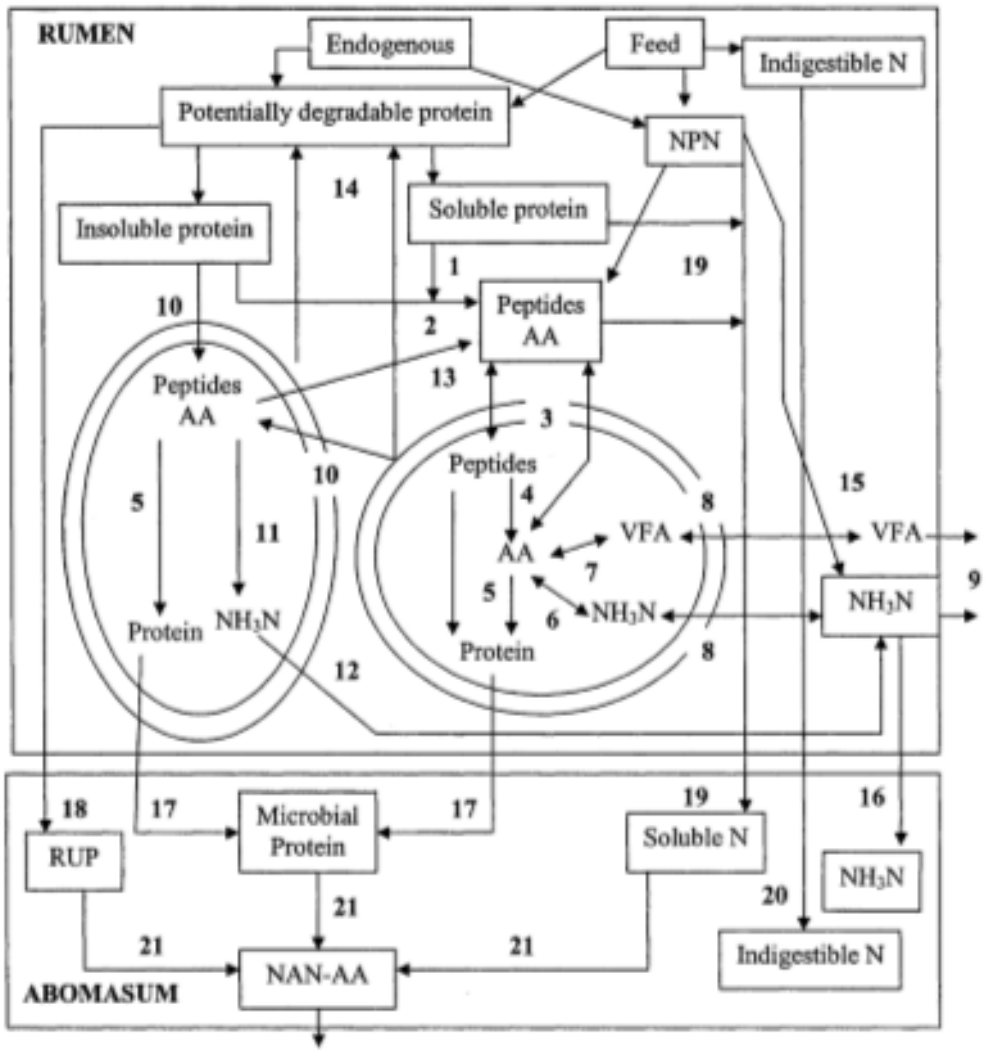

Figure 2-1. Schematic representation of N transactions in the rumen depicting major events [protozoa (oval), bacteria (circle), pools of major N fractions (box), and fluxes (arrows)]: (1) extracellular proteolysis (adsorption of soluble proteins to bacteria), (2) extracellular proteolysis (adsorption of bacteria to insoluble proteins), (3) bacterial uptake of small peptides and AA, (4) intracellular peptidolysis, (5) synthesis of protein, (6) intracellular deamination/amination, (7) intracellular fermentation of carbon skeletons to VFA, (8) excretion/assimilation of NH_3N by bacteria, (9) absorption through rumen walls, (10) engulfment of particulate matter followed by proteolysis, (11) intracellular deamination, (12) excretion of NH_3N by protozoa, (13) excretion, secretion, or release through cellular lysis of peptides and AA (14) excretion, secretion, or release through cellular lysis of complex N compounds (15) rapid conversion of simple NPN sources to NH_3N, (16) outflow of NH_3N with liquids, (17) outflow of microbial protein, (18) outflow of RUP, (19) outflow of soluble-N fractions, (20) outflow of indigestible-N fractions, and(21) contribution to absorbable-AA pool.

Appendix 2: Methyl donor metabolism (McFadden et al., 2020)

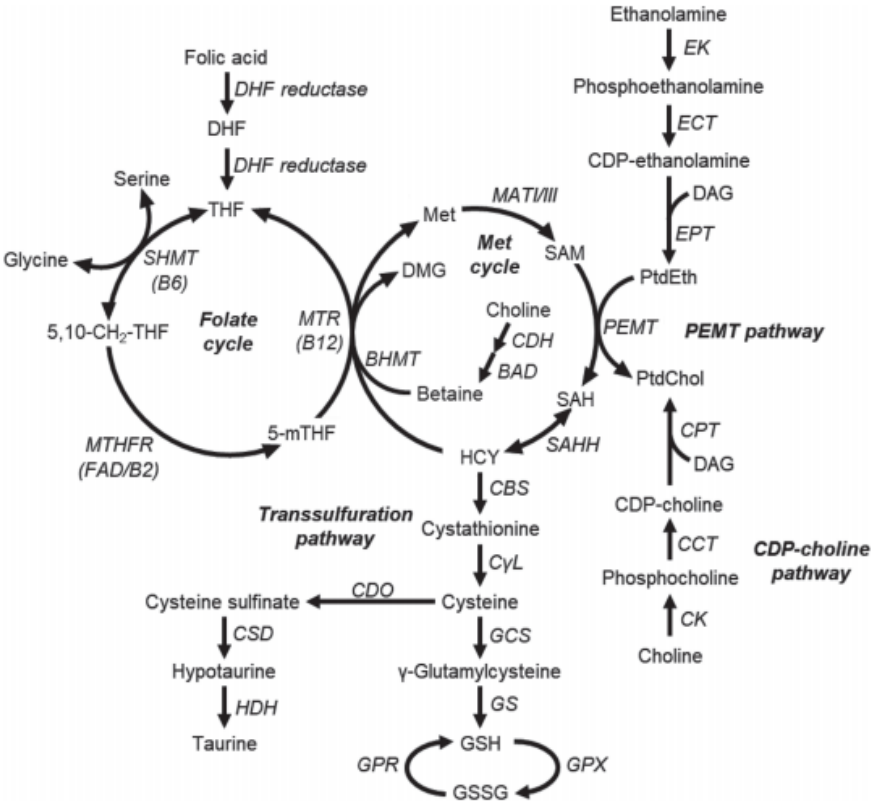

Figure 1. One-carbon metabolism, the transsulfuration pathway, and phosphatidylcholine (PtdChol) synthesis. BAD = betaine aldehyde dehydrogenase; BHMT = betaine-homocysteine methyltransferase; CBS = cystathionine β-synthase; CCT = CTP:phosphocholine cytidylyltransferase; CDH = choline dehydrogenase; CDO = cysteine dioxygenase; CDP = cytidine diphosphate; CγL = cystathionine γ-lyase; CK = choline kinase; CPT = cholinephosphotransferase; CSD = cysteine sulfinic acid decarboxylase; DAG = diacylglycerol; DHF = dihydrofolate; DMG = dimethylglycine; ECT = CTP:phosphoethanolamine cytidylyltransferase; EK = ethanolamine kinase; EPT = ethanolaminephosphotransferase; FAD = flavin adenine dinucleotide; GCS = γ-glutamylcysteine synthase; GPR = glutathione-disulfide reductase; GPX = glutathione peroxidase; GS = glutathione synthetase; GSH = reduced glutathione; GSSG = oxidized glutathione; HCY = homocysteine; HDH = hypotaurine dehydrogenase; MATI/III = methionine adenosyltransferase I/III (liver specific isoenzymes); 5-mTHF = 5-methyltetrahydrofolate; MTHFR = methylenetetrahydrofolate reductase; MTR = methionine synthase; PEMT = phosphatidylethanolamine *N*-methyltransferase (other types of methyltransferases may utilize SAM; only PEMT is shown for simplicity); PtdEth = phosphatidylethanolamine; SAH = *S*-adenosylhomocysteine; SAM = *S*-adenosylmethionine (3 SAM are required to convert PtdEth to PtdChol); SHMT = serine hydroxymethyltransferase; THF = tetrahydrofolate. Associated B vitamin cofactors are denoted in parentheses.