UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
CAMPUS DE BOTUCATU

NITROGEN METABOLISM AND EXCRETION IN HOLSTEIN DAIRY HEIFERS FED DIFFERENT LEVELS OF METABOLIZABLE PROTEIN

MARIA HELENA DE OLIVEIRA

Thesis presented to the Graduate Program in Animal Science in fulfillment of the requirements for the degree of Doctor in Animal Sciences.

BOTUCATU-SP
June, 2024

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DOCTORAL THESIS

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Atestamos que **MARIA HELENA DE OLIVEIRA**, RA nº: ZNP210102, RG nº 48.809.033-7, expedido pela SSP/SP, defendeu, no dia 10/06/2024, a tese intitulada **NITROGEN METABOLISM AND EXCRETION IN HOLSTEIN DAIRY HEIFERS FED DIFFERENT LEVELS OF METABOLIZABLE PROTEIN**, junto ao Programa de Pós Graduação em Zootecnia, Curso de Doutorado, tendo sido 'APROVADA'.

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Carlos Pazini Junior
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BIOGRAPHY

Maria Helena de Oliveira was born on August 22, 1993, in Laranjal Paulista, São Paulo, Brazil. She began her education at "E.M. João Salto" elementary school in Laranjal Paulista in 2000 and completed her high school studies at "Colégio Criar" in Tietê, São Paulo, in 2010. Maria holds a Bachelor's degree in Animal Science from the Federal University of Lavras, Brazil, earned in 2018. Subsequently, she obtained a Master's degree in Animal Science in 2021 from São Paulo State University, School of Veterinary Medicine and Animal Science - Campus Botucatu. In March 2021, she started her Ph.D. in Animal Science at the same institution. From September 2022 to June 2024, Maria completed an international internship at Virginia Polytechnic Institute and State University in Blacksburg, VA, USA. During this period, she conducted the research presented in this Thesis.

DEDICATION

To my parents, Edilene Ponce do Amaral and Marcelo Valdrighi de Oliveira, for their unconditional love and support.

To my best friends, my siblings Ana Elisa de Oliveira Alho and Marcelo Valdrighi de Oliveira Filho.

To my husband Júlio Henrique Pena, for his unwavering support and love.

To my grandparents *(in memoriam)*, Luiz Ponce do Amaral and Eliza Melo Coelho do Amaral, my eternal guardian angels.

I love you!

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"We ain't what we oughta be.
We ain't what we want to be.
We ain't what we gonna be.
But, thank God, we ain't what we were."

Martin Luther King Jr.

METABOLISMO E EXCREÇÃO DE NITROGÊNIO EM NOVILHAS HOLANDESAS ALIMENTADAS COM DIFERENTES NÍVEIS DE PROTEÍNA METABOLIZÁVEL

RESUMO - Apesar dos avanços, a otimização do metabolismo de nitrogênio em bovinos leiteiros continua sendo um desafio. No Capítulo 1, uma revisão abrangente da literatura sintetiza o conhecimento atual e identifica lacunas de pesquisa relacionadas à complexa interação de fatores que influenciam o metabolismo e a utilização de proteínas em ruminantes, com foco particular no uso eficiente de nitrogênio dietético. No Capítulo 2, o objetivo foi avaliar os efeitos de três níveis de proteína metabolizável na fermentação ruminal, taxas de entrada de aminoácidos e concentração plasmática de aminoácidos, além do metabolismo e excreção de ureia. Seis novilhas Holandesas dotadas de canulas ruminais foram alocadas em um delineamento em quadrado latino duplicado 3 × 3 com três novilhas cada. Os tratamentos dietéticos foram equilibrados para fornecer níveis semelhantes de proteína bruta (16%) e níveis crescentes de proteína metabolizável para medir as diferenças na eficiência de uso de nitrogênio. Nos primeiros 10 dias de cada período, as novilhas foram alimentadas uma vez ao dia; nos últimos quatro dias de cada período, as novilhas foram alimentadas a cada duas horas (95% da ingestão) para estabilizar o metabolismo dos animais entre as coletas. Coletas pontuais de urina, fezes, conteúdo ruminal, saliva e sangue foram realizadas do dia onze ao dia quatorze de cada período. Aumento da ingestão de proteína degradável no rúmen em uma dieta de Baixa proteína metabolizável resultou em maior produção de ácidos graxos voláteis de cadeia ramificada por meio da degradação de aminoácidos de cadeia ramificada, indicando uma utilização ineficiente de nitrogênio. Novilhas alimentadas com dieta de Alta proteína metabolizável apresentaram maior eficiência de nitrogênio e menor excreção de nitrogênio na urina, juntamente com maior balanço de nitrogênio devido ao maior fornecimento de proteína não degradavel no rúmen. Em conclusão, dietas de Alta proteína metabolizável otimizam a utilização de nitrogênio e melhoram a eficiência dietética.

Palavras-chave: Aminoácidos, Eficiência de N, Balanço de N.

NITROGEN METABOLISM AND EXCRETION IN HOLSTEIN DAIRY HEIFERS FED DIFFERENT LEVELS OF METABOLIZABLE PROTEIN

ABSTRACT - Despite advancements, the optimization of nitrogen (N) metabolism in dairy cattle remains a challenge. In Chapter 1 a comprehensive literature review synthesizes current knowledge and identify research gaps concerning the complex interplay of factors influencing protein metabolism and utilization in ruminants, particularly focusing on the efficient use of dietary nitrogen. In chapter 2 the objective was to assess the effects of 3 levels of metabolizable protein (MP) on ruminal fermentation, amino acids (AA) entry rates and plasma AA concentration, and urea-N metabolism and excretion. Six rumen-cannulated Holstein heifers were allocated in a duplicated 3 × 3 Latin square design with three cows each. Dietary treatments were balanced to provide similar crude protein CP intakes (16%) and increasing MP levels to measure differences in N efficient use. For the first 10 days of each period, heifers were fed once daily; for the last four days of each period, heifers were fed every two hours (95% of intake) to stabilize animals' metabolism between collections. Spot collections of urine, feces, rumen content, saliva, and blood were conducted from day eleven to day fourteen of each period. Increased ruminally degraded protein (RDP) intake on a Low MP diet led to elevated branched-chain volatile fatty acid (BCVFA) production through branched-chain amino acid (BCAA) breakdown, indicating inefficient nitrogen utilization. Heifers fed a High MP diet presented greater N efficiency and lower N urine output along with greater N balance due to greater rumen undegradable protein (RUP) supply. In conclusion, High MP diets optimize nitrogen utilization and enhance dietary efficiency.

Keywords: Amino acids, N efficiency, N balance.

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LIST OF ABBREVIATIONS AND SYMBOLS

AA	Amino acid
ADF	Acid detergent fiber
ADH	Antidiuretic hormone
ADG	Average daily gain
Ala	Alanine
AQP	Aquaporins
Asn	Asparagine
Asp	Aspartic acid
BCAA	Branched-chain amino acids
BCVFA	Branched-chain volatile fatty acids
BUN	Blood urea nitrogen
BW	Body weight
CHO	Carbohydrates
CP	Crude protein
DDG	Distillers Dried Grains
DDGS	Distillers Dried Grains with Solubles
ddH_2O	Double-distilled water
DM	Dry matter
DMI	DM intake
EAA	Essential AA
ED	Effective degradability
GIT	Gastrointestinal tract
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
H	Hydrogen
h	hour
His	Histidine
HCL	Hydrochloric acid
Ile	Isoleucine
k_d	Degradation rate
k_p	Passage rate

Leu	Leucine
Lys	Lysine
MCP	Microbial protein
ME	Metabolizable energy
Met	Methionine
MP	Metabolizable protein
MRL	Metabolic research laboratory
mRNA	messenger Ribonucleic acid
MUN	Milk urea nitrogen
N	Nitrogen
N_2O	Nitrous Oxide
NDF	Neutral detergent fiber
NEAA	Nonessential AA
NH_2	Amine
NH_3	Ammonia
NH_4^+	Ammonium
NO	Nitric oxide
NO_2	N dioxide
NO_2^-	Nitrite ions
NO_3^-	Nitrate ions
NNP	Non-protein N
Phe	Phenylalanine
Pro	Proline
RDP	Ruminally degraded protein
RUP	Ruminally undegraded protein
Ser	Serine
THI	Temperature humidity index
Thr	Threonine
TMR	Total mixed ratio
TP	True protein
Tyr	Tyrosine
UT	Urea transporters
Val	Valine
VFA	Volatile fatty acids

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CHAPTER 1

INITIAL CONSIDERATIONS

The prediction of N metabolism in cattle remains a challenge due to the complex microbial metabolic activity in the rumen and its impact on later processes in the intestines and body tissues (Hristov *et al.*, 2019). Briefly, dietary N is ingested as CP sources which is then divided according to its site of degradation and absorption as RDP and RUP (NRC, 2001). RDP supports microbial crude protein (MCP) synthesis in the rumen, while RUP bypasses this process. Together, MCP and RUP constitute the MP pool, which is essential for providing metabolizable AA to the ruminant.

In dairy operations, only 25% to 30% of dietary N is converted into milk protein, and less than 2% contributes to animal growth, leaving a substantial 68% to 73% to be excreted as waste in manure and urine (Arriola Apelo *et al.*, 2014). However, evolutionary adaptations allows the recycling of N surplus as urea, optimizing N utilization while minimizing environmental N excretion (Batista *et al.*, 2017). Recycled urea re-enters the rumen where it is hydrolyzed to ammonia (NH_3) that can be incorporated to MCP. This process increases the supply of AA to the small intestine providing an opportunity for the excess of N to be used towards animal metabolism rather than being irreversibly lost into the environment (Lapierre *et al.*, 2001). Nonetheless, the latest revision of the dairy cattle requirements (NASEM, 2021) adopts a direct method to estimate MCP synthesis, relying on the intake of RDP and rumen degraded carbohydrates without fully consider recycling process in the MCP prediction model. As these equations are based on direct *in vivo* observations it inherently reflects the specific conditions under which they were observed. However, it does not adequately capture the extent of N recycling under different dietary conditions, which then results in an N loss to the environment. Given this limitation, there is a clear need for additional research investigating N efficiency to enhance our understanding of the recycling processes, ultimately leading to improvements in protein prediction by nutritional models. Therefore, the overall objective of this literature review is to synthesize information on the complex interplay of factors influencing protein metabolism and utilization in ruminants.

1 LITERATURE REVIEW

1.1 Proteins

Proteins are large molecules essential for maintaining life. They fulfill diverse roles, including serving as structural components, enzymes, hormones, and storing genetic information (Dietzen, 2018). Each protein is composed of a unique composition of AA

connected by peptide bonds which allow them to vary in size, shape, digestibility, and solubility. AA themselves are made up of four key components: a hydrogen atom (H), an amine group (NH_2), a carboxyl group (COOH), and a unique R group (Wu, 2021). Notably, although a species may have over 10,000 different proteins, cattle and other mammals only require 20 AA for the synthesis of all proteins (Wu, 2021). Then, they were classified into essential AA (EAA) and nonessential AA (NEAA). An EAA cannot be synthesized by the animal or are synthesized at insufficient rate to meet animal requirement. Conversely, NEAA can be produced in adequate amounts by the organism, provided an ample supply of N (NASEM, 2021).

2 Dietary crude protein

Ruminants can produce their proteins using AA obtained straight from the diet or synthetized by ruminal microorganisms by using different N sources, such as TP, peptides, or non-protein N (NPN). NPN serves as a readily available N source for ruminal microbes, whereas peptides and TP offer a spectrum of digestibility and bioavailability characteristics. Peptides (AA linked by peptide bonds) are more complex than free AA but simpler than full-length proteins. Their role is particularly pertinent given their intermediate rate of degradation and absorption, which can influence the efficiency of protein utilization and the synthesis of microbial protein in the rumen.

The gross measurement of feed protein is CP, which is typically represented as a percentage of dry matter (DM). It is calculated as N x 6.25, assuming an average of 16 g of N per 100 g of TP (Jones, 1941). However, by considering any N present in the sample, it overestimates the actual protein content in the feed and fails to distinguish between TP and NPN compounds, as well as its difference in degradation and across feed types (Schwab *et al.*, 2017). Therefore, it is crucial to characterize rumen protein degradation to better understand and address these discrepancies. To this end, an *in situ* technique has been adopted as the reference method for evaluating the kinetics of ruminal protein degradation (NRC, 2001), providing information on ruminal breakdown processes prior to small intestine digestion. Notably, nowadays this method is still considered as reference method for such end (NASEM, 2021). Briefly, ground feed samples, processed to pass through a 2 mm screen, are placed into a Nylon or Dacron polyester bag with a pore size between 40 to 60 µm. These bags are then placed into the rumen of a ruminally cannulated cow. Incubation requires a minimum of five time points, including at least 0 and 48 hours (with additional time of 72 for forages), so the degradations can fit a nonlinear mathematical model. After the designated incubation period,

bags are removed, and the amount of undigested CP is determined (NASEM, 2021). Further, the *in situ* ruminal degradation model categorizes feed proteins into three distinct fractions: A, B, and C. Fraction A represents NPN alongside a small amount of TP that is either highly soluble or has a reduced particle size, leading to its immediate disappearance. Fraction C represents CP completely undegradable within the rumen, as determined by the residual CP found in the *in situ* bag, after a specified degradation interval. The remaining CP is grouped into Fraction B, comprising proteins that are potentially degradable within the rumen environment. The degradation dynamics of Fraction B are determined by its degradation rate (k_d), which is derived from the linear slope over time of each time point, expressed as percentage of the initially incubated material. However, since feed particles can exit the rumen undegraded, the degradation extent observed in the bag does not accurately reflect the true degradation under normal rumen conditions. Therefore, to better represent degradation dynamics of B fraction, the rate of passage (k_p) is included in the calculation based on flow marker evaluation, to account for feedstuff variability. To this end, a recent study has revised prediction equations for RUP and MCP and stablished *kp* for forage and concentrate feedstuff, as 4.95 and 7.11%, respectively (Hanigan *et al.*, 2021a), to correct for discrepancies in outflow rates among these feed classes. Thus, adjusting the degradation rate according to the outflow rates, taking into account the specific characteristics of the protein being studied, allows for a more accurate estimation of what is known as effective degradability (Ørskov *et al.*, 1979).

Upon this theoretical framework, it’s possible to classify ruminant protein degradation by a dual parallel, where part can be ruminally degraded while the other part bypasses the rumen undegraded. Consequently, feedstuff protein will be categorized as RDP and RUP. RDP is composed totally by A fraction along with potentially degradable B fraction. Conversely, B fraction that is not ruminally degraded and proteins completely undegradable (C fraction) are defined as RUP. In other words, RDP serves as a substrate for the rumen's microbial population, influencing the pool of AA derived from MCP available for intestinal absorption, while RUP bypasses ruminal degradation and directly contributes to AA absorbed in the intestine. Combined, MCP and RUP form MP, playing a crucial role in supporting the animal's growth, production, and overall health.

Formulating a total mixed ratio (TMR) for cows involves balancing feedstuffs to ensure rumen health and meet productivity targets. A well-formulated TMR typically comprises 50 to 60% forages and 40 to 50% concentrates with feedstuffs' varying in ruminal degradation, passage rates, digestibility, absorbable peptides, and amino acids profile. Adequate diet formulation is critical for maximizing the synthesis of MCP and ensuring the delivery of

metabolizable AA along with RUP. Since MCP represents more than 50% of the N flow to the duodenum (Sok, 2017), it is the main contributor to MP. However, prediction of MCP synthesis is a complex task, influenced by the dynamic interplay of several factors such DM intake (**DMI**), k_d of dietary components in the rumen, and k_p of digesta. Further, the synthesis of MCP is dependent on the availability of ruminal NH_3-N, which is largely produced from the breakdown of RDP. The transfer efficiency of NH_3-N incorporation into MCP is generally 70% under standard feeding conditions (Li *et al.*, 2019). In addition, rumen microbes have the capability to synthesize EAA through the process of transamination, thus providing the majority of the AA requirements for ruminants (Hartinger *et al.*, 2018). For RUP, its digestibility in the intestine also presents an area of uncertainty, necessitating a deeper understanding of the differential digestibility of AA. Current nutrition models often use the kinetics of CP degradation in the rumen to estimate the degradation rates of specific AA, presupposing uniform rates of degradation and digestion in the rumen (Van Duinkerken *et al.*, 2011), however, further research on AA digestibility would improve its prediction ensuring optimal nutrition and performance outcomes (Hanigan *et al.*, 2021a).

Cows require AA and not proteins per se for numerous physiological processes such as growth, maintenance, reproduction, and production as well as a source of N for the synthesis of NEAA. As previously mentioned, AA are nitrogenous compounds that can be ingested as TP and NPN (e.g., free AA). Although understanding of protein metabolism in ruminants has significantly advanced, gaps still exist in the literature. The major challenge lies in the complexity of the rumen as presents a diverse microbial population that interacts, compete, and works symbiotically to break down feed particles. Additionally, besides feed particles, these microbes can also use intermediate molecules for growth, which in turn results in MCP for the host animal. Furthermore, because of the development of an evolutionary adaptation, ruminants can recycle excess of blood urea to the rumen, what in exchange provide substrate for MCP synthesis if rumen RDP is limiting. Then, due to this mechanism the ruminant provides urea for microbial growth which in turn results in VFA and MCP, representing an efficient use of urea that could be redirected to urinary clearance. Thus, even though this ability can represent a significant source of substrate for microbial protein production, the latest revision of dairy cattle requirements (NASEM, 2021) adopts a direct approach to predict MCP synthesis based on RDP and rumen-degraded carbohydrates, neglecting the N derived from recycling processes. While these equations were derived from *in vivo* observations, it is inherent in the derivation to the extent of the observed conditions, however, it does not fully represent the true potential of MCP synthesis. With that said, predicting optimal protein supply requires an understanding of how

alterations on CP composition will alter MP and thus, the amount of AA available for absorption in the small intestine. By addressing these challenges, we can advance our predictions of protein utilization by ruminants, ultimately leading to improvements in N utilization, productivity, production costs, and sustainability.

3 RDP and MCP on AA Supply

As mentioned earlier, the rumen is colonized by several microbial species, including bacteria, protozoa, fungi, and archaea. Each of them presents distinct nutrient requirements and metabolic processes. Despite their differences, these microorganisms collectively play an important role in ruminant AA supply by utilizing different sources of N. Protein degradation starts either through the adsorption of soluble proteins to bacteria (Nugent *et al.*, 1981; Wallace, 1985) or through the attachment of bacteria to insoluble proteins (Broderick *et al.*, 1991). Subsequently, microbial proteases break down protein into peptides, free AA, and NH_3 that can be assimilated as MCP or deaminated into volatile fatty acids (**VFA**), carbon dioxide (**CO_2**), and NH_3 (Bach *et al.*, 2005). Rumen bacteria have the ability of converting NPN, such as urea and ammonium bicarbonate (**CH_5NO_3**) in MCP (NRC, 2001), which is hydrolyzed by microbial enzymes and became instantaneously available for ruminal microbes' incorporation. NH_3 is protonated in the ruminal acidic environment to NH_4^+ which are impermeable to cellular membranes. Therefore, in the rumen, NH_4^+ must be transferred into the bacterial cells by NH_4^+ transporter proteins (Pengpeng *et al.*, 2013).

Due to the high number of bonds in a single protein, many microbial species adhere to feed particles and work synergistic to break down and ferment proteins into peptides and AA (Bach *et al.*, 2005; Wallace *et al.*, 1997). Thus, microbial population can be classified based on their actions as proteolytic, peptidolytic, deaminating, or even ureolytic.

Proteolysis, peptidolysis, and deamination are three catabolic processes that occur during protein degradation to create peptides and AA. Briefly, proteolysis is the initial step, involving the breaking of peptide bonds within a protein molecule, thereby reducing large proteins to smaller peptides (Velásquez *et al.*, 2016). Peptidolysis occurs where the resulting peptides from the previous stage are further broken down into individual AA. Deamination is the process where AA are deaminated to release NH_3, which can then be utilized by the microbial population for protein synthesis. Furthermore, the ureolytic activity represents an important aspect of N utilization, where urea is hydrolyzed into NH_3 and CO_2 (Jin *et al.*, 2017). This reaction is essential for maintaining a balanced supply of NH_3, which is necessary for microbial protein synthesis. Ureolytic microorganisms facilitate this conversion, ensuring that urea,

whether from dietary sources, saliva, or ruminal wall, is effectively utilized rather than being excreted as waste.

3.1 Bacteria

Bacteria constitute the predominant and most abundant group of microorganisms in the rumen (Wright *et al.*, 2011), playing a central role in protein degradation. Proteolysis occurs in the form of nucleophile attacks on peptide bonds by proteases, which can be endoproteolytic or exoproteolytic (Velásquez *et al.*, 2016). The process normally occurs on the outside of cells intimately associated with the bacterial wall (e.g., protein adsorption in cellulosomes) or occurs freely with proteases being excreted into the rumen (Velásquez *et al.*, 2016). The principal bacterial strains exhibiting proteolytic activity include *Prevotella sp., Ruminobacter amylophilus, Butyrivibrio fibrisolvens, Streptococcus bovis, Selenomonas ruminantium*, and *Megasphaera elsdenii* (Nagaraja, 2016). Additionally, species such as *Eubacterium, Fusobacterium,* and *Lachnospira multipara* have shown some proteolytic activities with an overall relevant contribution (Hartinger *et al.*, 2018).

Peptidolysis is distinct from proteolysis as it targets smaller peptide chains rather than whole proteins, necessitating a different set of enzymes (Tan *et al.*, 2021). *Prevotella* exhibits a diverse proteolytic and even more pronounced peptidolytic activity with a wide range of peptidases (Henderson *et al.*, 2015). Its abundance in the rumen of lactating cows accounted for 42–60% of total bacteria (Stevenson *et al.*, 2007). Bacteria such as *Streptococcus bovis*, *Ruminobacter amylophilus, Lachnospira multipara, Fibrobacter succinogenes*, and *Eubacterium ruminantium* display mild peptidolytic activity, presenting a minor contribution to ruminal peptidolysis (Wallace *et al.*, 1991). Moreover, *Megasphaera elsdenii*, despite lacking peptidase activity, plays a crucial role in converting dipeptides to AA (Rychlik *et al.*, 2002; Wallace *et al.*, 1991).

Deamination is probably the most common mode of AA catabolism and almost all proteolytic bacteria are involved in deamination (Nagaraja, 2016). As minimal amounts of free AA are found in rumen, it is suggested that they undergo quick degradation (Nagaraja, 2016). Consequently, only a limited amount of AA is directly utilized for microbial protein synthesis (Hartinger *et al.*, 2018). Observations of low-abundance bacteria with high deaminating capacity (Chen *et al.*, 1989) suggest that ruminal AA deamination involves two bacterial fractions. The first one constitutes bacteria present in a high number with low or moderate deaminating activity of about 10–20 nmol NH_3/min/mg protein (Wallace, 1996). This fraction includes *Butyrivibrio fibrisolvens*, *Prevotella ruminicola,* and *Megasphaera elsdenii* (Rychlik

et al., 2002), underlining their pivotal role in ruminal N metabolism. The second fraction of deaminating rumen microorganisms comprises bacteria present in small numbers but exhibiting high deaminating activities exceeding 300 nmol NH_3/min/mg protein (Wallace, 1996). Bacteria such as *Peptostreptococcus anaerobius*, *Clostridium sticklandii*, *Clostridium aminophilum*, and *Fusobacterium necrophorum* are among the bacteria that are hyperammonia producers (Nagaraja, 2016), as they hydrolyze short peptides and deaminate AA. While NH_3 is crucial for cellulolytic rumen microbes (Russell *et al.*, 1992), an excess resulting from high deamination by hyperammonia producers can lead to poor ruminal N utilization efficiency and significant N losses (Patra *et al.*, 2014).

It is believed that the rapid urea hydrolysis in the rumen is primarily caused by urease activity of epimural bacteria; adherent bacteria found in ruminal wall (Nagaraja, 2016). There is no report of ciliated protozoa or fungi showing capability to break down urea. However, NH_3 a product of urea hydrolysis, serves as an important N source for most rumen microorganisms. This makes the breakdown of NPN sources, like urea, nitrates, or nucleic acids, beneficial for ruminal fermentation. Despite the critical role of ureolytic microbes, many of them remain unidentified (Hartinger *et al.*, 2018; Nagaraja, 2016).

Despite the representation of ruminal bacteria in the degradation of nutrients within the rumen, gaps in the understanding of microbial interactions and species identification remain. Traditional anaerobic cultivation methods attempeted to recreate the conditions of the rumen environment to culture all microbial species. However, due to the challenge of reproduce ruminal conditions it might have favored organisms with lower oxygen sensitivity when transferring rumen fluid from the animal to laboratory chambers (Matthews *et al.*, 2019). More recently, with the advent of sequencing technologies and the integration of bioinformatics, advancements in ruminal microbiota research initiated (Matthews *et al.*, 2019), offering deeper insights about these microbial species. Even so, the continued development and application of advanced omics can improve understanding on these complex microbial communities and their biochemical activities which might lead to advancements in N utilization efficiency.

3.2 Protozoa

Due to its size, protozoa accounts for up to 50 % of ruminal microbial biomass (Solomon *et al.*, 2020). Protozoa are mainly present in the rumen fluid phase and play an important role in protein and carbohydrates degradation. Once they are chemoattracted to released nutrients (Diaz *et al.*, 2014), rumen protozoa rely on live bacteria for nutrients necessary for life and growth (Park *et al.*, 2017). Instead of forming a complex with protein, protozoa act engulfing

large molecules, protein, carbohydrates or even ruminal bacteria, fungi and smaller protozoa (Belanche, A *et al.*, 2012), which are then digested inside the cell. Protein digestion releases peptides, which are broken down into free AA that are then incorporated into protozoal protein (NRC, 2001). Although protozoa also deaminate AA, they are unable to use NH_3 for the synthesis of new AA, consequently, a fraction of previously engulfed insoluble protein is later returned to the rumen fluid in the form of soluble protein, which become available in the rumen through cellular autolysis of these microorganisms (Dijkstra, 1994).

Entodinium accounts for more than 86% of total protozoa, following by *Dasytricha* and *Isotricha*, at a much lower relative abundance (Park *et al.*, 2019). They present considerable inter-species differences in their protease profiles (Lockwood *et al.*, 1988). Additionally, *Vestibuliferida* protozoa have numerous proteases that allow them to digest both insoluble and soluble proteins (Jouany, 1996). Cellulolytic protozoa, on the other hand, had the lowest proteolytic activity (Coleman, 1983). *Dasytricha ruminantium* exhibit significant proteolytic activity, accounting for up to 34% of protozoal cells and potentially impacting dietary protein breakdown and bacterial turnover (Belanche, A *et al.*, 2012). *Polyplastron multivesiculatum* had modest proteolytic activity and accounted for 10-20% of the overall protozoal population (Ushida *et al.*, 1985). The proteolytic activity of *Isotricha* and *Ophryoscolex caudatus* was lower, whereas *Epidinium caudatum ecaudatum* was higher (Lockwood *et al.*, 1988). Specifically, *Entodinium* species and *D. ruminantium*, play an important role in dipeptide breakdown (Newbold *et al.*, 1989). Furthermore, protozoa demonstrated minimal deamination and no urease activity (Hartinger *et al.*, 2018).

Due to the low passage rate of these microorganisms to the intestine, they contribute minimally to the flow of MCP to the intestine, approximately 10 to 30% of the total CP flow (Sylvester *et al.*, 2005). Additionally, because of the predation of other microbes, protozoa are considered to be prejudicial for ruminant N utilization efficiency (Hartinger *et al.*, 2018) and thus its overall contribution to protein metabolism needs to be further explored.

3.3 Fungi

Fungi account for approximately 10% of rumen microbial biomass (Krause *et al.*, 2013) and are important fiber degraders (Paul *et al.*, 2004), especially when ruminants are fed low-quality forages (Paul *et al.*, 2004). Protease activity was mostly cell-associated in *Neocallimastix patriciarum* and *Piromyces communis*, but extracellular in *Orpinomyces joyonii* (Yanke *et al.*, 1993). Rumen fungi had limited ability to breakdown proteins, however, numerous fungal isolates exhibited endo- and exopeptidase activity (Michel *et al.*, 1993),

suggesting that ruminal peptidolysis may be promoted by fungi. Furthermore, increased fungal variety during the increase in protein supply to dairy cows (Belanche, Alejandro *et al.*, 2012) may imply that ruminal fungus benefits from high protein inclusion. Moreover, while fungi may not be major players in protein degradation compared to bacteria, their presence and activities, hint at a nuanced role in ruminal protein metabolism. Thus, a better understanding about the mechanisms by which fungi contribute to protein degradation in the rumen is still necessary to stablish their significance in the overall protein metabolism.

3.4 Microbial Interactions

Reciprocal interactions between N compound-degrading rumen microorganisms occur, as distinct proteolytic bacteria grow better together than alone (Wallace, 1985). *Cl. aminophilum* and *Peptostreptococcus anaerobius* produced significantly greater amounts of NH_3 when cultivated in the presence of peptidolytic *Prevotella ruminicola* or *Prevotella bryantii* (Madeira *et al.*, 1997), showing their dependence on peptidolytics. Although excessive deamination is thought to be deleterious to efficient N utilization (Russell *et al.*, 1992), deaminating bacteria and links between cellulolytic and deaminating rumen microorganisms are required. Once potentially degradable proteins are protected by structural polysaccharides it becomes available to proteolytic bacteria via cellulose breakdown (Debroas *et al.*, 1993), thus proteolytic activities are used by bacteria such as *Streptococcus bovis* to get access to starch granules enclosed by protein matrices (Griswold *et al.*, 1999). This interaction highlights the beneficial interplay among microorganisms and their dependency, where the substrate being fermented may affect the predominant microbial population and modify subsequent degradations (Bach *et al.*, 2005).

Protozoa predate bacteria (Belanche, A *et al.*, 2012), but by digesting insoluble food proteins, protozoa encourage the formation of peptidolytic and deaminating bacteria, which use peptides and AA from protozoal proteolysis. As a result, protozoa may raise the deaminating activity of hyperammonia (Firkins, J. *et al.*, 2007) and hence decrease the efficiency of N utilization in the rumen by two mechanisms: bacterial and fungal predation and release of AA into the rumen. Similar interaction patterns have been observed between peptidolytic or deaminating bacteria and proteolytic fungi that produce N compounds from protein degradation (Wallace *et al.*, 1985). Rumen fungus may also play a role, as they breakdown cell wall components (Gordon *et al.*, 1998), allowing access to enclosed proteins. According to (Dehority *et al.*, 2000), fungi and bacteria have a general negative interaction in which each create inhibitory compounds to hinder the growth of the other. As a result, the presence, type, and

magnitude of the interaction between fungi and bacteria may differ between species or even strains (Bernalier *et al.*, 1991) and must be assessed individually.

Protein degradation by microorganisms is affected by multiple factors, such as the nature and solubility of the protein, its interactions with other nutrients, and the prevailing microbial population. Therefore, the microbial population, relies on the specific characteristics of the ration, passage rate, and rumen pH levels (Bach *et al.*, 2005). These authors hypothesize that a decrease in pH leads to a decline in cellulolytic bacteria, resulting in a reduction in fiber degradation. Consequently, this reduction limits the access of proteolytic bacteria to proteins, indirectly diminishing protein degradation. Moreover, cellulolytic bacteria are significantly impaired when there is a deficiency of RDP in the rumen, leading to reduced disappearance of fibrous carbohydrates, consequently decreasing the passage rate and, subsequently, dry matter intake (Russell *et al.*, 1992).

The efficiency of microbial growth is detrimental for ruminants (Russell *et al.*, 1992). As already stated, MCP is the major component of MP (NASEM, 2021), composed by an average of 82.4% AA (Sok, M *et al.*, 2017), with an approximate digestibility of 80% (NRC, 2001). Furthermore, the EAA profile of MCP is similar to that of milk and muscles (Schwab *et al.*, 2017) making it particularly important especially for high producing dairy cows, as casein is one of the main proteins found in milk. Nevertheless, rumen microbiota composition may be important for animal performance, as there is evidence of rumen microorganisms' ability to alter milk quality parameters (Jami *et al.*, 2014). The authors discovered strong relationships between milk fat production and the *Firmicutes* to *Bacteroidetes* ratio, which remained at the genus level (Jami *et al.*, 2014). Thus, this fact underlines the impact of rumen microbes on host physiology, and hence the significance of altering microbial composition and activity to improve nutrition and energy utilization (Hartinger *et al.*, 2018). In this sense, further understanding of interactions among rumen microbes, and how it may affect protein degradation and MCP synthesis would optimize overall protein nutrition and efficient N utilization.

4 RUP on AA Supply

As discussed previously, RUP bypasses ruminal hydrolysis, providing a direct source of AA to the animal through digestion in the small intestine (Putri *et al.*, 2021). It contributes to MP supplying AA necessary for tissue maintenance, growth (Silva, A. *et al.*, 2018), and production (Flis *et al.*, 2005). However, protein feedstuffs vary widely in their CP content and AA profile. Solvent extracted soybean meal is the most used protein source for dairy cows due

to its content of CP, which is approximately 52.6% of DM (NASEM, 2021). Additionally, it presents approximately 67% of RDP, % CP along with 6.16% of Lysine and 1.38% of Methionine (NASEM, 2021). However, as it is highly degraded ruminally, its profile is altered before reaching the small intestine. A widely used alternative to decrease its ruminal degradation is through heat process, which modifies the structure of the proteins, making them less degradable in the rumen (Mass *et al.*, 2000). This is the case with expeller soybean meal, which involves pressing the soybeans to remove the oil, producing heat, and increasing the RUP content. Consequently, more protein is protected from ruminal degradation, and makes it a valuable feed option for providing readily available AA for absorption in the small intestine. While expeller soybean meal still possesses a substantial amount of crude protein (47.6% of DM) (NASEM, 2021), the critical advantage is its improved lysine and methionine profile after rumen passage (Castro *et al.*, 2007). Castro *et al.* (2007) found that availability of lysine in soybean meal expellers were 65.6 % of original feed, which was higher than values found for solvent extracted soybean meal; 37.7% of original feed. Regarding Methionine, the same study showed an availability of 67.8 % of original feed for expeller soybean meal versus 31.3% of original feed for solvent extracted soybean meal (Castro *et al.*, 2007). This fact makes expeller soybean meal an attractive choice for high-producing dairy cows that have higher requirements for essential AA to support their milk production. Further, Distillers Dried Grains (DDG) and Distillers Dried Grains with Solubles (DDGS) are common byproducts utilized in ruminant nutrition. While they present greater RUP contents 47% RUP, % CP when compared with soybean meal, their amino acid profile tends to be vary between batches, with a notably deficient lysine content (2.81% CP) (NASEM, 2021). Thus, relying solely on DDG or DDGS as the primary protein source may not meet the precise amino acid requirements of lactating dairy cows. Moreover, animal-derived RUP sources like blood meal and feather meal also contribute significantly to dietary protein. Blood meal, characterized by its elevated lysine levels (8.77% CP) (NASEM, 2021), and can be considered option for addressing specific amino acid deficiencies in ruminant diets.

Nonetheless, in diet formulation approaches, the AA profile in RUP is commonly assumed to be the same as in the original feedstuff, while the intestinal digestibility of the RUP fraction is considered to be the same as the CP digestibility for all AA (NASEM, 2021). The issue is based on the lack of data available on AA disappearance *in situ*. Consequently, diets formulated solely based on CP content without considering the appropriateness of the AA profile may pose a risk of providing an imbalanced AA supply, especially if the feed exhibits a high RUP content but lacks the necessary AA profile to meet the animal's requirements. Then,

as the digestibility of RUP varies considerably among different feedstuffs, it directly impacting AA availability for absorption (White *et al.*, 2017). This variability not only influences N utilization efficiency but also raises concerns about increased N excretion in feces, contributing to environmental issues related to N excretion. Therefore, to ensure consistency and comparability across studies, it becomes imperative to provide information on the degradability and intestinal digestibility of total AA, (White *et al.*, 2017), enabling a more accurate prediction of total AA availability.

5 Nitrogen Metabolism in Ruminants

In summary, RDP undergoes hydrolysis by rumen microorganisms, leading to the production of NH_3, which is utilized for the synthesis of MCP. Conversely, RUP bypasses ruminal degradation. When NH_3 levels exceed the MCP synthesis requirements, its excess is absorbed across the rumen epithelium into the bloodstream. Similarly, NH_3 generated from AA catabolism in body's tissues will be released into the animals' circulation. As NH_3 is toxic for the organism, once in the bloodstream it is transported to the liver where it is converted into urea through the urea cycle or alternatively into glutamine. Then, urea will be excreted in urine or milk. A comprehensive representation of the protein metabolism in ruminants is illustrated in (Figure 1).

During low N supply, blood urea can return to rumen via saliva or ruminal epithelium, a process known as urea recycling. The mechanism of urea recycling in ruminants is an evolutionary advantage, but also optimizes N utilization while minimizing environmental N excretion (Batista *et al.*, 2017). The recycling process begins with the entry of urea produced in the liver into the rumen, which can occur through saliva or directly from the bloodstream through the ruminal wall (Calsamiglia *et al.*, 2010). In the rumen, recycled urea is again hydrolyzed to NH_3 and made available for microbial use. Approximately 15 to 40% of the total N intake can potentially be recycled (Lapierre *et al.*, 2001), thus enhancing the efficiency of N use.

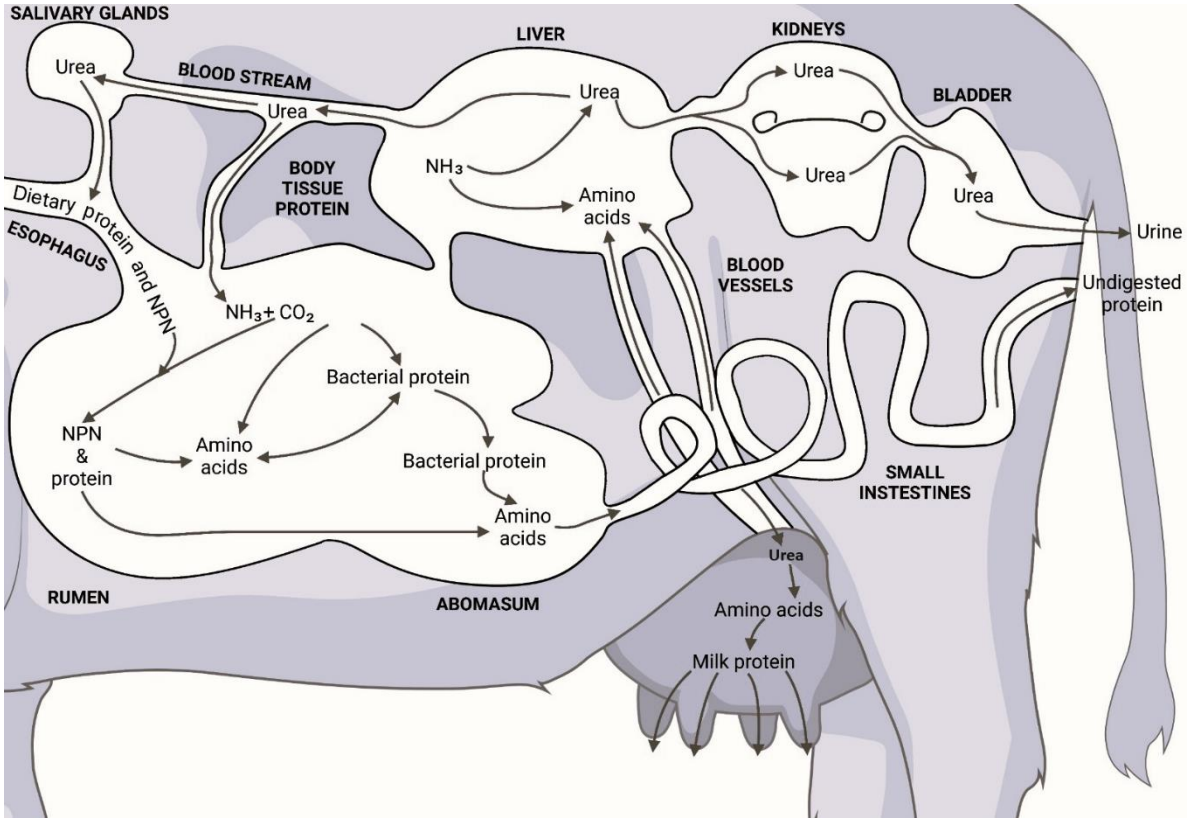


Figure 1. Dynamics of protein metabolism in ruminants. Adapted from J. Bryant and B. R., Moss Montana State University).

5.1 Hepatic urea synthesis and urea transport across tissues

As previously mentioned, NH_3 is a toxic compound for ruminants. Its interaction with hemoglobin forms methemoglobin, which has low oxygen transport efficiency. Hence, it must be removed by the liver before reaching the heart and other organs, including the central nervous system (Silva *et al.*, 2019). In the liver, specifically in the mitochondria of the hepatocytes, NH_3 is converted to urea through ureagenesis by the ornithine cycle (urea cycle) (Reynolds *et al.*, 2008). The liver can extract 70 to 95% of portal NH_3 (Tan *et al.*, 2004); which can be taken up through periportal or perivenous hepatocytes. The direct uptake of NH_3 by periportal hepatocytes is a "low-affinity/high-capacity" system, meaning that NH_3 can potentially escape extraction due to an NH_3 influx entering the liver (Watford, 2003). Additionally, periportal hepatocytes remove glutamine from circulation that will be further degraded to NH_3 (Watford, 2003). Since excessive amounts of NH_3 can saturate the periportal hepatocyte system, NH_3 can reach the perivenous hepatocytes. The perivenous hepatocytes extract NH_3 for glutamine synthesis (Watford, 2003), promoting another opportunity for NH_3 removal from the bloodstream. Thus, glutamine plays a crucial role as N transporter in the body. This process not only removes NH_3 from the blood, but also provides N for synthesis of new

proteins and other essential nitrogenous compounds. Detoxification of NH_3 in perivenous hepatocytes is a "high-affinity/low-capacity" system that prevents NH_3 from entering the peripheral circulation, where it can cause toxicity. Since the main pathway for NH_3 detoxification is via ureagenesis in periportal hepatocytes, there is a large output of urea from the liver, in contrast glutamine concentrations remain relatively unchanged (Watford, 2003).

Following synthesis in the liver, endogenous urea can have different fates in ruminants. Blood circulates throughout the body and urea is simultaneously taken up by various organs, which can occur via gradient concentration or by specific transport mechanisms. Once urea is a small water-soluble molecule, it is easily excreted by the kidneys through urine and by the mammary gland via milk (Powell *et al.*, 2014). However, because it is highly polar, urea has low permeability through lipid bilayers and is thus transported carrier-mediated across the epithelium (Abdoun *et al.*, 2006). Proteins facilitating this uptake are expressed in several organs, including the kidney (Bankir *et al.*, 2000; Klein *et al.*, 2011; Weiner *et al.*, 2015), mammary glands, rumen epithelium (Bankir *et al.*, 2004; Zhong *et al.*, 2022), salivary glands (Ludden *et al.*, 2009) and colon (Ludden *et al.*, 2009).

From 10 to 95% of endogenously produced urea can be secreted into the gastrointestinal tract (GIT) (Lobley *et al.*, 2000). This transit occurs via two pathways: 1) secretion in saliva; and 2) direct transfer from the bloodstream through the ruminal wall (Reynolds *et al.*, 2008). Salivary urea secretion can account for 3 to 20% of urea secreted into the GIT (Zhou *et al.*, 2017) and may be influenced by the dietary forage contents (Lapierre *et al.*, 2001). Facilitative transporters known as urea transporters (UT), specifically UT-B in the ductal system of salivary glands, drive this mechanism, showing a strong correlation with BUN levels (Muscher *et al.*, 2010). In fact, previous studies have shown that the saturation threshold of this transporter is between 65 to 74% of BUN in goats (Harmeyer *et al.*, 1980; Muscher *et al.*, 2010) and sheep (Cirio *et al.*, 2000). Consequently, the main pathway for urea secretion into the GIT is directly through the ruminal wall (Zhong *et al.*, 2022) by epithelial membrane transporters. These transporters enable urea passage through cell membranes, moving passively toward a concentration gradient (Zhong *et al.*, 2022). A comprehensive literature review has shown that urea transfer across the ruminal epithelium may involve UT and aquaporins (AQP) (Stewart *et al.*, 2005; Walpole *et al.*, 2015; Zhong *et al.*, 2022). UT-B transporter is located in all layers of the ruminal epithelial stratum, except the stratum corneum in cattle (Coyle *et al.*, 2016). Additionally, AQP responsible for transporting water across cell membranes, can facilitate solute movements, including urea (Zhong *et al.*, 2022). Once urea has been transported from the blood to the GIT, it is degraded by epimural bacteria into NH_3 and CO_2 (Stewart *et al.*,

2005). Again, the formed NH_3 can be incorporated into MCP and subsequently absorbed in the small intestine as AA. If this pathway occurs, AA will flow into the bloodstream where it can be used in anabolism or catabolism. AA catabolism will generate NH_3 that will flow to liver by circulation, where it will be converted to urea and released to bloodstream. Contrastingly, if NH_3 from recycling is not incorporated into MCP, it is conducted to the bloodstream and reaches the liver where it is also converted to urea and released to bloodstream, which offers another possibility for recycling.

In the kidneys, the volume and composition of urine are primarily influenced by the renin-angiotensin system, which is regulated by a decrease in blood volume, blood pressure, or renal filtration rate (Hoorn *et al.*, 2020). Additionally, a range of hormones such as aldosterone and antidiuretic hormone (ADH) perform water reabsorption depending on osmotic pressure. Then, urea uptake is also related to UT, which are influenced by ADH, apical expression of UT-A1 and UT-A3 (SLC14A2) in the terminal collecting duct (Klein *et al.*, 2011). UT-B1 is found in erythrocytes and along the descending renal vasa recta, accumulating urea from the bloodstream. Thus, UT-B1 participates in maintaining osmotic balance between BUN and interstitium and enables intrarenal urea recycling (Bankir *et al.*, 2000; Weiner *et al.*, 2015). However, whether UT-A2 and UT-B1 are independent or dependent on ADH remains unclear (Sands *et al.*, 2009; Weiner *et al.*, 2015). Generally, between 30 and 50% of primarily ultrafiltered urea is excreted in urine (Klein *et al.*, 2011; Weiner *et al.*, 2015). Furthermore, dietary N restriction increases renal urea recovery, increasing the chance of reabsorbed urea being recycled to the rumen and consequently reused for MCP synthesis, enhancing animal N efficiency (Røjen *et al.*, 2011; Starke *et al.*, 2012).

In mammary glands, BUN is secreted with milk, resulting in milk urea N (MUN). Once MUN is highly correlated with BUN (R^2 = 0.84; (Broderick *et al.*, 1997) it is commonly used as an indicator of dietary protein intake. Gustafsson *et al.* (1993) described that BUN peak occurs 1.5 to 2 hours after the peak of ruminal NH_3. Consequently, MUN concentration seems to reflect the level of ingested N exceeding ruminal microbial N incorporation, and thus ruminal NH_3 concentration, favoring hepatic urea synthesis (Broderick *et al.*, 1997). Differences between MUN concentration of cows presenting the same milk production volume and consuming the same diet exists and may be related to smaller kidney size (Marini *et al.*, 2004) and lower renal urea excretion capacity. Additionally, since no differences were found in hepatic metabolism of cows with high and low MUN concentrations (Prahl *et al.*, 2022), a lower abundance of ureolytic bacteria in the rumen of cows excreting more urea (Honerlagen *et al.*, 2022) would be one of the reasons for this phenomenon.

The diffusion of urea through mammary ducts, from plasma to milk and vice versa, follows a daily dynamic in response to food intake and milking frequency (Spek *et al.*, 2016). A recent study aimed to investigate mRNA expression of genes encoding urea transporters in mammary gland; however, no mammary gland urea transporter was identified to explain divergent MUN concentrations, indicating that higher milk urea concentrations are predominantly driven by higher BUN (Prahl *et al.*, 2023) as previously thought (Broderick *et al.*, 1997).

Regarding the intestinal epithelium, mRNA expression of UT-B was found in the colon of sheep (Ludden *et al.*, 2009) could be the underlying mechanism responsible for urea transport in this region. Through infusions of carbohydrates into the ileum of sheep, it was observed an increase in fecal N excretion and a decrease in urine N excretion (Oncuer *et al.*, 1990; Ørskov *et al.*, 1970; Thornton *et al.*, 1970). However, microbial protein produced in the large intestine cannot contribute anabolically to the animal due to the inability to digest protein and absorb the constituent AA in these regions (Lapierre *et al.*, 2001). Then, even without an anabolic benefit to ruminants, the ability of a ruminant to recycle N to the final portions of the intestine can reduce N excretion to the environment. Secretion of urea to the hindgut will result in the production of hydrolyzed N-NH_3, which can be used for microbial protein synthesis (Oncuer *et al.*, 1990; Ørskov *et al.*, 1970; Thornton *et al.*, 1970). On the other hand, urinary urea N excreted into the environment is rapidly volatilized. Thus, microbial N excreted in feces is organically bound, being less labile compared to urea N (Thornton *et al.*, 1970), and therefore may reduce the environmental footprint of ruminant production systems (Abdoun *et al.*, 2006).

6 N Efficiency and Excretion

The balance of energy and N in the diet, are factors that impact N efficiency, as the degradation of protein is highly dependent on the presence of fermentable energy in the rumen and the end products of the protein degradation (peptides, AA, and NH_3-N), which are incorporated into microbial protein. Moreover, recycling is particularly evident under N limiting conditions, such as when animals are fed low CP diet, or low RDP diets. Under these circumstances, microbial fermentation in the rumen will use this N source for MCP synthesis, which decreases N excretion while increasing N efficiency, all without any adverse impacts on milk production (Bahrami-Yekdangi *et al.*, 2016; Kaufman *et al.*, 2017).

N losses in dairy cows represent a major point of inefficiency within dairy production systems, with significant implications for environmental sustainability and profitability. These losses are broadly categorized into pre-absorptive and post-absorptive, each originating from

different stages of the digestive process and carrying distinct environmental footprints. Pre-absorptive losses are primarily associated with N compounds in the rumen that are not incorporated into MCP or absorbed in the small intestine. This includes ruminal NH_3 surplus as well as AA from MCP, RUP, and endogenous proteins that are not absorbed and thus excreted via feces. Post-absorptive losses, on the other hand, encompass AA absorbed by the small intestine but not utilized for milk protein synthesis or other productive purposes by the mammary glands or various body tissues, which are then catabolized and excreted in urine. These losses represent the bulk of N inefficiency in dairy cows and are notably the least studied (Pszczolkowski *et al.*, 2020). N excretion in ruminants is a byproduct of protein digestion and metabolism that has direct implications for both farm productivity and the environment. When animals excrete urea through urine, it rapidly hydrolyzes into NH_3, which can convert to form NH_4^+ in the atmosphere. Those process plays a role in the generation numerous volatile or aqueous compounds, such as nitrous oxide (N_2O), nitric oxide (NO), N dioxide (NO_2), nitrite ions (NO_2^-), and nitrate ions (NO_3^-) (Beeckman *et al.*, 2018). N_2O acts as a potent greenhouse gas and contributes to carbon footprint of milk (Hristov, 2023). These N forms can also undergo transformations into other reactive N types and subsequently enter the terrestrial environments via leaching (Beeckman *et al.*, 2018), impacting watersheds, aquifers, and other water sources. In this context, enhancing N efficiency presents a step towards both excellence in animal production and global ecological integrity.

Thus, we hypothesized that altering the proportion of MP in a fixed CP level would alter N utilization patterns, ultimately leading to changes in AA absorption and N excretion.

Therefore, our goal is to understand how the supply of 3 different dietary proportions of MP can affect: 1) ruminal fermentation, 2) AA entry rates and absorption, and 3) urea-N metabolism and excretion.

The present work will result in Chapter 2, entitled **"Nitrogen utilization and rumen fermentation dynamics of Holstein heifers fed different proportions of metabolizable protein"**, which will be submitted to the **"Journal of Dairy Science".**

REFERENCES

ABDOUN, Khalid; STUMPFF, Friederike; MARTENS, Holger. Ammonia and urea transport across the rumen epithelium: a review. Animal Health Research Reviews, v. 7, n. 1-2, p. 43-59, 2006. https://doi.org/10.1017/s1466252307001156

ARRIOLA APELO, S. I.; BELL, A. L.; ESTES, K.; ROPELEWSKI, J. *et al.* Effects of reduced dietary protein and supplemental rumen-protected essential amino acids on the nitrogen efficiency of dairy cows. **Journal of Dairy Science**, 97, n. 9, p. 5688-5699, 2014. https://doi.org/10.3168/jds.2013-7833

BACH, A.; CALSAMIGLIA, S.; STERN, M. D. Nitrogen metabolism in the rumen. **Journal of dairy science**, 88, p. E9-E21, 2005. https://doi.org/10.3168/jds.S0022-0302(05)73133-7

BAHRAMI-YEKDANGI, M.; GHORBANI, G.; KHORVASH, M.; KHAN, M. *et al.* Reducing crude protein and rumen degradable protein with a constant concentration of rumen undegradable protein in the diet of dairy cows: Production performance, nutrient digestibility, nitrogen efficiency, and blood metabolites. **Journal of animal science**, 94, n. 2, p. 718-725, 2016. https://doi.org/10.2527/jas.2015-9947

BANKIR, Lise. Active urea transport in lower vertebrates and mammals. Urea Transporters, p. 193-226, 2014. https://doi.org/10.1007/978-94-017-9343-8_13

BATISTA, E.; DETMANN, E.; VALADARES FILHO, S.; TITGEMEYER, E. *et al.* The effect of CP concentration in the diet on urea kinetics and microbial usage of recycled urea in cattle: a meta-analysis. **Animal**, 11, n. 8, p. 1303-1311, 2017. https://doi.org/10.1017/S1751731116002822

BEECKMAN, F.; MOTTE, H.; BEECKMAN, T. Nitrification in agricultural soils: impact, actors and mitigation. **Current opinion in Biotechnology**, 50, p. 166-173, 2018. https://doi.org/10.1016/j.copbio.2018.01.014

BELANCHE, A.; DE LA FUENTE, G.; MOORBY, J.; NEWBOLD, C. J. Bacterial protein degradation by different rumen protozoal groups. **Journal of animal science**, 90, n. 12, p. 4495-4504, 2012. https://doi.org/10.2527/jas.2012-5118

BELANCHE, A.; DOREAU, M.; EDWARDS, J. E.; MOORBY, J. M. *et al.* Shifts in the rumen microbiota due to the type of carbohydrate and level of protein ingested by dairy cattle are associated with changes in rumen fermentation. **The Journal of nutrition**, 142, n. 9, p. 1684-1692, 2012. https://doi.org/10.3945/jn.112.159574

BEQUETTE, B.; SUNNY, N.; EL-KADI, S.; OWENS, S. Application of stable isotopes and mass isotopomer distribution analysis to the study of intermediary metabolism of nutrients. **Journal of animal science**, 84, n. suppl_13, p. E50-E59, 2006. https://doi.org/10.2527/2006.8413_supple50x

BERNALIER, A.; FONTY, G.; GOUET, P. Cellulose degradation by two rumen anaerobic fungi in monoculture or in coculture with rumen bacteria. **Animal Feed Science and Technology**, 32, n. 1-3, p. 131-136, 1991. https://doi.org/10.1016/0377-8401(91)90016-L

BRODERICK, G.; WALLACE, R.; ØRSKOV, E. Control of rate and extent of protein

degradation. *In*: **Physiological aspects of digestion and metabolism in ruminants**: Elsevier, 1991. p. 541-592. https://doi.org/10.1016/B978-0-12-702290-1.50030-8

BRODERICK, G. A.; REYNAL, S. M. Effect of source of rumen-degraded protein on production and ruminal metabolism in lactating dairy cows. **Journal of Dairy Science**, 92, n. 6, p. 2822-2834, 2009. https://doi.org/10.3168/jds.2008-1865

CALDER, A. G.; GARDEN, K. E.; ANDERSON, S. E.; LOBLEY, G. E. Quantitation of blood and plasma amino acids using isotope dilution electron impact gas chromatography/mass spectrometry with U-13C amino acids as internal standards. **Rapid Communications in Mass Spectrometry**, 13, n. 21, p. 2080-2083, 1999. https://doi.org/10.1002/(sici)1097-0231(19991115)13:21%3C2080::aid-rcm755%3E3.0.co;2-o

CALSAMIGLIA, S.; FERRET, A.; REYNOLDS, C.; KRISTENSEN, N. B. *et al.* Strategies for optimizing nitrogen use by ruminants. **Animal**, 4, n. 7, p. 1184-1196, 2010. https://doi.org/10.1017/S1751731110000911

CAMACHO, L. F.; DA SILVA, T. E.; RODRIGUES, J. P. P.; FRANCO, M. D. O. *et al.* A Standard Procedure for In Vitro Digestion Using Rumen Fermenters: A Collaborative Study. **Animals**, 12, n. 20, p. 2842, 2022. https://doi.org/10.3390/ani12202842

CASTRO, S. B.; PHILLIP, L.; LAPIERRE, H.; JARDON, P. *et al.* Ruminal degradability and intestinal digestibility of protein and amino acids in treated soybean meal products. **Journal of Dairy Science**, 90, n. 2, p. 810-822, 2007. https://doi.org/10.3168/jds.s0022-0302(07)71565-5

CHANEY, A. L.; MARBACH, E. P. Modified Reagents for Determination of Urea and Ammonia. **Clinical Chemistry**, 8, n. 2, p. 130-132, 1962. https://doi.org/10.1093/clinchem/8.2.130

CHEN, G.; RUSSELL, J. More monensin-sensitive, ammonia-producing bacteria from the rumen. **Applied and environmental microbiology**, 55, n. 5, p. 1052-1057, 1989. https://doi.org/10.1128/aem.55.5.1052-1057.1989

CHOLEWIŃSKA, P.; CZYŻ, K.; NOWAKOWSKI, P.; WYROSTEK, A. The microbiome of the digestive system of ruminants – a review. **Animal Health Research Reviews**, 21, n. 1, p. 3-14, 2020. https://doi.org/10.1017/s1466252319000069

CIRIO, A. et al. Determination of parotid urea secretion in sheep by means of ultrasonic flow probes and a multifactorial regression analysis. Journal of animal science, v. 78, n. 2, p. 471-476, 2000. https://doi.org/10.2527/2000.782471x

CLARK, J. H. Lactational responses to postruminal administration of proteins and amino acids. **Journal of Dairy Science**, 58, n. 8, p. 1178-1197, 1975. https://doi.org/10.3168/jds.S0022-0302(75)84696-0

COLEMAN, G. Hydrolysis of fraction 1 leaf protein and casein by rumen entodiniomorphid protozoa. **Journal of applied bacteriology**, 55, n. 1, p. 111-118, 1983. https://doi.org/10.1111/j.1365-2672.1983.tb02654.x

COYLE, J.; MCDAID, S.; WALPOLE, C.; STEWART, G. S. UT-B urea transporter

localization in the bovine gastrointestinal tract. **The Journal of membrane biology**, 249, p. 77-85, 2016. https://doi.org/10.1007/s00232-015-9850-5

DEBROAS, D.; BLANCHART, G. Interactions between proteolytic and cellulolytic rumen bacteria during hydrolysis of plant cell wall protein. **Reproduction Nutrition Development**, 33, n. 3, p. 283-288, 1993. https://doi.org/10.1051/rnd:19930310

DEHORITY, B. A.; TIRABASSO, P. A. Antibiosis between ruminal bacteria and ruminal fungi. **Applied and Environmental Microbiology**, 66, n. 7, p. 2921-2927, 2000. https://doi.org/10.1128%2Faem.66.7.2921-2927.2000

DIAZ, H.; BARR, K.; GODDEN, K.; PLANK, J. *et al*. Eukaryotic inhibitors or activators elicit responses to chemosensory compounds by ruminal isotrichid and entodiniomorphid protozoa. **Journal of Dairy Science**, 97, n. 4, p. 2254-2269, 2014. https://doi.org/10.3168/jds.2013-7698

DIETZEN, D. J. Amino acids, peptides, and proteins. *In*: **Principles and Applications of Molecular Diagnostics**: Elsevier, 2018. p. 345-380. https://doi.org/10.1016/B978-0-12-816061-9.00013-8

DIJKSTRA, J. Simulation of the dynamics of protozoz in the rumen. **British Journal of Nutrition**, 72, n. 5, p. 679-699, 1994. https://doi.org/10.1079/BJN19940071

ESTES, K. A.; WHITE, R. R.; YODER, P. S.; PILONERO, T. *et al.* An in vivo stable isotope–based approach for assessment of absorbed amino acids from individual feed ingredients within complete diets. **Journal of Dairy Science**, 101, n. 8, p. 7040-7060, 2018. https://doi.org/10.3168/jds.2017-13447

FLIS, S. A.; WATTIAUX, M. A. Effects of parity and supply of rumen-degraded and undegraded protein on production and nitrogen balance in Holsteins. Journal of Dairy Science, v. 88, n. 6, p. 2096-2106, 2005. https://doi.org/10.3168/jds.S0022-0302(05)72886-1

FIRKINS, J.; YU, Z.; MORRISON, M. Ruminal nitrogen metabolism: perspectives for integration of microbiology and nutrition for dairy. **Journal of dairy science**, 90, p. E1-E16, 2007. https://doi.org/10.3168/jds.2006-518

GETAHUN, D.; ALEMNEH, T.; AKEBEREGN, D.; GETABALEW, M. *et al.* Urea metabolism and recycling in ruminants. **Biomed J Sci Tech Res**, 20, n. 1, p. 14790-14796, 2019. http://dx.doi.org/10.26717/BJSTR.2019.20.003401

GORDON, G. L.; PHILLIPS, M. W. The role of anaerobic gut fungi in ruminants. **Nutrition research reviews**, 11, n. 1, p. 133-168, 1998. https://doi.org/10.1079/nrr19980009

GRISWOLD, K. E.; WHITE, B. A.; MACKIE, R. I. Proteolytic activities of the starch-fermenting ruminal bacterium, Streptococcus bovis. **Current microbiology**, 39, p. 180-186, 1999. https://doi.org/10.1007/s002849900442

HANIGAN, M. D.; SOUZA, V. C.; MARTINEAU, R.; DALEY, V. L. *et al.* Predicting ruminally undegraded and microbial protein flows from the rumen. **J Dairy Sci**, 104, n. 8, p. 8685-8707, Aug 2021. https://doi.org/10.3168/jds.2020-19672

HARTINGER, T.; GRESNER, N.; SÜDEKUM, K.-H. Does intra-ruminal nitrogen recycling waste valuable resources? A review of major players and their manipulation. **Journal of animal science and biotechnology**, 9, p. 1-21, 2018. https://doi.org/10.1186/s40104-018-0249-x

HENDERSON, G.; COX, F.; GANESH, S.; JONKER, A. *et al.* Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. **Scientific reports**, 5, n. 1, p. 14567, 2015. https://doi.org/10.1038/srep14567

HONERLAGEN, H.; REYER, H.; SEGELKE, D.; MÜLLER, C. B. M. *et al.* Ruminal background of predisposed milk urea (MU) concentration in Holsteins. **Frontiers in Microbiology**, 13, p. 939711, 2022. https://doi.org/10.3389/fmicb.2022.939711

HOORN, E. J.; GRITTER, M.; CUEVAS, C. A.; FENTON, R. A. Regulation of the renal NaCl cotransporter and its role in potassium homeostasis. **Physiological reviews**, 100, n. 1, p. 321-356, 2020. https://doi.org/10.1152/physrev.00044.2018

HRISTOV, A. N. Perspective: Could dairy cow nutrition meaningfully reduce the carbon footprint of milk production? **Journal of dairy science**, 106, n. 11, p. 7336-7340, 2023. https://doi.org/10.3168/jds.2023-23461

HRISTOV, A. N.; BANNINK, A.; CROMPTON, L. A.; HUHTANEN, P. *et al.* Invited review: Nitrogen in ruminant nutrition: A review of measurement techniques. **Journal of Dairy Science**, 102, n. 7, p. 5811-5852, 2019. https://doi.org/10.3168/jds.2018-15829

HUANG, X.; ESTES, K. A.; YODER, P. S.; WANG, C. *et al.* Assessing availability of amino acids from various feedstuffs in dairy cattle using a stable isotope-based approach. **Journal of Dairy Science**, 102, n. 12, p. 10983-10996, 2019. https://doi.org/10.3168/jds.2019-16597

JAMI, E.; WHITE, B. A.; MIZRAHI, I. Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency. **PloS one**, 9, n. 1, p. e85423, 2014. https://doi.org/10.1371/journal.pone.0085423

JIN, D.; ZHAO, S.; ZHENG, N.; BECKERS, Y. *et al.* Differences in ureolytic bacterial composition between the rumen digesta and rumen wall based on ureC gene classification. **Frontiers in Microbiology**, 8, p. 248317, 2017. https://doi.org/10.3389/fmicb.2017.00385

JONES, D. B. **Factors for converting percentages of nitrogen in foods and feeds into percentages of proteins**. US Department of Agriculture Washington, DC, 1941.

JOUANY, J.-P. Effect of rumen protozoa on nitrogen utilization by ruminants. **The Journal of nutrition**, 126, p. 1335S-1346S, 1996. https://doi.org/10.1093/jn/126.suppl_4.1335s

KAUFMAN, J. D.; KASSUBE, K. R.; RÍUS, A. G. Lowering rumen-degradable protein maintained energy-corrected milk yield and improved nitrogen-use efficiency in multiparous lactating dairy cows exposed to heat stress. **Journal of Dairy Science**, 100, n. 10, p. 8132-8145, 2017. https://doi.org/10.3168/jds.2017-13026

KLEIN, Janet D. Expression of urea transporters and their regulation. Urea Transporters, p. 79-107, 2014. https://doi.org/10.1007/978-94-017-9343-8_6

KRAUSE, D.; NAGARAJA, T.; WRIGHT, A.; CALLAWAY, T. Board-invited review: rumen microbiology: leading the way in microbial ecology. **Journal of animal science**, 91, n. 1, p. 331-341, 2013. https://doi.org/10.2527/jas.2012-5567

LAPIERRE, H.; LOBLEY, G. Nitrogen recycling in the ruminant: A review. **Journal of Dairy Science**, 84, p. E223-E236, 2001. https://doi.org/10.3168/jds.S0022-0302(01)70222-6

LAPIERRE, H.; PACHECO, D.; BERTHIAUME, R.; OUELLET, D. *et al.* What is the true supply of amino acids for a dairy cow? **Journal of dairy science**, 89, p. E1-E14, 2006. https://doi.org/10.3168/jds.S0022-0302(06)72359-1

LI, M. M.; TITGEMEYER, E. C.; HANIGAN, M. D. A revised representation of urea and ammonia nitrogen recycling and use in the Molly cow model. **Journal of Dairy Science**, 102, n. 6, p. 5109-5129, 2019/06/01/ 2019. https://doi.org/10.3168/jds.2018-15947

LOBLEY, G.; BREMNER, D.; ZUUR, G. Effects of diet quality on urea fates in sheep as assessed by refined, non-invasive [15N15N] urea kinetics. **British Journal of Nutrition**, 84, n. 4, p. 459-468, 2000. doi:10.1017/S0007114500001768

LOCKWOOD, B. C.; COOMBS, G. H.; WILLIAMS, A. G. Proteinase activity in rumen ciliate protozoa. **Microbiology**, 134, n. 9, p. 2605-2614, 1988. https://doi.org/10.1099/00221287-134-9-2605

LUDDEN, P. A. et al. Effect of protein supplementation on expression and distribution of urea transporter-B in lambs fed low-quality forage. Journal of animal science, v. 87, n. 4, p. 1354-1365, 2009. https://doi.org/10.2527/jas.2008-1399

MADEIRA, H.; PENG, L.; MORRISON, M. Biochemical and mutational analysis of a gingipain-like peptidase activity from Prevotella ruminicola B (1) 4 and its role in ammonia production by ruminal bacteria. **Applied and environmental microbiology**, 63, n. 2, p. 670-675, 1997. https://doi.org/10.1128%2Faem.63.2.670-675.1997

MARINI, J.; KLEIN, J.; SANDS, J.; VAN AMBURGH, M. Effect of nitrogen intake on nitrogen recycling and urea transporter abundance in lambs. **Journal of animal science**, 82, n. 4, p. 1157-1164, 2004. https://doi.org/10.2527/2004.8241157x

MATTHEWS, Chloe et al. The rumen microbiome: a crucial consideration when optimising milk and meat production and nitrogen utilisation efficiency. Gut microbes, v. 10, n. 2, p. 115-132, 2019. https://doi.org/10.1080%2F19490976.2018.1505176

MAXIN, G.; OUELLET, D. R.; LAPIERRE, H. Ruminal degradability of dry matter, crude protein, and amino acids in soybean meal, canola meal, corn, and wheat dried distillers grains. **Journal of Dairy Science**, 96, n. 8, p. 5151-5160, 2013. https://doi.org/10.3168/jds.2012-6392

MICHEL, V.; FONTY, G.; MILLET, L.; BONNEMOY, F. *et al.* In vitro study of the proteolytic activity of rumen anaerobic fungi. **FEMS Microbiology Letters**, 110, n. 1, p. 5-9, 1993. https://doi.org/10.1111/j.1574-6968.1993.tb06287.x

NAGARAJA, T. Microbiology of the rumen. **Rumenology**, p. 39-61, 2016.

NASEM. **National Academies of Sciences; Medicine**. 2021. 0309677777.

NEWBOLD, C. J.; MCKAIN, N.; WALLACE, R. J. The role of protozoa in ruminal peptide metabolism. **Biochemistry and molecular biology of'anaerobic'protozoa.**, p. 42-55, 1989.

NRC. **National Research Council. Nutrient requirements of dairy cattle: 2001**. National Academies Press, 2001. 0309069971.

NUGENT, J. H.; MANGAN, J. L. Characteristics of the rumen proteolysis of fraction I (18S) leaf protein from lucerne (Medicago sativa L). **British Journal of Nutrition**, 46, n. 1, p. 39-58, 1981. https://doi.org/10.1079/bjn19810007

ONCUER, A.; MILNE, J.; WHITELAW, F. The effect of a hindgut fermentation on urea metabolism in sheep nourished by intragastric infusion. **Experimental Physiology: Translation and Integration**, 75, n. 5, p. 689-700, 1990. https://doi.org/10.1113/expphysiol.1990.sp003447

ØRSKOV, E.; FRASER, C.; MASON, V.; MANN, S. Influence of starch digestion in the large intestine of sheep on caecal fermentation, caecal microflora and faecal nitrogen excretion. **British Journal of Nutrition**, 24, n. 3, p. 671-682, 1970. doi:10.1079/BJN19700068

ØRSKOV, E. R.; MCDONALD, I. The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. **The Journal of Agricultural Science**, 92, n. 2, p. 499-503, 1979. https://doi.org/10.1017/S0021859600063048

PARK, T.; MAO, H.; YU, Z. Inhibition of rumen protozoa by specific inhibitors of lysozyme and peptidases in vitro. **Frontiers in microbiology**, 10, p. 497428, 2019. https://doi.org/10.3389/fmicb.2019.02822

PARK, T.; MEULIA, T.; FIRKINS, J. L.; YU, Z. Inhibition of the rumen ciliate Entodinium caudatum by antibiotics. **Frontiers in microbiology**, 8, p. 254418, 2017. https://doi.org/10.3389/fmicb.2017.01189

PATRA, A. K.; YU, Z. Effects of vanillin, quillaja saponin, and essential oils on in vitro fermentation and protein-degrading microorganisms of the rumen. **Applied microbiology and biotechnology**, 98, p. 897-905, 2014. https://doi.org/10.1007/s00253-013-4930-x

PAUL, S.; KAMRA, D.; SASTRY, V.; SAHU, N. *et al.* Effect of administration of an anaerobic gut fungus isolated from wild blue bull (Boselaphus tragocamelus) to buffaloes (Bubalus bubalis) on in vivo ruminal fermentation and digestion of nutrients. **Animal feed science and technology**, 115, n. 1-2, p. 143-157, 2004. http://dx.doi.org/10.1016/j.anifeedsci.2004.01.010

PENGPENG, W.; TAN, Z. Ammonia assimilation in rumen bacteria: a review. **Animal biotechnology**, 24, n. 2, p. 107-128, 2013. https://doi.org/10.1080/10495398.2012.756402

POWELL, J. M.; ROTZ, C. A.; WATTIAUX, M. A. Potential use of milk urea nitrogen to abate atmospheric nitrogen emissions from Wisconsin dairy farms. Journal of Environmental Quality, v. 43, n. 4, p. 1169-1175, 2014. https://doi.org/10.2134/jeq2013.09.0375

PRAHL, M. C.; MÜLLER, C. B.; ALBRECHT, D.; KOCH, F. *et al.* Hepatic urea, creatinine and uric acid metabolism in dairy cows with divergent milk urea concentrations. **Scientific Reports**, 12, n. 1, p. 17593, 2022. https://doi.org/10.1038/s41598-022-22536-y

PSZCZOLKOWSKI, V. L.; ARRIOLA APELO, S. I. The market for amino acids: understanding supply and demand of substrate for more efficient milk protein synthesis. **J Anim Sci Biotechnol**, 11, n. 1, p. 108, Nov 12 2020. https://doi.org/10.1186/s40104-020-00514-6

PUTRI, E. M.; ZAIN, M.; WARLY, L.; HERMON, H. Effects of rumen-degradable-to-undegradable protein ratio in ruminant diet on in vitro digestibility, rumen fermentation, and microbial protein synthesis. **Veterinary world**, 14, n. 3, p. 640, 2021. https://doi.org/10.14202%2Fvetworld.2021.640-648

RØJEN, B. A.; THEIL, P. K.; KRISTENSEN, N. B. Effects of nitrogen supply on inter-organ fluxes of urea-N and renal urea-N kinetics in lactating Holstein cows. **Journal of Dairy Science**, 94, n. 5, p. 2532-2544, 2011. https://doi.org/10.3168/jds.2010-3949

ROMAN-GARCIA, Y.; WHITE, R. R.; FIRKINS, J. L. Meta-analysis of postruminal microbial nitrogen flows in dairy cattle. I. Derivation of equations. **Journal of Dairy Science**, 99, n. 10, p. 7918-7931, 2016. https://doi.org/10.3168/jds.2015-10661

RUSSELL, J. B.; O'CONNOR, J.; FOX, D.; VAN SOEST, P. *et al.* A net carbohydrate and protein system for evaluating cattle diets: I. Ruminal fermentation. **Journal of animal science**, 70, n. 11, p. 3551-3561, 1992. https://doi.org/10.2527/1992.70113551x

RYCHLIK, J. L.; LAVERA, R.; RUSSELL, J. B. Amino acid deamination by ruminal Megasphaera elsdenii strains. **Current Microbiology**, 45, p. 340-345, 2002. https://doi.org/10.1007/s00284-002-3743-4

SAVARI, M.; KHORVASH, M.; AMANLOU, H.; GHORBANI, G. R. *et al.* Effects of rumen-degradable protein:rumen-undegradable protein ratio and corn processing on production performance, nitrogen efficiency, and feeding behavior of Holstein dairy cows. **Journal of Dairy Science**, 101, n. 2, p. 1111-1122, 2018. https://doi.org/10.3168/jds.2017-12776

SCHWAB, C. G.; BRODERICK, G. A. A 100-Year Review: Protein and amino acid nutrition in dairy cows. **Journal of Dairy Science**, 100, n. 12, p. 10094-10112, 2017. https://doi.org/10.3168/jds.2017-13320

STARKE, S.; MUSCHER, A.; HIRSCHHAUSEN, N.; PFEFFER, E. *et al.* Expression of urea transporters is affected by dietary nitrogen restriction in goat kidney. **Journal of Animal Science**, 90, n. 11, p. 3889-3897, 2012. https://doi.org/10.2527/jas.2011-4262

SILVA, A. L.; DETMANN, E.; RENNÓ, L. N.; PEDROSO, A. M. *et al.* Effects of rumen undegradable protein on intake, digestibility and rumen kinetics and fermentation characteristics of dairy heifers. **Animal Feed Science and Technology**, 244, p. 1-10, 2018/10/01/ 2018. https://doi.org/10.1016/j.anifeedsci.2018.07.019

SILVA, L.; DIXON, R.; COSTA, D. Nitrogen recycling and feed efficiency of cattle fed protein-restricted diets. **Animal Production Science**, 59, n. 11, p. 2093-2107, 2019. http://dx.doi.org/10.1071/AN19234

SOK, M.; OUELLET, D.; FIRKINS, J.; PELLERIN, D. *et al.* Amino acid composition of rumen bacteria and protozoa in cattle. **Journal of dairy science**, 100, n. 7, p. 5241-5249, 2017. https://doi.org/10.3168/jds.2016-12447

SOLOMON, R.; WEIN, T.; LEVY, B.; REISS, V. *et al.* Rumen protozoa shape microbiome composition and metabolic output of fermentation. **bioRxiv**, p. 2020.2005. 2015.080218, 2020. https://doi.org/10.1101/2020.05.15.080218

SOUZA, V. C.; AGUILAR, M.; VAN AMBURGH, M.; NAYANANJALIE, W. A. D. *et al.* Milk urea nitrogen variation explained by differences in urea transport into the gastrointestinal tract in lactating dairy cows. **Journal of Dairy Science**, 104, n. 6, p. 6715-6726, 2021. https://doi.org/10.3168/jds.2020-19787

SPANGHERO, M.; KOWALSKI, Z. M. Updating analysis of nitrogen balance experiments in dairy cows. **Journal of Dairy Science**, 104, n. 7, p. 7725-7737, 2021. https://doi.org/10.3168/jds.2020-19656

SPEK, J.; DIJKSTRA, J.; BANNINK, A. Influence of milk urea concentration on fractional urea disappearance rate from milk to blood plasma in dairy cows. **Journal of dairy science**, 99, n. 5, p. 3880-3888, 2016. https://doi.org/10.3168/jds.2015-9421

STEVENSON, D. M.; WEIMER, P. J. Dominance of Prevotella and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. **Applied microbiology and biotechnology**, 75, p. 165-174, 2007. https://doi.org/10.1007/s00253-006-0802-y

STEWART, G. S.; SMITH, C. P. Urea nitrogen salvage mechanisms and their relevance to ruminants, non-ruminants and man. **Nutrition research reviews**, 18, n. 1, p. 49-62, 2005. https://doi.org/10.1079/NRR200498

SYLVESTER, J.; KARNATI, S.; YU, Z.; NEWBOLD, C. J. *et al.* Evaluation of a real-time PCR assay quantifying the ruminal pool size and duodenal flow of protozoal nitrogen. **Journal of dairy science**, 88, n. 6, p. 2083-2095, 2005. https://doi.org/10.3168/jds.S0022-0302(05)72885-X

TACOMA, R.; FIELDS, J.; EBENSTEIN, D. B.; LAM, Y. W. *et al.* Ratio of dietary rumen degradable protein to rumen undegradable protein affects nitrogen partitioning but does not affect the bovine milk proteome produced by mid-lactation Holstein dairy cows. **Journal of Dairy Science**, 100, n. 9, p. 7246-7261, 2017. https://doi.org/10.3168/jds.2017-12647

TAN, P.; LIU, H.; ZHAO, J.; GU, X. *et al.* Amino acids metabolism by rumen microorganisms: Nutrition and ecology strategies to reduce nitrogen emissions from the inside to the outside. **Science of the Total Environment**, 800, p. 149596, 2021. https://doi.org/10.1016/j.scitotenv.2021.149596

THAI, K. E.; TAYLOR, M. W.; FERNANDES, T.; AKINADE, E. A. *et al.* Topiramate alters the gut microbiome to aid in its anti-seizure effect. **Frontiers in Microbiology**, 14, 2023. https://doi.org/10.3389/fmicb.2023.1242856

THORNTON, R.; BIRD, P.; SOMERS, M.; MOIR, R. Urea excretion in ruminants. III. The role of the hind-gut (caecum and colon). **Australian Journal of Agricultural Research**, 21, n. 2, p. 345-354, 1970. https://doi.org/10.1071/AR9700345

USHIDA, K.; JOUANY, J.-P. Effect of protozoa on rumen protein degradation in sheep. **Reproduction Nutrition Développement**, 25, n. 6, p. 1075-1081, 1985. https://doi.org/10.1051/rnd:19850807

VAN DUINKERKEN, G.; BLOK, M.; BANNINK, A.; CONE, J. *et al.* Update of the Dutch protein evaluation system for ruminants: the DVE/OEB2010 system. **The Journal of Agricultural Science**, 149, n. 3, p. 351-367, 2011. https://doi.org/10.1017/S0021859610000912

VELÁSQUEZ, A.; VIERA, M. J. R.; MARNET, P.-G. Empirical attributes and limitations of methodologies for predicting the degradability of ruminal protein. **Ciencia e investigación agraria: revista latinoamericana de ciencias de la agricultura**, 43, n. 2, p. 171-189, 2016. http://dx.doi.org/10.4067/S0718-16202016000200001

WALLACE, R. J. Adsorption of soluble proteins to rumen bacteria and the role of adsorption in proteolysis. **British journal of nutrition**, 53, n. 2, p. 399-408, 1985. https://doi.org/10.1079/BJN19850047

WALLACE, R. J. Ruminal microbial metabolism of peptides and amino acids. **The Journal of nutrition**, 126, p. 1326S-1334S, 1996. https://doi.org/10.1093/jn/126.suppl_4.1326s

WALLACE, R. J.; JOBLIN, K. N. Proteolytic activity of a rumen anaerobic fungus. **FEMS microbiology letters**, 29, n. 1-2, p. 19-25, 1985. https://doi.org/10.1111/j.1574-6968.1985.tb00828.x

WALLACE, R. J.; MCKAIN, N. A survey of peptidase activity in rumen bacteria. **Microbiology**, 137, n. 9, p. 2259-2264, 1991. https://doi.org/10.1099/00221287-137-9-2259

WALLACE, R. J.; MCKAIN, N.; BRODERICK, G. A.; RODE, L. M. *et al.* Peptidases of the rumen bacterium, Prevotella ruminicola. **Anaerobe**, 3, n. 1, p. 35-42, 1997. https://doi.org/10.1006/anae.1996.0065

WALSH, R. G.; HE, S.; YARNES, C. T. Compound-specific δ¹³C and δ¹⁵N analysis of amino acids: a rapid, chloroformate-based method for ecological studies. **Rapid Communications in Mass Spectrometry**, 28, n. 1, p. 96-108, 2014. https://doi.org/10.1002/rcm.6761

WANG, C.; LIU, J.; YUAN, Z.; WU, Y. *et al.* Effect of level of metabolizable protein on milk production and nitrogen utilization in lactating dairy cows. **Journal of Dairy Science**, 90, n. 6, p. 2960-2965, 2007. https://doi.org/10.3168/jds.2006-129

WATFORD, Malcolm. The urea cycle: Teaching intermediary metabolism in a physiological setting. Biochemistry and Molecular Biology Education, v. 31, n. 5, p. 289-297, 2003. https://doi.org/10.1002/bmb.2003.494031050249

WEINER, I. David; MITCH, William E.; SANDS, Jeff M. Urea and ammonia metabolism and

the control of renal nitrogen excretion. Clinical Journal of the American Society of Nephrology, v. 10, n. 8, p. 1444-1458, 2015. Doi: 10.2215/CJN.10311013

WHITE, R. R.; KONONOFF, P. J.; FIRKINS, J. L. Technical note: Methodological and feed factors affecting prediction of ruminal degradability and intestinal digestibility of essential amino acids. **Journal of Dairy Science**, 100, n. 3, p. 1946-1950, 2017. https://doi.org/10.3168/jds.2016-12008

WRIGHT, A.-D. G.; KLIEVE, A. V. Does the complexity of the rumen microbial ecology preclude methane mitigation? **Animal feed science and technology**, 166, p. 248-253, 2011. https://doi.org/10.1016/j.anifeedsci.2011.04.015

WU, G. **Amino acids: biochemistry and nutrition**. CRC Press, 2021. 1003092748. https://doi.org/10.1201/9781003092742

YANKE, L.; DONG, Y.; MCALLISTER, T.; BAE, H. *et al.* Comparison of amylolytic and proteolytic activities of ruminal fungi grown on cereal grains. **Canadian journal of microbiology**, 39, n. 8, p. 817-820, 1993. https://doi.org/10.1139/m93-121

ZHOU, J. W.; ZHONG, C. L.; LIU, H.; DEGEN, A. A. *et al.* Comparison of nitrogen utilization and urea kinetics between yaks (Bos grunniens) and indigenous cattle (Bos taurus)1. **Journal of Animal Science**, 95, n. 10, p. 4600-4612, 2017. https://doi.org/10.2527/jas2017.1428

CHAPTER 2

"Nitrogen metabolism and excretion in Holstein dairy heifers fed different levels of metabolizable protein."

Written and formatted according to the "Journal of dairy Science".

ABSTRACT

This study aimed to assess the effects of 3 levels of MP on ruminal fermentation, AA entry rates and plasma AA concentration, and urea-N metabolism and excretion. Six rumen-cannulated Holstein heifers (435 ± 27 kg BW on experiment onset) were allocated in a duplicated 3 × 3 Latin square design with 3 cows each. Dietary treatments were balanced to provide similar CP intakes and increasing MP levels targeted as, 1) Low MP (9.85% MP), 2) Medium MP (10.9% MP), and 3) High MP (12.1% MP), while maintaining iso-protein diets (16% CP). For the first 10 d of each 14-d period, heifers were fed 1 x/d; for the last 4 d of each period, heifers were fed 12 x/d at 95% to establish a steady state. Spot collections of urine, feces, rumen content, saliva, and blood were conducted from d 11 to d 14 of each period. On d 14, stable-isotope U-[13C]-labeled AA was infused (83mg/min) into the jugular vein for 720 minutes. No alterations were observed in animals' DMI, ADG, daily activities, or ruminal pH. High MP supply resulted in greater N efficient use by increasing N balance and reducing N excretion, mainly due to the lesser RDP imposed on this treatment. Greater RDP supply on Low MP diet resulted in greater BCVFA by BCAA breakdown denoting a wasteful use of dietary N. His entry rates increased linearly while Met entry rates increased quadratically as the level of MP increased due to a greater supply of MP from RUP in this dietary treatment. Moreover, greater plasma AA uptakes were observed for heifers fed High MP due to greater MP and energy supply in this dietary treatment when compared to Low MP. Overall, the findings presented herein point out to possibility of lowering RDP supply for dairy growing cattle than previously recognized.

Key Words: amino acids, N efficiency, N balance.

INTRODUCTION

The understanding of nitrogen (N) metabolism in ruminants is still one of the major challenges due to the complex microbial metabolic activity in the rumen and its impact on later processes in the intestines and body tissues (Hristov et al., 2019; Lapierre et al., 2020). Dietary N, supplied as CP and NPN, undergoes different degradation processes - a portion is degraded in the rumen, while another portion bypasses ruminal degradation. RDP is essential for microbial growth contributing to the formation of microbial crude protein (MCP). Both MCP and RUP constitute MP that is digested into AA, absorbed into the bloodstream, and used for animals' growth, maintenance, reproduction, and production (NASEM, 2021). Further, due to an evolutionary mechanism N surplus from microbial degradation of protein and body tissue catabolism of AA (Batista et al., 2017; Reynolds & Kristensen, 2008) can be recaptured through urea recycling, where N returns to the rumen to support MCP synthesis, enhancing MP supply and improving N use efficiency.

An update of the nutritional requirements of dairy cattle (NASEM, 2021) was recently published and provided library upgrades and new insights about dairy cattle feed requirements. Yet, it uses a direct approach for predicting MCP synthesis based on RDP and rumen-degradable carbohydrates without including urea recycling in the MCP model. As these equations were derived from in vivo observation, it is intrinsic in the derivation to the extent of the conditions observed. Nonetheless, despite the significant role of urea recycling in enhancing N use efficiency in dairy systems, the current predictions fail to capture its magnitude in ruminants' diets. It is important to note that the committee does recognize the critical aspect of urea recycling in dairy nutrition; however, limited studies have prevented the full incorporation of this mechanism in dietary requirement guidelines (NASEM, 2021). Most available literature has focused on

understanding mechanisms of N metabolism by varying levels of CP (Kaufman et al., 2017; Reynal & Broderick, 2005), preventing a comprehensive understanding of the effects of dietary ingredient manipulation on N metabolism. To the best of our knowledge, literature available on the N efficiency of dairy heifers while maintaining fixed CP levels evaluated intake, performance, N balance, mammary gland development, hormonal status (Silva et al., 2018a), along with digestibility and ruminal kinetics (Silva et al., 2018b), without reporting metabolizable AA. As such, further studies on fixed CP levels would improve the understanding of AA absorption and utilization to fully describe efficient N metabolism of dairy heifers. We hypothesized that maintaining a high CP level in the diet and increasing the supply of MP by increasing RUP, will stimulate catabolism of AA surplus, and the ammonia produced from this metabolism will be used to compensate the lack of N in the rumen rather than being excreted in urine. Therefore, we aim to investigate how and to what extent the supply of 3 different dietary levels of MP would affect ruminal fermentation, AA entry rates and absorption, urea-N metabolism, and N excretion in Holstein dairy heifers.

MATERIALS AND METHODS

Experiment 1: Metabolism trial

Experimental Design, Animals, and Diets

The animal procedures were conducted at the Virginia Tech Kentland Dairy Farm and approved by the Virginia Tech Animal Care and Use Committee (IACUC #132-22). Heifers were ruminally cannulated 6 months before starting the trial. Each heifer was housed in the same individual tie stall for the duration of the experiment. All heifers were allocated in the same room of Metabolic Research Laboratory, Virginia Tech (MRL), with room temperature of 18.33°C ± 0.14 and THI 60.34 ± 21.7. Heifers were allowed to exercise in a free pen for 1 hour daily, unless

during collection days. Heifers were fitted with ear tag accelerometers (CM; CowManager; Harmelen, Netherlands). Data were continuously registered, summarized by hour, and classified as eating, ruminating, not active, active, highly active, and average ear temperature, which was done by the proprietary algorithm.

Six ruminally canulated Holstein heifers (435 ± 27 kg BW at experimental onset) were allocated in a duplicated 3 × 3 Latin square design with 2 squares of cows with 3 cows each. Heifers within each square were randomly assigned to the treatment sequence. The squares were conducted at different times because of animal availability and correspond to the blocks. Heifers were blocked by BW during the acclimation period (14 d before starting the experimental period) and were weighted at d 1, 7, and 14 of each period. Each square was conducted with 3 periods of 14 days, 10 days for adaptation, and 4 days for collections each; timeline is presented in Error! R eference source not found.. In the initial 10 d of each period, heifers were fed once a day ad libitum with a target refusal of 5%. Then, from days 11 to 14 of each period, heifers were fed every 2 h, equivalent to 95% of the average intake from d 5 to d 10, to establish a steady state of AA absorption from the gut during sampling period.

Treatments were formulated to provide increasing levels of MP, 1) Low MP (9.85% MP), 2) Medium MP (10.9% MP), and 3) High MP (12.1% MP), while maintaining iso-protein diets (16% CP). Diets were formulated using the NASEM model (2021) and surpassed CP levels (Error! R eference source not found.). The MP levels tested herein were achieved by altering the inclusion of RDP and RUP, which were selected expecting to generate considerable differences in microbial protein synthesis and AA provided by RUP. Due to the constant CP level in the diet, we were not certain to see great changes in N excretion, however, any change would be a response to N recycling due to the limitation on RDP imposed to the treatment High MP. The diets were

formulated to fulfill the requirements of a Holstein first lactation cow, with 500 kg of BW, 700 kg mature weight, at 150 DIM, and 60 days pregnant. However, we considered a heifer would eat 70% of the estimated DMI. Kentland Farm supplied grass hay and corn silage; grain mixes were purchased from Rockingham Milling Company (Harrisonburg, VA).

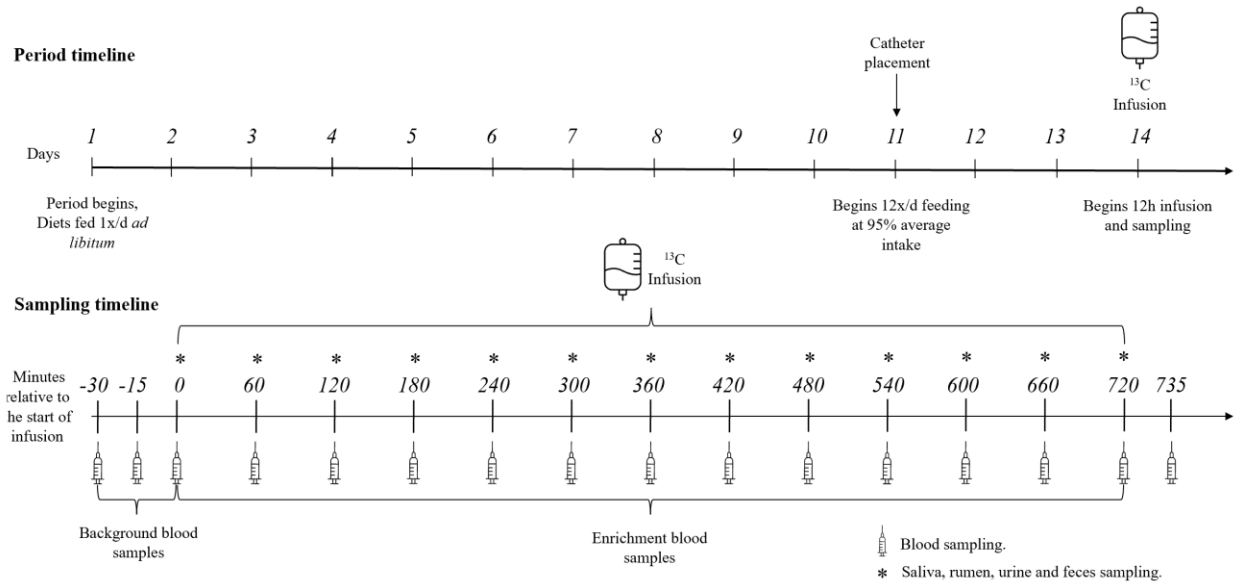


Figure 1. Experimental timeline for each period.

Each treatment diet was individually mixed once daily using a stationary mixer. The DM content of corn silage and grass hay was measured twice weekly, by drying samples in an oven for 12 h at 100ºC. Dietary adjustments were made in response to observed changes in DM content, if any. Feed Ingredients, TMR, and refusals were sampled over the last four days of each period, composited by animals and period, and stored at -20ºC for further analysis. A subsample of composited ingredients from each period was sent to Cumberland Valley Analytical Services (Waynesboro, PA, USA). Forages were analyzed using NIR (package Basic NIR) for DM, soluble protein, lignin, starch, sugar, fat, and ash with wet chemistry analyses for minerals, CP, ADF, NDF, and fatty acid profile following protocols described in Supplementary Table 1. Grains were

analyzed by wet chemistry for DM, CP, adjusted protein, soluble protein, ADF, NDF, ash, minerals, starch, and fatty acid profile (Supplementary Table 1). The other subsample of feed ingredients was dried and stored for *in situ* incubation, which will be discussed following.

Table 1. Ingredients and nutrient composition of dietary treatments.

Item	Low MP	Medium MP	High MP
Ingredient			
Corn Silage, %	56.5	56.5	56.5
Grass Hay, %	8.70	8.70	8.70
Corn grain dry, fine grind, %	5.63	6.16	5.36
Citrus pulp, dry, %	7.45	5.07	4.59
Soybean meal, %	19.3	10.9	0.66
Soybean meal expellers, SoyPlus %	0.00	9.97	21.9
Minerals[1], %	2.14	2.32	2.00
Trace mineral premix[2], %	0.04	0.04	0.04
Vitamins[3], %	0.24	0.27	0.23
Nutrients composition[4]			
MP, % of DM	10.5 ± 0.26	11.4 ± 0.33	12.1 ± 0.49
CP, % of DM	16.1 ± 0.52	15.9 ± 0.54	15.7 ± 0.62
RDP, % of DM	11.4 ± 0.41	9.75 ± 0.34	7.85 ± 0.35
RUP, % of DM	4.65 ± 0.18	6.10 ± 0.26	7.85 ± 0.34
ME, Mcal/d	28.79 ± 4.19	28.68 ± 4.14	27.91 ± 3.54
Fatty Acids, % of DM	2.41 ± 1.75	2.41 ± 1.76	2.53 ± 1.63
NDF, % of DM	31.0 ± 1.31	31.4 ± 1.00	32.2 ± 0.71
ForNDF, % of DM	26.3 ± 0.71	26.3 ± 0.75	26.3 ± 0.73
Starch, % of DM	27.5 ± 1.07	28.0 ± 1.54	26.9 ± 2.06

[1]Contained (% DM) Sodium bicarbonate, 29.70 %; Limestone, 24.75 %; Sodium sulfate, 17.33 %; Calcium phosphate (di), 2.48 %; Sodium Chloride (salt), 9.90 %; Clay, 11.39 %; Selenium Yeast Premix (Bainbridge, GA), 1.98 %; Active ingredients (Ultrasorb R, ABVista), 1.98 %; Zinc sulfate, 0.50 %. [2]Contained (% DM) Calcium, 8.02%; Copper 40,080 ppm; Zinc, 16,032 10ppm; Manganese, 15,030 10ppm; Cobalt, 1,603 ppm; Iodine, 3,507 ppm; Iron, 3.01%.[3]Contained 56.5 % of Vitamin ADE mix (Vitamin A, 26,485 KIU/kg; Vitamin D, 8,828 KIU/kg; Vitamin E, 44,141 mg/kg) and 43.5% of Vitamin E (60000 IU; Vitamin E, 132,422 mg/kg).
[4]Calculated using the NASEM (2021) model library of input data means of feed composition of the offered diet.

Catheterizations, Infusions, and Sample Collections

On the morning of d 10 of each period, heifers were tranquilized with 0.15 µL/kg of xylazine (XylaMed, Bimeda, Oakbrook Terrace, IL) administered into the coccygeal vein and 1 mL of 2% lidocaine was intradermally administered at each catheter insertion site. Two jugular catheters were fitted: one for isotope infusions (75 cm x 13 ga internal diameter Micro-Renathane, Braintree Scientific Inc., Braintree, MA - 55 cm inserted into the jugular) and another for blood sampling (60 cm x 13 ga internal diameter Micro-Renathane, Braintree Scientific Inc., Braintree, MA - 20 cm inserted into the jugular). This catheterization technique was adapted from the methodology described by Estes et al. (2018). To ensure blood samples were taken post-circulation, the infusion catheter tip was approximately 40 cm cauda (downstream) from the tip of the blood sampling catheter. The catheters were alternately placed on opposite sides of the neck in successive periods.

On d 14 animals were subjected to a continuous jugular infusion of 1 g of U-[^{13}C]-labeled AA derived from algae (#426199, Sigma-Aldrich, St. Louis, MO, USA) and 30 mg of U-[$^{13}C_6$]-labeled His (#201740-88-7, Cambridge Isotope Laboratories, Inc. Andover, MA, USA) were dissolved in 500 mL of 0.9% saline and filtered through 0.22-µm membrane filters into sterile infusion bags. Blood samples (10 ml) were collected from the jugular catheter at heparinized tubes every 6 h from d 11 to d 13 and on d 14 at – 30, -15, 0, 180, 360, 540, 720, and 735 min relative to the start of the algae infusion. Plasma was extracted from blood samples by centrifugation for 15 min at 1600 × g and 4 °C. All samples were processed within 30 min and immediately stored at -20º C for subsequent analysis. At the end of each period, catheters were carefully removed, the sites were cleaned, and an antibiotic ointment (Triple Antibiotic Ointment, Bacitracin Zinc, 400 units; Neomycin, 3.5 mg; and Polymyxin B Sulfate, 5000 units; Equate – Walmart Inc.,

Bentonville, AR, USA) was applied.

To evaluate the metabolism of urea and ammonia, spot samples of saliva and rumen content were collected every 6 h from d 11 to d 13 and on d 14 at 0, 180, 360, 540, and 720 min relative to the start of algae infusion. For saliva collection, a compressed cotton was clipped to a mosquito straight forceps which was placed into heifer's mouth to be chewed for approximately 1 min, then the soaked cotton was transferred into a 20 ml syringe and saliva was extracted by squeezing it into a collection tube, and stored at −20 °C. Rumen samples were taken from 3 different rumen sections (anterior, medial, and dorsal) through rumen cannulas, and filtered through four layers of cheesecloth. Thereafter, an aliquot of 10 ml of rumen fluid was acidified using 1.7 mL of metaphosphoric acid (250 g/L), and stored at −20 °C for further analysis. Fecal spot samples were collected every 6 h from d 11 to d 13 and on d 14 at 0, 180, 360, 540, and 720 min relative to the start of algae infusion and samples were frozen at −20°C. Urine spot samples were obtained simultaneously with fecal sampling by sub-vulvar stimulation, 50% sulfuric acid solution was added (10 mL of urine and 100 µL of acid), and samples were frozen at −20°C.

N excretions were determined by the total collection of feces during 24-h period on d 13 to 14. Feces were collected concurrent with defecation and weighed. Fecal aliquots (equal fresh weight basis) were immediately frozen during the collection period and a composite sample was formed per cow. Total urinary output was collected in buckets, simultaneously with fecal sampling. A 50% sulfuric acid solution (100 mL) was added to 20-L buckets to prevent N volatilization, and urine was added to it during the collection period (Souza et al., 2021). At the end of the 24-h collection period, composite urine samples were diluted frozen at −20°C.

Analyses

Ruminal pH was evaluated immediately after collection with a HI98100 Hanna Checker® Plus pH (Hanna Instruments, Woonsocket, RI, USA). The analysis of VFA concentrations in

rumen fluid was conducted after centrifugation (1500 × g for 5 min). The upper layer (1ml) was acidified with 0.17 mL of metaphosphoric acid (25%, w/v), and 0.13 mL of internal standard (5 mmol, 4-methyl-valeric acid, 277827, Sigma, St. Louis, MO), vortex, and rest for 30 min (4 °C). Then samples were centrifuged at 3000 × g for 15 min. The supernatant was collected and used for VFA determination by gas chromatography according to Thai et al. (2023). Ammonia and Urea concentration from plasma, rumen fluid, saliva, and urine were accessed colorimetrically according to the methodology described by Chaney and Marbach (1962).

Samples of TMR, refusals, and both urine and feces from the total collection were analyzed for N content via combustion using a Vario EL cube analyzer (Elementar, Langenselbold, Germany) for the evaluation of N balance, which was calculated as follows:

$$N\ Balance = N\ Intake - (N\ Urine + N\ Feces)$$

Where: *N Intake* = the total N consumed from the diet (g/d). *N Urine* and *N Feces* are the amounts of N excreted in urine (g/d) and feces (g/d), respectively.

To evaluate the blood ^{13}C AA enrichment, plasma deproteinized samples (1.5 ml as described previously) of each time point were desalted using ion exchange chromatography (BioRad Resin AG 50W-X8*, 100 to 200 mesh; Bio-Rad, Hercules, CA) and eluted using ammonium hydroxide (2N) into salinized glass vials to measure the ^{13}C-labeled AA. Desalted samples were freeze-dried and derivatized as described by (Walsh et al., 2014). Using an isotope-ratio mass spectrometer coupled to a GC by a combustion oven, the isotopic ratios of 13C-labeled AA were determined (Thermo Fisher Scientific, Waltham, MA).

For plasma free AA concentration, a composite sample per heifer and period were processed as follows, 1 mL was deproteinized by mixing with 8% sulfosalicylic acid and centrifuged at 16000 x g for 15 min at 4 °C. The supernatant was then stored at -20ºC. Gravimetric

measurements were made of deproteinized plasma samples, along with the addition of SSA, and the results were recorded for use in calculating concentration. Deproteinized plasma samples (250 μL) were gravimetrically mixed with 250 μL of two external tracers. Tracers were obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA) unless otherwise specified. The first tracer contained 0.3 g/L of U-[13C]- AA derived from algae (#426199, Sigma-Aldrich, St. Louis, MO, USA), 0.35 mM of [15N3]-L-histidine (#NLM-1513), 0.1 mM of [15N]-labeled L-threonine (#NLM-742), and 0.1 mM of [2H3]-labeled L-methionine (#DLM-431) dissolved in 0.1 N HCl. The second tracer consisted of 0.15 mM of [15N]-labeled L-asparagine (#NLM-120), and 0.15 mM of [15N]-labeled L-glutamine (#NLM-557) dissolved in ddH2O. Followed by desalting of the samples through ion chromatography with 0.5 g resin (AG 50 W-X8 resin, Bio-Rad Life Science, Hercules, CA, USA). Subsequently, they were lyophilized and derivatized following the method described by Calder et al. (1999). For the separation of AA derivatives, gas chromatography was employed (Thermo Trace 1310 GC with a TriPlus RSH autosampler, Thermo Fisher Scientific, Waltham, MA, USA), followed by quantification using a single quadrupole mass spectrometer with selective ion monitoring (ISQ LT system, Thermo Fisher Scientific, Waltham, MA, USA). Concentrations were determined using isotope ratios and a gravimetrically produced calibration curve made up of rising masses of unlabeled AA and constant masses of both external tracer solutions. Concentrations were determined using SAS (SAS Institute Inc., Cary, NC, USA). To subtract the naturally occurring background from both the standard curve and all observations, two backgrounds were produced for the curve. The background included large quantities of unlabeled AA concentrations and no exogenous tracer.

Amino Acid Entry Rate Derivation

The model represented the metabolism of a single AA. Modeling was based on a set of

ordinary differential equations derived over time from state variables and initial parameters input, using the fourth order Runge-Kutta method. It was coded in R Studio (version 2023.12.1) with R 3.3.0 (R Core Team, Vienna, Austria). The model contained 3 primary-state variables representing a pool of AA with a fast turnover rate, a pool of AA in tissue protein with a slow turnover rate, and a pool of AA in tissue protein with a very slow turnover rate, with a linear increasing pool for protein deposited as gain, the same primary-state variable was assumed for total AA or labeled AA. A description of the model is in Supplementary Figure 1. The fast, slow, and very slow turnover pools are defined as described by Estes et al. (2018), the protein deposited as gain was determined based on BW change during the period of infusion. We utilized the function modFit from package FME (Soetaert and Petzoldt, 2010) to fit the model to observed enrichment of 13C of each AA, using an optimized to run model equations and return minimal residuals between predicted and observed enrichment of ^{13}C with modCost function from package FME (Soetaert and Petzoldt, 2010), based on Nelder-Mead algorithm to converge towards the optimal solve, to derive adjustable variables entry rate (g/d), the mass action constants (min^{-1}) from fast to slow turnover pool. In the present trial, the model could not solve for Thr and Val entry rates.

Statistical Methods

Data collected across multiple collection time points were aggregated by treatment before being incorporated into the statistical analysis. Then, it was analyzed using the "lme4" package in R software (v. 4.2.1; R Studio v. 2023.06.1; Kuznetsova et al., 2017), according to a replicated Latin Square design using the following model:

$$Y_{ijk} = \mu + T_i + P_j + S_k + C(S)_{l(k)} + \mathcal{E}_{ijkl}$$

where Y_{ijk} represents the dependent variable, μ is the overall population mean, T_i represents the

effect of treatment (i = 3), P_j represents the fixed effect of period (j = 6), S_k represents the random effect of square (k = 2), $C(S)_{l(k)}$ represents the random effect of cow nested in the square (l = 6), $\mathcal{E}_{ijkl}$ represents the error term. Outliers were removed if they exceeded 2 standard deviations from the average. Treatments were compared with linear and quadratic orthogonal polynomial contrasts. The presence of a linear component refers to a difference between the initial and final levels, whereas a quadratic component shows that the intermediary level does not fit into a linear relationship (Gill, 1978). Treatment differences were considered significant if $P \leq 0.05$, and as trends for $P \leq 0.10$. Data are reported as least square means with standard error of the means (SEM).

Experiment 2: In Situ Incubation

Incubation

After experiment 1, 3 cannulated heifers were used to evaluate the degradation kinetics of all feed ingredients by in situ ruminal incubation. Throughout this experiment, heifers were fed the Medium MP diet. Incubations were conducted following the recommendation of NASEM (2021). Briefly, dried samples were ground to 2 mm (Wiley Mill, Thomas Scientific, Swedesboro, NJ). Subsequently, 5 g of sample were placed into Dracon bags (10 x 20 cm, 50 µm porosity; Ankom Technology, Macedon, NY), sealed, and incubated in the rumen of each heifer (1 replicate per heifer) for 0, 4, 8, 12, 16, 24, 48, 72, 96, 120 and 240h. Incubated bags were removed from the rumen, immersed in cold water to stop fermentation, followed by rinsing under cold running water. Further washing was conducted in a household washer by a delicate cold cycle without detergent until water clarity was achieved. The bags with samples were then freeze-dried and weighed to determine the DM degradation. The residue was analyzed for N content via combustion using a Vario EL cube analyzer (Elementar, Langenselbold, Germany). Soybean meal was used as

standard subtract and blank bags were used to access the infiltration of particle matter.

DM and N in situ degradation

The kinetics of DM and N degradation were estimated as described by (Huang et al., 2019). Considering A fraction (% of initial content) represents DM or N escaping from the bag at time 0, C fraction (% of initial content) represents the remaining content at 48 hours for grains and 72 hours for forages (NASEM 2021). The degradation kinetics of each feed ingredient were evaluated individually, then the results were used to calculate the DM and N degradation kinetics, effective degradability of DM, and RUP of each dietary treatment used in experiment 1. Effective degradability (ED) of DM and RUP was predicted according to NRC (2001) with passage rates of concentrate 7.11 %/h and 4.95 %/h for forages defined by (Hanigan et al., 2021).

Statistical Methods

DM and N degradation kinetics of each dietary treatment were analyzed using the "lm" package in R software (v. 4.2.1; R Studio v. 2023.06.1; Kuznetsova et al., 2017), by the following model:

$$Y_{ij} = \mu + T_i + C_j + \mathcal{E}_{ij}$$

where Y_{ij} represents the dependent variable, μ = the overall population mean, T_i represents the fixed effect of treatment (i = 3), C_j represents the random effect of cow (j = 3), $\mathcal{E}_{ij}$ represents the error term. Treatments were compared with linear and quadratic orthogonal polynomial contrasts as previously described. Treatment differences were considered significant if $P \leq 0.05$. Data are reported as least square means with SEM.

RESULTS

Diet and performance

Feed ingredients and nutrient composition of dietary treatments are presented in **Table 1**. Ruminal disappearance kinetics of each dietary treatment on DM and CP basis are summarized in **Table 2**. As the supply of MP escalated, a linear increase in A and C fractions of DM was observed ($P < 0.01$), whereas B fraction declined linearly ($P < 0.01$). Consequently, both K_d and ED decreased linearly (1.28 and 2.1 percentual units, respectively) from the lower to the highest MP level. The same pattern was observed for CP *in situ* degradation, while A and C fractions increased linearly ($P < 0.01$ and $P = 0.04$, respectively), B fraction presented a linear reduction ($P < 0.01$). As a result, CP K_d decreased (1.12 percentual units; $P < 0.01$) while RUP increased (2.7 percentual units, $P < 0.01$) from the lower to the higher MP treatment. The proportion of MP did not affect DMI, ADG, or the activities in the study (

Table 3) but tended to decrease DM digestibility as the level of MP increased ($P = 0.07$). Heifers exhibited an average DMI of 10.9 kg/d of DM, which resulted in 1.60 kg/d of ADG. Activities were not affected by MP supply; the average daily durations of ruminating and eating time were 474 min and 151 min, respectively, across dietary treatments. Additionally, non-active, active, and high active behaviors were 419 min, 162 min, and 231 min, respectively. The average ear temperature recorded across the dietary treatments was 26.4 ºC.

Ruminal parameters

Ruminal pH, total VFA, and VFA molar proportions are presented in **Table 4**. Ruminal pH remained consistent across different levels of MP, averaging 6.4 ($P = 0.75$). However total VFA concentration exhibited a negative quadratic effect ($P < 0.01$) and tended ($P = 0.07$) to decrease

Table 2. *In situ* DM and N degradation of dietary treatments.

	Treatments				P^2	
Item	Low MP	Medium MP	High MP	SEM[1]	Linear	Quadratic
DM						
A Fraction, %	53.9	54.4	54.7	0.24	<0.01	0.55
B Fraction, %	34.3	33.4	32.8	0.52	<0.01	0.74
C Fraction, %	11.9	12.2	12.5	0.42	<0.01	0.90
Kd, %/h	4.93	4.34	3.65	0.194	<0.01	0.53
ED, %	69.8	68.9	67.7	0.34	<0.01	0.26
Crude Protein						
A Fraction, %	50.4	51.9	53.2	0.69	<0.01	0.73
B Fraction, %	38.2	36.7	35.3	0.48	<0.01	0.83
C Fraction, %	11.73	12.35	13.18	0.432	0.04	0.81
Kd, %/h	4.76	4.09	3.34	0.215	<0.01	0.68
RUP, %	34.8	36.4	38.4	0.63	<0.01	0.54

[1]Standard error of the mean.
[2]Probability corresponds to the null hypothesis, with linear and quadratic contrasts.

linearly with increasing MP (6 mmol/L). Acetate molar proportion increased (linear and quadratic components, $P = 0.03$), and propionate tended to decrease linearly (0.5%; $P = 0.07$) at high MP compared to low MP. In contrast, iso-butyrate, iso-valerate, and valerate presented a linear decrease ($P < 0.01$; $P < 0.01$ and $P = 0.02$, respectively), while butyrate showed a quadratic reduction (0.3%; $P < 0.01$) at higher MP levels. There was a linear increase in Acetate:Propionate ratio in response to the incremental levels of MP (0.18; $P < 0.01$).

When compared to low and medium MP rumen urea concentration decreased at high MP (linear and quadratic effects, $P = 0.04$). Similarly, rumen ammonia decreased at greater MP levels in a linear and quadratic pattern ($P < 0.01$).

N metabolism and efficiency

Urea and ammonia concentrations of rumen fluid, saliva, plasma and urine as well as N balance and outputs are presented in ***Consequently,*** for Low MP N surplus was excreted rather

than effectively utilized (Lobley et al., 2000), increasing the N load to the environment. Furthermore, the lower BUN and excretion outputs along with greater N balance for High MP, indicates that decreasing dietary RDP increased N efficiency (Bahrami-Yekdangi et al., 2016; Kaufman et al., 2017). Thus, even though RDP is the main source of N for MCP synthesis, recycled urea-N may buffer the rumen if dietary RDP supply is insufficient (Batista et al., 2017; Firkins et al., 2007), increasing N incorporation into MCP.

Table *5*. Saliva urea, plasma urea, and urine urea decreased linearly ($P < 0.01$) in response to the incremental levels of MP. Whereas saliva ammonia exhibited a quadratic reduction ($P = 0.05$) at High MP supply. Plasma ammonia increased both linear and quadratic ($P = 0.04$), averaging 3.03 mg/dL at the higher MP level. While N intake had a positive linear and quadratic effect ($P < 0.01$), fecal and urine output presented a linear reduction and quadratic effect ($P < 0.01$), which resulted in a linear increase in N balance for High MP supply (16 g/d; $P = 0.01$).

Table 3. Dry matter intake, performance, activities, and ear temperature of dairy heifers fed Low, Medium, and High MP diets.

	Treatments				P^2	
Item	Low MP	Medium MP	High MP	SEM[1]	Linear	Quadratic
DMI, kg/d	11.02	10.92	10.75	0.331	0.47	0.92
DM Apparent Digestibility, %	73.4	75.6	70.2	0.95	0.17	0.07
MP supply, kg/d[3]	1.16	1.26	1.31	0.065	0.03	0.58
BW, kg	517	519	519	15.0	0.37	0.35
ADG, kg/d	1.48	1.71	1.62	0.099	0.58	0.46
Ruminating, min	458	476	489	9.1	0.17	0.90
Eating, min	149	152	153	2.97	0.53	0.95
Non-Active, min	431	411	416	12.4	0.37	0.41
Active, min	163	167	156	5.14	0.61	0.48
High Active, min	237	232	223	9.15	0.59	0.93
Ear Temperature, °C	26.88	26.09	26.10	0.343	0.31	0.53

[1]Standard error of the mean.

[2]Probability corresponds to the null hypothesis, with linear and quadratic contrasts.
[3]Estimates derived from NASEM (2021).

Plasma AA entry rates are presented in **Table 6.** His entry rates increased linearly (P = 0.04) while Met entry rates had a positive quadratic effect as the level of MP increased (P = 0.06). Plasma EAA and NEAA concentrations are presented in **Table 7**.Plasma concentration of Lys, Met, Thr, and Ala decreased linearly (P = 0.03; P = 0.01; P = 0.01, and P = 0.02, respectively), while His, Gly, and Ser tended to decrease linearly in response to the incremental levels of MP (P = 0.08, P = 0.0, and P = 0.09, respectively).

DISCUSSION

Diets, performance, and rumen fermentation

The present study was performed to evaluate the effects of 3 different dietary MP levels relative to a fixed CP content on ruminal fermentation, AA entry rates, N excretion, and N efficiency in dairy heifers. To this end, dietary treatments were specifically designed to vary in the supply of RDP and RUP, as follows: above recommendations of RDP (Low MP), meeting recommendation of RDP (Medium MP), and bellow recommendation of RDP (High MP). Therefore, RUP was adjusted accordingly to maintain the overall 16% CP on a DM basis, which kept all diets above MP requirements (NASEM, 2021). The formulated diets (**Table 1**) differed mostly in the inclusions of soybean meal and soybean meal expellers. Briefly, greater proportions of soybean meal were fed for Low MP and greater soybean meal expellers for High MP as the latter ingredient is known for its ruminal degradation resistance properties (Boucher et al., 2009; Mass et al., 2000). The aim was to reduce dietary N available for microbial fermentation thus inducing greater N recycling reliance in this dietary treatment. Consequently, metabolic events differed mostly between heifers receiving the lowest vs. highest supply of MP tested herein.

In situ ruminal incubation is the reference method to calculate rumen CP degradation in several protein evaluation systems for ruminants (Hristov et al., 2019). In this study it was used to assess degradation kinetics of each dietary treatment (**Table 2**), allowing for the estimation of ED and RUP. Soybean meal was used as a standard subtract, and none of the incubation time points needed to be removed from the dataset. Variations in DM and N kinetics across dietary treatments reflect the fermentation processes, as influenced by intrinsic characteristics of the feed ingredients. Specifically, lesser ED and greater RUP observed in High MP diets is attributed to the greater inclusion of soybean meal expellers as a replacement for soybean meal, as previously stated. As expected, soybean meal expellers bypassed ruminal degradation (Boucher et al., 2009; Mass et al., 2000), increasing the RUP level in this dietary treatment and consequently MP. As such, an expected greater proportion of rumen undegradable matter (C fraction) and lower *Kd* (Castro et al., 2007; Kalscheur et al., 2006) were observed for High MP.

Herein, the RUP content expected for each dietary treatment was 29, 39, and 51 % of RUP in CP, respectively for Low, Medium, and High MP. In contrast, the observed values of RUP were 34.9, 36.4, and 38.5 %, respectively. Diets were formulated based on the available NASEM (2021) feed library, resulting in greater RUP values predicted compared to RUP estimated with N in situ degradation data. Only one value of soybean meal expeller is available in the NASEM (2021) feed library, which was used on diet formulation. However, this value probably corresponds to soybean meal expeller with greater RUP content than Soyplus, which was actually fed to heifers (Mass et al., 2000). SoyPlus is produced via an expeller process, applying pressure to extract oil from soybeans. This process generates heat, resulting in a ruminal-protected product compared to regular soybean meal (Mass et al., 2000). In contrast, other types of protection technology can be used, such as the addition of sulfite liquors to soybean meal followed by a heating process (Mass

et al., 2000). The sulfite liquors, which are byproducts of wood pulp processing, act as a chemical treatment altering the proteins' structure and promoting greater ruminal resistance properties. Once no sugars are added to the protection to increase condensation products in SoyPlus, a lower concentration of RUP is found in this commodity when compared to sulfite liquor-protected products (Mass et al., 2000). This fact evidences the importance of properly addressing the feed ingredients composition on the software prior to diet formulation.

The MP proportions did not affect DMI (**Table 3**), which remained consistent at 10.9 % DM. Similar results have been observed by Silva et al. (2018b) since no change in DMI was observed in heifers fed diets with increasing RUP levels relative to CP (15% of DM). Moreover, heifers fed increasing levels of dietary RUP relative to a fixed CP (17.6% of DM) did not present changes in DMI (Silva et al., 2018a). In agreement, Benchaar et al. (2023) reported no difference in DMI when lactating Holstein and Ayrshire cows were fed incremental levels of MP relative to CP of 13.1, 15.4, and 16.5%. For Erickson et al. (2023), the lack of statistical difference in DMI of dairy cows consuming increasing levels of CP can be attributed to the short duration of the treatment period or to the small magnitude of dietary changes, which might have happened herein. Additionally, when varying MP contents of diets, DMI are found to increase only when animals are deficient in ME (Newbold, 1994; Sinclair et al., 2014), which was not observed herein. In this study, despite no statistical difference for ADG, it presented a greater numerical difference of 13.4% and 8.60% for Medium MP and High MP, respectively, when compared to Low MP. It suggests that greater MP contents along with greater starch and energy supply, respectively for Medium and High MP, potentially facilitated greater whole-body protein synthesis for these dietary treatments when compared to Low MP. However, the duration of the experimental periods may have prevented the detection of significant statistical differences. Moreover, the duration of

eating and ruminating time remained constant across dietary treatments, which is similar to results observed by Savari et al. (2018) when multiparous cows were fed different RDP:RUP ratios based on a constant CP level across treatment. Furthermore, no variation in ruminal pH was observed. Herein, the consistent DMI, along with no alteration in eating and ruminating times as well as in ruminal pH across treatments suggest maintenance of similar dietary conditions. Combined, this data underscores the establishment of a stable feeding pattern within the experimental regime, where animals were fed every 2 hours on collection days. This approach effectively kept consistent intake, supporting stable rumination and fermentation processes (Beauchemin, 2018).

VFA are the main product of rumen microbial activity. Greater total VFA (mmol) (**Table 4**) was observed at Medium MP supply (quadratic component, P <0.01), indicating an increased fermentation end-product accumulation (Cholewińska et al., 2020). This result is mainly driven by greater butyrate molar percentage due to slightly greater starch fed to this dietary treatment. For High MP, increased Acetate, and a tendency to decreased Propionate were observed, which in consequence resulted in greater Acetate:Propionate ratio. The latter was a response to a slight change in dietary NDF fed for treatments with higher levels of MP, resulting in increased overall acetate production (Lean et al., 2014). Moreover, Valerate and BCVFA (iso-Butyrate and iso-Valerate) linearly decreased along with increased MP supply due to the decrease in RDP. When ruminal ammonia is limited, BCVFA production occurs upon ruminal protein catabolism of BCAA (Val, Leu, and Ile) (Firkins, 2021); these AA will then be deaminated and their carbon skeleton will be incorporated into VFA rather than being used for protein synthesis (Bach et al., 2005). Thus, it represents an inefficient utilization of AA, which was expected herein due to the RDP surplus imposed on Low MP treatment. Similarly, Erickson et al. (2024) when fed late lactation dairy cows with different levels of CP, observed greater BCVFA production in the treatment with

the greater CP content. Moreover, for Broderick and Reynal (2009), greater total BCVFA were observed for lactating Holstein cows when greater dietary RDP from soybean meal was fed instead of partially replaced with urea (1.2, 2.4, or 3.7% RDP), supporting the greater AA utilization at the expense of N source. Overall, these results along with no alteration in ruminal pH shows that although fermentation processes were not impaired, differences in fermentation end-products across treatments occurred. It indicates that a supply of N and rumen degradable carbohydrates were achieved even though different metabolic routes might have supported microbial fermentation. In other words, for Low MP, due to an adaptative response, greater AA breakdown supported microbial fermentation, however an inefficient use of dietary AA occurred.

Table 4. Ruminal pH and VFA of dairy heifers fed Low, Medium, and High MP diets.

	Treatments				*P*[2]	
Item	Low MP	Medium MP	High MP	SEM[1]	Linear	Quadratic
pH	6.4	6.4	6.4	0.02	0.75	0.75
Total VFA, mmol/L	108	114	102	1.6	0.07	<0.01
Acetate, %	63.4	63.1	64.5	0.19	0.01	0.03
Propionate, %	17.7	17.7	17.2	0.12	0.07	0.30
iso-Butyrate, %	1.55	1.46	1.39	0.016	<0.01	0.93
Butyrate, %	12.8	13.3	12.5	0.10	0.10	<0.01
iso-Valerate, %	1.24	1.13	1.04	0.019	<0.01	0.63
Valerate, %	1.93	1.89	1.84	0.019	0.02	0.86
Caproate, %	1.35	1.33	1.41	0.024	0.18	0.13
Acetate:Propionate	3.57	3.57	3.75	0.032	<0.01	0.14

[1]Standard error of the mean.
[2]Probability corresponds to the null hypothesis, with linear and quadratic contrasts.

N metabolism and efficiency

For High MP, the low dietary supply of soluble N for ruminal degradation decreased rumen ammonia and urea contents (**Table 5**). In ruminants, endogenous urea flows with the bloodstream and can enter the rumen through secretion in saliva, or direct transfer from the bloodstream through

the ruminal wall (Reynolds & Kristensen, 2008; Zhong et al., 2022). BUN concentration is correlated with salivary urea and saturation of salivary urea transports, such as UT-B transports, indicating a limitation in the capacity of saliva urea secretion (Muscher et al., 2010). Therefore, due to the transfer limitation to this channel, the primary mechanism of urea transfer is direct transport across the rumen wall (Zhong et al., 2022) which allows urea to move passively towards a concentration gradient (Zhong et al., 2022). Ammonia is the primary N source for MCP synthesis by ruminal microorganisms; with a minimum concentration requirement of 5 mg/dL (Satter & Roffler, 1975). For High MP treatment, an average of 4.51 mg/dL of ammonia in the rumen was observed. In vitro studies showed that due to a mechanism of active ammonia transport into rumen microbes, high intracellular ammonia concentration could be reached even when ammonia concentration in the medium was close to zero (Marini & Van Amburgh, 2003; Russell & Strobel, 1987). Thus, it might indicate that a greater portion of N recycled towards animal metabolism was being immediately hydrolyzed and incorporated into MCP rather than being excreted, as observed in the High MP treatment. Consequently, lower rumen urea concentration found for High MP demonstrates that rapid incorporation to MCP was probably occurring, as previously mentioned, allowing greater utilization of endogenous urea flux, increasing N efficient use, and decreasing N excretion. Further, the lesser plasma ammonia concentration observed for High MP treatment was efficiently cleared by the liver, and along with the enhanced hepatic extraction of certain AA (Raggio et al., 2004) led to a greater N efficient use for heifers fed this dietary treatment. For Low MP treatment, greater ruminal ammonia indicates that ruminal microorganisms have reached their capacity to incorporate ammonia into MCP, which was expected due to the greater RDP level supplied in this dietary treatment. Additionally, greater plasma urea concentrations suggest an excess of N in Low MP. Based on the same numerical salivary urea content for Low and Medium

MP, it indicates that saturation of salivary urea transporters for Low MP has occurred, which is supported by studies in goats, where it was found that for every 1 µM increase in BUN concentration, there was a corresponding 0.73 µM increase in salivary urea content (Muscher et al., 2010). Consequently, for Low MP N surplus was excreted rather than effectively utilized (Lobley et al., 2000), increasing the N load to the environment. Furthermore, the lower BUN and excretion outputs along with greater N balance for High MP, indicates that decreasing dietary RDP increased N efficiency (Bahrami-Yekdangi et al., 2016; Kaufman et al., 2017). Thus, even though RDP is the main source of N for MCP synthesis, recycled urea-N may buffer the rumen if dietary RDP supply is insufficient (Batista et al., 2017; Firkins et al., 2007), increasing N incorporation into MCP.

Table 5. Parameters of N metabolism and balance in dairy heifers fed Low, Medium, and High MP diets.

	Treatments				P^2	
Item	Low MP	Medium MP	High MP	SEM[1]	Linear	Quadratic
Urea Nitrogen						
Rumen, mg/dL	0.72	0.78	0.21	0.074	<0.01	0.04
Saliva, mg/dL	13.0	13.0	10.3	0.44	<0.01	0.12
Plasma, mg/dL	19.7	17.8	14.5	0.42	<0.01	0.41
Urine, g/L	21.5	19.8	19.6	0.30	<0.01	0.23
Ammonia Nitrogen						
Rumen, mg/dL	7.93	7.72	4.51	0.213	<0.01	<0.01
Saliva, mg/dL	3.23	2.00	2.54	0.213	0.18	0.05
Plasma, mg/dL	2.35	3.07	3.03	0.104	<0.01	0.04
Urine, mg/dL	2.79	2.77	2.97	0.069	0.26	0.42
Nitrogen						
Intake, g/d	250	278	261	3.0	0.02	<0.01
Apparent Digestibility, %	69.4	71.3	68.4	1.26	0.75	0.37
Fecal output, g/d	85.1	78.6	81.4	0.92	0.01	<0.01
Urine output, g/d	90.9	96.1	74.9	2.25	<0.01	<0.01
N Balance, g/d	88.0	103	104	2.97	0.01	0.19

[1]Standard error of the mean.
[2]Probability corresponds to the null hypothesis, with linear and quadratic contrasts.

When heifers were fed different levels of RUP relative to a fixed CP, 51% of RUP at 15% of CP it resulted in greater N retention along with lesser MCP synthesis than heifers fed 38% of RUP (Silva et al., 2018b). Therefore, since milk protein content positively responds to ruminal MCP synthesis efficiency (Cant et al., 2018), further studies evaluating similar conditions in dairy cows would elucidate its impact on nutrient utilization and milk synthesis.

It is known that MCP is composed of an average of 82.4% of AA in its CP (Sok et al., 2017). Additionally, due to its high apparent digestibility and EAA composition (Schwab & Broderick, 2017), it is more efficient in providing AA required by ruminants for growth and production rather than RUP. Therefore, AA influx and plasma AA concentration were evaluated herein. Greater plasma entry rates of His was observed as the supply of MP increased (**Table 6**), due to a greater concentration of this AA in particular from RUP (Supplementary Table 2), rather than in MCP (NRC, 2001). Plasma AA concentrations reflect the balance between the MP supply and utilization of AA, and thus its elevation typically occurs only when absorption or protein turnover release exceeds body utilization rates (Clark, 1975).

Table 6. Plasma AA entry rates of dairy heifers fed Low, Medium, and High MP diets.

	Treatments				*P*[2]	
Item	Low MP	Medium MP	High MP	SEM[1]	Linear	Quadratic
Essential AA						
His, g/d	96	114	114	4.21	0.04	0.14
Ile, g/d	168	183	198	7.6	0.21	0.97
Leu, g/d	136	139	151	5.0	0.40	0.74
Lys, g/d	169	167	178	8.0	0.62	0.67
Met, g/d	25.4	19.8	26.3	1.49	0.79	0.06
Phe, g/d	111	132	119	5.8	0.61	0.21
Non-essential AA						
Ala, g/d	139	148	152	4.2	0.44	0.84
Gly, g/d	429	424	401	4.5	0.47	0.77
Pro, g/d	122	132	122	3.9	0.97	0.22
Ser, g/d	373	336	344	20.9	0.77	0.76

Tyr, g/d	120	138	129	7.89	0.74	0.58

[1]Standard error of the mean.
[2]Probability corresponds to the null hypothesis, with linear and quadratic contrasts.

Overall, plasma AA concentration presented a linear decrease (Lys, Met, Thr, and Ala) or a tendency for a linear decrease (His, Gly, and Ser) as levels of MP increased (**Table 7**). Previous studies have suggested a negative correlation between the AA profile of RUP and plasma concentrations of Ala, Thr, Ser, and Gly (Swanepoel et al., 2016). This correlation was observed across data collected from 20 dairy commercial facilities. According to the authors, it indicates that greater dietary RUP levels, at the expense of MCP, do not supply adequate amounts of these AA in particular. However, we refute this hypothesis once no such increase was observed in the entry rates of Ala, Thr, Ser, and Gly, in the High MP treatment, where greater RUP levels were fed. Alternatively, excess of circulating AA could also have been removed by mass action kinetics in the liver (Hanigan et al., 1998). Further, Raggio et al. (2004) found greater hepatic removal of His, Met, Phe, and Thr in lactating cows fed increased MP diets, which is proved by the fact that enzymes responsible for AA catabolism of His, Met, and Phe are almost exclusively restricted to hepatic tissues (Lobley, 2003; Raggio et al., 2004). These results align with the data observed herein, except for Phe, which only had a numerical increase in plasma AA concentration at High MP supply. Therefore, considering the intricate interactions among AA, it is prudent to evaluate them collectively rather than in isolation (Swanepoel et al., 2015), as well as the antagonistic and synergistic relationships that exist among them (Haque et al., 2013).

Based on our findings, lesser plasma AA concentration in High MP is likely attributed to the combination of increased dietary energy supply and high MP, which could have enhanced AA uptakes to support whole-body protein synthesis. However, as previously discussed, the short duration of periods employed in this experiment might have prevented a statistical difference in

ADG among treatments. Notably, a greater numerical difference was observed, with High MP treatment presenting greater ADG than Low MP. Similarly, when heifers at different physiological stages were fed for 84 d with the same RUP supply that High MP treatment (51%), they presented greater BW, ADG, and feed efficiency regardless of physiological stage (Silva et al., 2018a). Thus, if an extended duration of periods were used, it's plausible that such differences would have become more evident.

Moreover, when available energy intake and AA are fed it promotes greater whole-body protein synthesis in growing calves (Rius et al., 2012) and multiparous cows (Kaufman et al., 2018). The latter is likely observed along with greater circulating insulin levels, which is considered an anabolic signal of increased whole-body protein synthesis, increasing uptakes of circulating AA and consequently decreasing plasma AA concentration (Kaufman et al., 2018; Silva et al., 2018a).

Table 7. Plasma AA concentrations of dairy heifers fed Low, Medium, and High MP diets.

	Treatments				*P*[2]	
Item	Low MP	Medium MP	High MP	SEM[1]	Linear	Quadratic
Essential AA						
Arg, µM	91.2	86.1	84.5	2.19	0.19	0.67
His, µM	67.2	55.0	50.4	3.79	0.08	0.65
Ile, µM	136	134	138	2.4	0.79	0.59
Leu, µM	162	167	173	3.4	0.20	0.94
Lys, µM	113	104	99.9	3.08	0.03	0.65
Met, µM	38.2	36.2	34.9	0.58	0.01	0.71
Phe, µM	48.8	48.3	50.3	1.09	0.60	0.61
Thr, µM	29.4	26.1	22.0	1.53	0.01	0.84
Val, µM	286	285	291	4.6	0.68	0.74
Non-essential AA						
Ala, µM	226	200	191	6.1	0.02	0.50
Asn, µM	78.5	77.3	76.8	0.81	0.22	0.76
Asp, µM	2.13	2.12	1.98	0.267	0.82	0.90
Gln, µM	196	193	185	3.0	0.13	0.64
Glu, µM	69.8	61.4	65.6	3.14	0.55	0.32
Gly, µM	177	162	162	4.6	0.08	0.33

Pro, µM	79.1	73.5	72.8	2.00	0.11	0.45
Ser, µM	65.5	60.3	58.7	1.90	0.09	0.57
Tyr, µM	39.7	36.9	38.1	1.24	0.51	0.35

[1]Standard error of the mean.
[2]Probability corresponds to the null hypothesis, with linear and quadratic contrasts.

Therefore, the findings presented herein outline a greater efficient N utilization pathway observed by animals fed High MP diets. As such, increased costs associated with feeding ingredients with greater RUP content must be taken into consideration to increase animals' efficiency and sustainability of dairy systems. Further studies using lactating animals are necessary for a more comprehensive understanding of these processes and their implications.

CONCLUSION

Our results described responses on performance, rumen fermentation and metabolism of dairy heifers fed increasing levels of MP within a fixed level of CP. No alterations were observed in animals' performance and activities across dietary treatments. Lesser RDP content promoted greater N utilization for metabolic functions, enhancing N efficiency and decreasing N excretion via urine. In contrast, greater RDP supply increased BCVFA by BCAA breakdown, outlining a wasteful use of dietary N. Overall, the findings presented herein point out that requirements of RDP supply for dairy growing cattle could be lower than previously recognized. However, to fully understand the implications of these strategies, additional studies are warranted to assess their long-term impacts, as well as their applicability to lactating animals. Nonetheless, by feeding the same CP level across treatments, we could prove that N efficiency is variable, and BUN has the potential to boost MCP synthesis when a shortfall of RDP is imposed. These results reaffirm the potential of improvements in N efficiency prediction and encourage these strategies in the mitigation of environmental impact through reduced N excretion. As such, this outcome must be

properly addressed by protein evaluation systems to successfully predict animals' needs as well as promote financial and environmental improvements.

REFERENCES

Bach, A., Calsamiglia, S., & Stern, M. D. (2005). Nitrogen metabolism in the rumen. Journal of Dairy Science, 88, E9-E21. https://doi.org/10.3168/jds.2008-1884

Bahrami-Yekdangi, M., Ghorbani, G., Khorvash, M., Khan, M., & Ghaffari, M. (2016). Reducing crude protein and rumen degradable protein with a constant concentration of rumen undegradable protein in the diet of dairy cows: Production performance, nutrient digestibility, nitrogen efficiency, and blood metabolites. Journal of animal science, 94(2), 718-725. https://doi.org/10.2527/jas.2015-9947

Batista, E., Detmann, E., Valadares Filho, S., Titgemeyer, E., & Valadares, R. (2017). The effect of CP concentration in the diet on urea kinetics and microbial usage of recycled urea in cattle: a meta-analysis. animal, 11(8), 1303-1311. https://doi.org/10.1017/S1751731116002822

Beauchemin, K. (2018). Invited review: Current perspectives on eating and rumination activity in dairy cows. Journal of Dairy Science, 101(6), 4762-4784. https://doi.org/10.3168/jds.2017-13706

Benchaar, C., Hassanat, F., Beauchemin, K. A., Ouellet, D. R., Lapierre, H., & Côrtes, C. (2023). Effect of metabolizable protein supply on milk performance, ruminal fermentation, apparent total-tract digestibility, energy and nitrogen utilization, and enteric methane production of Ayrshire and Holstein Cows. Animals, 13(5), 832. https://doi.org/10.3390/ani13050832

Boucher, S. E., Calsamiglia, S., Parsons, C. M., Stein, H. H., Stern, M. D., Erickson, P. S., Utterback, P. L., & Schwab, C. G. (2009). Intestinal digestibility of amino acids in rumen undegradable protein estimated using a precision-fed cecectomized rooster bioassay: I. Soybean meal and SoyPlus. Journal of Dairy Science, 92(9), 4489-4498. https://doi.org/10.3168/jds.2008-1884

Broderick, G. A., & Reynal, S. M. (2009). Effect of source of rumen-degraded protein on production and ruminal metabolism in lactating dairy cows. Journal of Dairy Science, 92(6), 2822-2834. https://doi.org/10.3168/jds.2008-1865

Calder, A. G., Garden, K. E., Anderson, S. E., & Lobley, G. E. (1999). Quantitation of blood and plasma amino acids using isotope dilution electron impact gas chromatography/mass spectrometry with U-13C amino acids as internal standards. Rapid Communications in Mass Spectrometry, 13(21), 2080-2083. https://doi.org/10.1002/(sici)1097-0231(19991115)13:21<2080::aid-rcm755>3.0.co;2-o

Cant, J. P., Kim, J. J., Cieslar, S. R., & Doelman, J. (2018). Symposium review: Amino acid uptake by the mammary glands: Where does the control lie? Journal of Dairy Science, 101(6), 5655-5666. https://doi.org/10.3168/jds.2017-13844

Castro, S. B., Phillip, L., Lapierre, H., Jardon, P., & Berthiaume, R. (2007). Ruminal degradability and intestinal digestibility of protein and amino acids in treated soybean meal products. Journal of Dairy

Science, 90(2), 810-822. https://doi.org/10.3168/jds.2009-2883

Chaney, A. L., & Marbach, E. P. (1962). Modified Reagents for Determination of Urea and Ammonia. Clinical Chemistry, 8(2), 130-132. https://doi.org/10.1093/clinchem/8.2.130

Cholewińska, P., Czyż, K., Nowakowski, P., & Wyrostek, A. (2020). The microbiome of the digestive system of ruminants – a review. Animal Health Research Reviews, 21(1), 3-14. https://doi.org/10.1017/s1466252319000069

Clark, J. H. (1975). Lactational responses to postruminal administration of proteins and amino acids. Journal of Dairy Science, 58(8), 1178-1197. https://doi.org/10.3168/jds.S0022-0302(75)84696-0

Erickson, M., Barros, T., Aguerre, M., Colmenero, J. O., Bertics, S., & Wattiaux, M. (2024). Reducing dietary crude protein: effects on digestibility, N balance, and blood metabolites in late-lactation Holstein cows. Journal of Dairy Science. https://doi.org/10.3168/jds.2023-24079

Erickson, M. G., Zanton, G. I., & Wattiaux, M. A. (2023). Dynamic lactation responses to dietary crude protein oscillation in diets adequate and deficient in metabolizable protein in Holstein cows. Journal of Dairy Science, 106(12), 8774-8786. https://doi.org/10.3168/jds.2023-23603

Estes, K. A., White, R. R., Yoder, P. S., Pilonero, T., Schramm, H., Lapierre, H., & Hanigan, M. D. (2018). An in vivo stable isotope–based approach for assessment of absorbed amino acids from individual feed ingredients within complete diets. Journal of Dairy Science, 101(8), 7040-7060. https://doi.org/10.3168/jds.2017-13447

Firkins, J. (2021). Invited Review: Advances in rumen efficiency. Applied Animal Science, 37(4), 388-403. https://doi.org/10.15232/aas.2021-02163

Firkins, J. L., Yu, Z., & Morrison, M. (2007). Ruminal Nitrogen Metabolism: Perspectives for Integration of Microbiology and Nutrition for Dairy. Journal of Dairy Science, 90, E1-E16. https://doi.org/10.3168/jds.2006-518

Gill, J. L. (1978). Design and analysis of experiments in the animal and medical sciences. (No Title).

Hanigan, M., Cant, J., Weakley, D., & Beckett, J. (1998). An evaluation of postabsorptive protein and amino acid metabolism in the lactating dairy cow. Journal of Dairy Science, 81(12), 3385-3401. https://doi.org/10.3168/jds.S0022-0302(98)75903-X

Hanigan, M. D., Souza, V. C., Martineau, R., Daley, V. L., & Kononoff, P. (2021). Predicting ruminally undegraded and microbial protein flows from the rumen. J Dairy Sci, 104(8), 8685-8707. https://doi.org/10.3168/jds.2020-19672

Haque, M. N., Rulquin, H., & Lemosquet, S. (2013). Milk protein responses in dairy cows to changes in postruminal supplies of arginine, isoleucine, and valine. Journal of Dairy Science, 96(1), 420-430. https://doi.org/10.3168/jds.2012-5610

Hristov, A. N., Bannink, A., Crompton, L. A., Huhtanen, P., Kreuzer, M., McGee, M., Nozière, P.,

Reynolds, C. K., Bayat, A. R., Yáñez-Ruiz, D. R., Dijkstra, J., Kebreab, E., Schwarm, A., Shingfield, K. J., & Yu, Z. (2019). Invited review: Nitrogen in ruminant nutrition: A review of measurement techniques. Journal of Dairy Science, 102(7), 5811-5852. https://doi.org/10.3168/jds.2018-15829

Huang, X., Estes, K. A., Yoder, P. S., Wang, C., Jiang, N., Pilonero, T., & Hanigan, M. D. (2019). Assessing availability of amino acids from various feedstuffs in dairy cattle using a stable isotope-based approach. Journal of Dairy Science, 102(12), 10983-10996. https://doi.org/10.3168/jds.2019-16597

Kalscheur, K., Vi, R. B., Glenn, B., & Kohn, R. (2006). Milk production of dairy cows fed differing concentrations of rumen-degraded protein. Journal of Dairy Science, 89(1), 249-259. https://doi.org/10.3168/jds.s0022-0302(06)72089-6

Kaufman, J., Pohler, K., Mulliniks, J., & Ríus, A. (2018). Lowering rumen-degradable and rumen-undegradable protein improved amino acid metabolism and energy utilization in lactating dairy cows exposed to heat stress. Journal of Dairy Science, 101(1), 386-395. https://doi.org/10.3168/jds.2017-13341

Kaufman, J. D., Kassube, K. R., & Ríus, A. G. (2017). Lowering rumen-degradable protein maintained energy-corrected milk yield and improved nitrogen-use efficiency in multiparous lactating dairy cows exposed to heat stress. Journal of Dairy Science, 100(10), 8132-8145. https://doi.org/10.3168/jds.2017-13026

Kuznetsova, A., Brockhoff, P. B., & Christensen, R. H. (2017). lmerTest package: tests in linear mixed effects models. Journal of Statistical Software, 82, 1-26. https://doi.org/https://doi.org/10.18637/jss.v082.i13

Lapierre, H., Martineau, R., Hanigan, M. D., van Lingen, H. J., Kebreab, E., Spek, J. W., & Ouellet, D. R. (2020). Review: Impact of protein and energy supply on the fate of amino acids from absorption to milk protein in dairy cows. animal, 14(S1), s87-s102. https://doi.org/10.1017/S1751731119003173

Lean, I. J., Golder, H. M., & Hall, M. B. (2014). Feeding, evaluating, and controlling rumen function. Veterinary Clinics: Food Animal Practice, 30(3), 539-575. https://doi.org/10.1016/j.cvfa.2014.07.003

Lobley, G., Bremner, D., & Zuur, G. (2000). Effects of diet quality on urea fates in sheep as assessed by refined, non-invasive [15N15N] urea kinetics. British Journal of Nutrition, 84(4), 459-468. https://doi.org/10.1017/s0007114500001768

Lobley, G. E. (2003). Protein turnover—what does it mean for animal production? Canadian Journal of Animal Science, 83(3), 327-340. https://doi.org/10.4141/a03-019

Marini, J. C., & Van Amburgh, M. E. (2003). Nitrogen metabolism and recycling in Holstein heifers12.

Journal of animal science, 81(2), 545-552. https://doi.org/10.2527/2003.812545x

Mass, R., Jordon, D., Scott, T., & Klopfenstein, T. J. (2000). Metabolizable protein estimates of treated soybean meal products. Nebraska Beef Cattle Reports, 379.

Muscher, A., Schröder, B., Breves, G., & Huber, K. (2010). Dietary nitrogen reduction enhances urea transport across goat rumen epithelium. Journal of animal science, 88(10), 3390-3398. https://doi.org/10.2527/jas.2010-2949

NASEM. (2021). National Academies of Sciences; Medicine.

Newbold, J. (1994). Practical application of the metabolisable protein system. Practical application of the metabolisable protein system., 231-264.

NRC. (2001). Nutrient Requirements of Dairy Cattle. National Research Council, Washington, DC.

Raggio, G., Pacheco, D., Berthiaume, R., Lobley, G. E., Pellerin, D., Allard, G., Dubreuil, P., & Lapierre, H. (2004). Effect of Level of Metabolizable Protein on Splanchnic Flux of Amino Acids in Lactating Dairy Cows. Journal of Dairy Science, 87(10), 3461-3472. https://doi.org/10.3168/jds.s0022-0302(04)73481-5

Reynal, S. M., & Broderick, G. A. (2005). Effect of Dietary Level of Rumen-Degraded Protein on Production and Nitrogen Metabolism in Lactating Dairy Cows. Journal of Dairy Science, 88(11), 4045-4064. https://doi.org/10.3168/jds.s0022-0302(05)73090-3

Reynolds, C., & Kristensen, N. B. (2008). Nitrogen recycling through the gut and the nitrogen economy of ruminants: an asynchronous symbiosis. Journal of animal science, 86(suppl_14), E293-E305. https://doi.org/10.2527/jas.2007-0475

Rius, A. G., Weeks, H. A., Cyriac, J., Akers, R. M., Bequette, B. J., & Hanigan, M. D. (2012). Protein and energy intakes affected amino acid concentrations in plasma, muscle, and liver, and cell signaling in the liver of growing dairy calves. Journal of Dairy Science, 95(4), 1983-1991. https://doi.org/10.3168/jds.2011-4688

Russell, J. B., & Strobel, H. J. (1987). Concentration of ammonia across cell membranes of mixed rumen bacteria. Journal of Dairy Science, 70(5), 970-976. https://doi.org/10.3168/jds.s0022-0302(87)80101-7

Satter, L., & Roffler, R. (1975). Nitrogen requirement and utilization in dairy cattle. Journal of Dairy Science, 58(8), 1219-1237. https://doi.org/10.3168/jds.s0022-0302(75)84698-4

Savari, M., Khorvash, M., Amanlou, H., Ghorbani, G. R., Ghasemi, E., & Mirzaei, M. (2018). Effects of rumen-degradable protein:rumen-undegradable protein ratio and corn processing on production performance, nitrogen efficiency, and feeding behavior of Holstein dairy cows. Journal of Dairy Science, 101(2), 1111-1122. https://doi.org/10.3168/jds.2017-12776

Schwab, C. G., & Broderick, G. A. (2017). A 100-Year Review: Protein and amino acid nutrition in dairy

cows. Journal of Dairy Science, 100(12), 10094-10112. https://doi.org/10.3168/jds.2017-13320

Silva, A., Detmann, E., Dijkstra, J., Pedroso, A., Silva, L., Machado, A., Sousa, F., Dos Santos, G., & Marcondes, M. (2018a). Effects of rumen-undegradable protein on intake, performance, and mammary gland development in prepubertal and pubertal dairy heifers. Journal of Dairy Science, 101(7), 5991-6001. https://doi.org/10.3168/jds.2017-13230

Silva, A. L., Detmann, E., Rennó, L. N., Pedroso, A. M., Fontes, M. M. S., Morais, V. C., Sguizzato, A. L. L., Abreu, M. B., Rotta, P. P., & Marcondes, M. I. (2018b). Effects of rumen undegradable protein on intake, digestibility and rumen kinetics and fermentation characteristics of dairy heifers. Animal Feed Science and Technology, 244, 1-10. https://doi.org/https://doi.org/10.1016/j.anifeedsci.2018.07.019

Sinclair, K., Garnsworthy, P., Mann, G., & Sinclair, L. (2014). Reducing dietary protein in dairy cow diets: Implications for nitrogen utilization, milk production, welfare and fertility. animal, 8(2), 262-274. https://doi.org/10.1017/S1751731113002139

Soetaert, K. E. R., & Petzoldt, T. (2010). Inverse modelling, sensitivity and Monte Carlo analysis in R using package FME. Journal of statistical software, 33(3). https://doi.org/10.18637/jss.v033.i03

Sok, M., Ouellet, D., Firkins, J., Pellerin, D., & Lapierre, H. (2017). Amino acid composition of rumen bacteria and protozoa in cattle. Journal of Dairy Science, 100(7), 5241-5249. https://doi.org/10.3168/jds.2016-12447

Souza, V. C., Aguilar, M., Van Amburgh, M., Nayananjalie, W. A. D., & Hanigan, M. D. (2021). Milk urea nitrogen variation explained by differences in urea transport into the gastrointestinal tract in lactating dairy cows. Journal of Dairy Science, 104(6), 6715-6726. https://doi.org/10.3168/jds.2020-19787

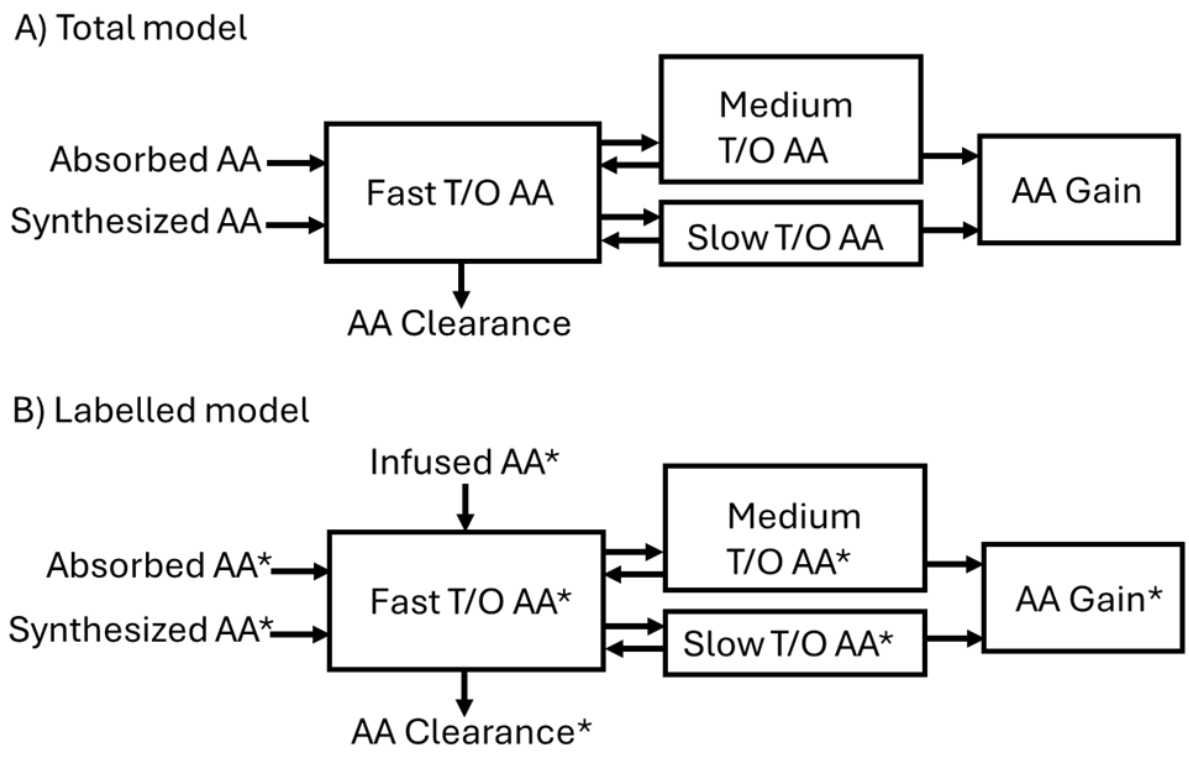
Swanepoel, N., Robinson, P., & Erasmus, L. J. (2016). Rumen microbial protein flow and plasma amino acid concentrations in early lactation multiparity Holstein cows fed commercial rations, and some relationships with dietary nutrients. Livestock Science, 190, 58-69. https://doi.org/10.1016/j.livsci.2016.06.001

Thai, K. E., Taylor, M. W., Fernandes, T., Akinade, E. A., & Campbell, S. L. (2023). Topiramate alters the gut microbiome to aid in its anti-seizure effect. Frontiers in Microbiology, 14. https://doi.org/10.3389/fmicb.2023.1242856

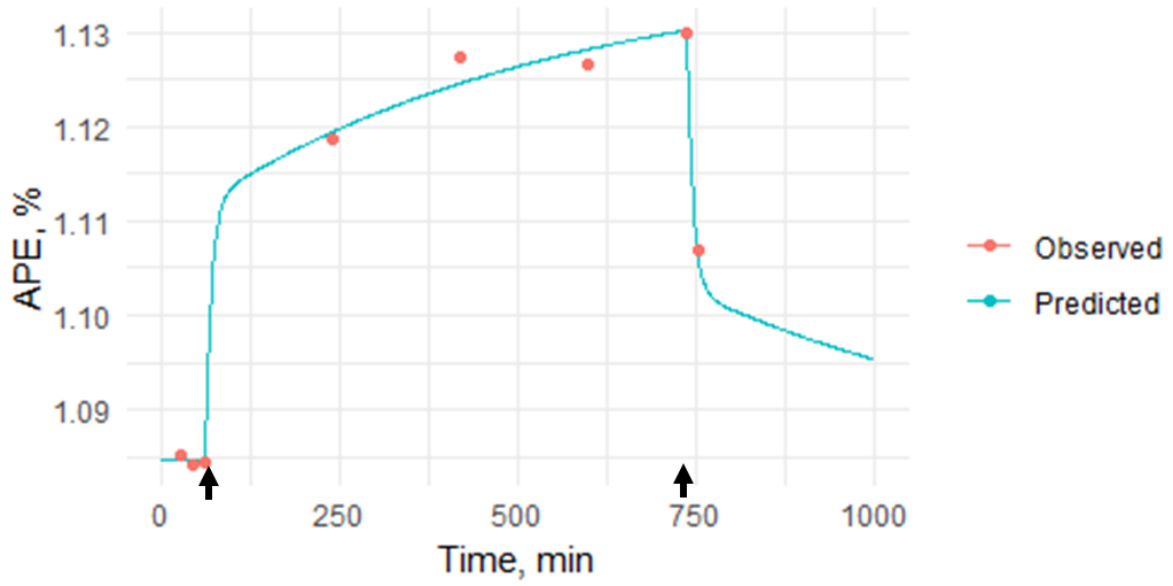
Walsh, R. G., He, S., & Yarnes, C. T. (2014). Compound-specific δ¹³C and δ¹⁵N analysis of amino acids: a rapid, chloroformate-based method for ecological studies. Rapid Communications in Mass Spectrometry, 28(1), 96-108. https://doi.org/10.1002/rcm.6761

Zhong, C., Long, R., & Stewart, G. S. (2022). The role of rumen epithelial urea transport proteins in urea

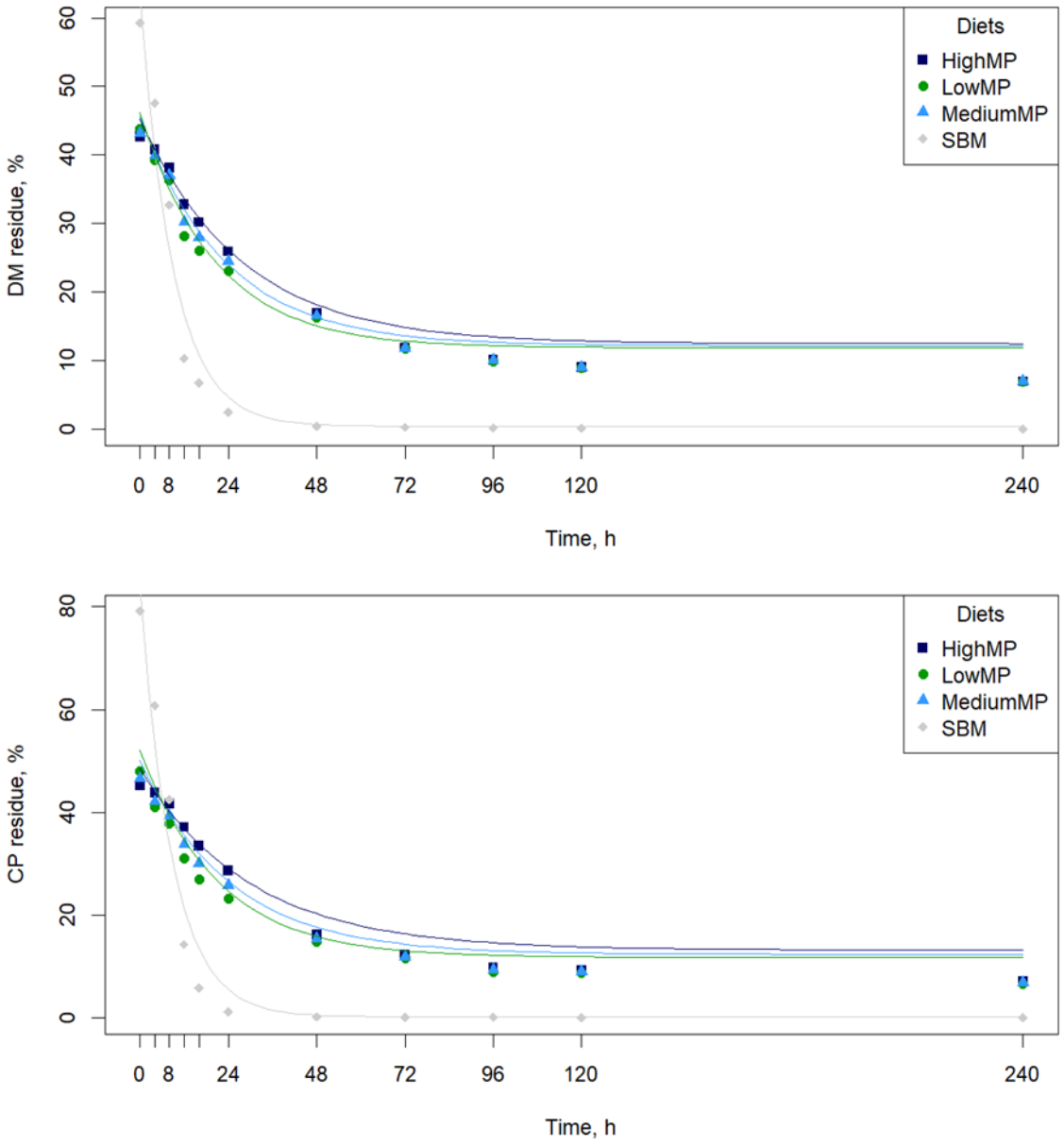
nitrogen salvage: A review. Animal Nutrition, 9, 304-313. https://doi.org/10.1016%2Fj.aninu.2022.01.008



Supplementary Figure 1. A schematic representation of the model used to derive AA entry rates. The model was fit to plasma AA enrichment data for each AA.



Supplementary Figure 1. Predicted and observed Lys atomic percentage enrichment (13C/12C) over infusion time for one infusion. Arrows indicate the infusion start and end time.



Supplementary Figure 2. In situ DM and CP degradation of dietary treatments. SBM = soybean meal as a reference.

Supplementary Table 1. Citations for relevant Cumberland Valley analytical wet chemistry procedures for feed analyses.

Analysis	Citation	Method Number	Modifications or Further Details
ADF	(Horwitz and Latimer, 2000)	973.18	Whatman 934-AH glass micro-fiber filters with 1.5 µM particle retention used in place of fritted glass crucible
Ash	(Horwitz and Latimer, 2000)	942.05	1.5 g sample weight, 4 h ash time, hot weigh
CP	(Horwitz and Latimer, 2000)	990.03	Leco FP-528 Nitrogen Combustion Analyzer (Leco, 3000 Lakeview Av, St. Joseph, MI, USA)
DM	Step 1:(Goering and Van Soest, 1970); Step 2: (Undersander et al., 1993)	-	Second step, modified to Modified to 105 °C for 3 h
Fatty Acid Profiles	(Sukhija and Palmquist, 1988)	-	-
Minerals	(Horwitz and Latimer, 2000)	985.01	Ash 0.35g sample for 1 h at 535 °C. Digest in open crucibles for 20 min in 15% nitric acid on hot plate. Samples diluted to 50 mL.; Analysis on Perkin Elmer 5300 DV ICP (Perkin Elmer, 710 Bridgeport Avenue, Shelton, CT, USA)
NDF	(Van Soest et al., 1991)	-	Whatman 934-AH glass micro-fiber filters with 1.5 µM particle retention
Starch	(Hall, 2009)	-	Corrected for free glucose as described in citation.

Supplementary Table 2. Predicted duodenal AA flows of diets (NASEM, 2021).

	Metabolizable EAA					
	From RUP		From MiCP		Total	
AA, g/d	Mean	SD	Mean	SD	Mean	SD
Low MP						
Arg	29.5	4.59	40.3	3.56	69.8	6.94
His	11.8	2.04	16.2	1.47	28.3	2.88
Ile	22.0	3.29	51.3	4.72	73.3	6.89
Leu	38.2	5.71	68.2	6.11	106	10.0
Lys	26.3	4.27	69.5	6.16	95.7	8.52
Met	6.67	1.03	19.5	1.87	26.2	2.40
Phe	23.3	3.67	46.5	4.42	70.0	6.54
Thr	18.8	2.99	46.0	4.00	64.8	5.91
Trp	6.00	0.63	10.0	0.89	16.2	1.72
Val	6.00	0.63	50.7	4.46	74.5	6.89
Medium MP						
Arg	39.7	5.54	37.0	4.47	76.5	10.2
His	15.3	2.25	14.8	1.94	30.3	3.78
Ile	28.3	3.98	47.3	5.65	75.5	9.50
Leu	48.2	7.08	62.5	7.74	110.8	14.3
Lys	34.7	4.76	64.0	7.97	98.3	12.5
Met	8.50	1.38	17.7	2.34	26.3	3.27
Phe	30.0	4.34	42.5	5.24	72.5	9.50
Thr	24.0	3.29	42.3	5.20	66.2	8.75
Trp	7.83	1.17	9.33	1.21	17.0	2.10
Val	7.83	1.17	46.3	5.65	76.8	10.1
High MP						
Arg	52.5	6.19	30.3	3.39	82.7	9.33
His	20.0	2.00	12.3	1.37	32.2	3.66
Ile	36.8	4.12	38.8	4.36	75.5	8.26
Leu	61.3	6.92	51.5	5.68	113	12.5
Lys	45.2	5.04	52.5	5.68	97.7	10.9
Met	10.8	1.33	14.7	1.63	25.7	2.66
Phe	39.0	4.73	35.0	4.00	73.8	8.21
Thr	30.7	3.61	34.7	3.67	65.2	7.19
Trp	10.0	1.41	7.67	1.03	17.7	2.07
Val	10.0	1.41	38.2	3.97	77.2	8.57

REFERENCES (SUPPLEMENTARY MATERIAL)

Goering, H. K. and P. J. Van Soest. 1970. Forage fiber analyses (apparatus, reagents, procedures, and some applications). No. 379. US Agricultural Research Service.

Hall, M. B. 2009. Determination of starch, including maltooligosaccharides, in animal feeds: comparison of methods and a method recommended for AOAC collaborative study. J. AOAC Int. 92:42-49. https://doi.org/10.1093/jaoac/92.1.42.

Horwitz, W. and G. W. Latimer. 2000. Official methods of analysis of AOAC International. Vol. 1. AOAC international Gaithersburg.

Sukhija, P. S. and D. Palmquist. 1988. Rapid method for determination of total fatty acid content and composition of feedstuffs and feces. J. Agric. Food Chem. 36:1202-1206. https://doi.org/10.1021/jf00084a019.

Undersander, D., D. Mertens, and N. Thiex. 1993. Forage analysis procedures. National Forage Testing Association, Omaha, NE. 25.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583-3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2.

CHAPTER 3

IMPLICATIONS

This study will provide valuable insights for producers, researchers, and nutritionists by offering updated understanding of the mechanisms involved in nitrogen utilization in Holstein heifers. We demonstrated that adjusting the ratio between RDP and RUP can significantly impact the efficient utilization of nitrogen by ruminants, even with high level of CP. Increasing the proportion of RUP in diets high in protein content can effectively reduce nitrogen excretion into the environment, offering a promising strategy for enhancing environmental sustainability. However, the feasibility of implementing such diets must be carefully considered, particularly due to the elevated costs associated with incorporating high-quality amino acids found in RUP sources.

The findings presented herein underscore the critical role of nitrogen recycling in meeting microbial protein synthesis requirements, thereby contributing to a more sustainable dairy cattle industry. Nonetheless, further research is warranted to better understand the nuanced response of nitrogen utilization to different proportions of RDP and RUP in feed formulations, thereby improving the accuracy of predicting dairy cattle's nutritional protein requirements. Furthermore, this refinement holds promise for updating predictions of protein and other nutrient requirements for ruminants more broadly.

Given the intricate interplay of ruminal and post-ruminal factors, it is crucial to acknowledge that current techniques for evaluating protein metabolism lack precision and standardization. Previous studies align with our findings, indicating discrepancies between predicted and observed RDP and RUP values in diets, as recommended by the NASEM (2021). Therefore, refining evaluation methodologies and updating food libraries to include a broader range of ingredient evaluations are imperative steps towards advancing our understanding of protein metabolism in ruminants.