# RUMEN-PROTECTED METHIONINE SUPPLEMENTATION IMPROVES LACTATION, OXIDATIVE STATUS, AND IMMUNE RESPONSE DURING A SUBCLINICAL MASTITIS CHALLENGE IN LACTATING DAIRY COWS

BY

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THESIS ACCEPTANCE PAGE

Anita Paz

This thesis is approved as a creditable and independent investigation by a candidate for the master's degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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*-Anita Paz Lopez*

CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| 4EBP1 | eIF4E-binding protein 1 |
| AA | Amino acid |
| APPs | Acute phase proteins |
| APR | Acute phase response |
| BMEC | Bovine mammary ephitilial cells |
| CFU | Colony forming unit |
| CM | Clinical mastitis |
| CMT | California mastitis test |
| CON | Control |
| d | Days |
| DAMP | Damage-Associated Molecular Patterns |
| DIM | Days in milk |
| DM | Dry matter |
| DMI | Dry matter intake |
| EC | Electrical conductivity |
| GSH | Glutathione |
| h | Hour |
| IL-1 | Interleukin 1 |
| IL-6 | Interleukin 6 |
| IMI | Intramammary infection |
| Met | Methionine |
| MP | Metabolizable protein |
| mTOR | Mammalian target of rapamicin |
| PAMP | Pathogen-Associated Molecular Patterns |
| PMN | Polymorphonuclear Neutrophils |
| PRR | Patern recognition receptors |
| ROM | Reactive oxygen metabolites |
| ROS | Reactive oxygen species |

| | |
|---|---|
| RPM | Rumen protected methionine |
| S6RP | S6 ribosomal protein |
| SAA | Serum amyloid A |
| SAM | S-adenosyl methionine |
| SCC | Somatic cell count |
| SCCLS | Somatic cell count linear score |
| SCM | Subclinical mastitis |
| SM | Smartamine M |
| SMC | Subclinical mastitis challenge |
| TLR | Toll-like receptor |
| TNFα | Tumor necrosis factor-alpha |
| Trt | Treatment |

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# ABSTRACT

RUMEN-PROTECTED METHIONINE SUPPLEMENTATION IMPROVES LACTATION, OXIDATIVE STATUS, AND IMMUNE RESPONSE DURING A SUBCLINICAL MASTITIS CHALLENGE IN LACTATING DAIRY COWS

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Mastitis is a significant problem in the dairy industry, posing numerous challenges for dairy farmers worldwide. Mastitis refers to the inflammation of the mammary gland, primarily caused by bacterial infections. This condition affects the health and well-being of dairy cows and has severe economic implications for farmers and overall dairy production. The prevalence of mastitis substantially threatens milk quality, productivity, and profitability, making it a top concern in the dairy industry. Preventing mastitis in cows is crucial for maintaining the herd's health and ensuring high-quality milk production. Nutritional strategies have recently gained greater attention due to a direct impact on immune function and a large effect on mastitis prevention. This study evaluated the effects of rumen-protected methionine (RPM) during a subclinical mastitis challenge to enhance the immune system in dairy cows. Thirty-two multiparous Holstein cows were enrolled in a randomized complete block design and assigned to either a basal diet (CON; n=16) or a basal diet supplemented with rumen-protected methionine (SM; n=16, Smartamine M 0.09% DM). The dietary treatment was

administered to cows at -21 days relative to a SMC, and data were collected from 0 to 3 d relative to SMC. At 0 d relative to SMC, the mammary gland's rear right quarter was infused with 500,000 cfu of *Streptococcus uberis* (O140J). RPM did not prevent subclinical mastitis or showed lower SCC during SMC. However, it helped to maintain milk production, improved milk components and showed improvements in oxidative stress status. In addition, RPM supplementation during subclinical mastitis challenge might attenuate inflammation and enhance blood and milk immune cells protein synthesis through the mTOR pathway.

# CHAPTER 1. LITERATURE REVIEW

## Mastitis in dairy cattle

Dairy cows have been part of agriculture for thousands of years since at least 3100 BC (Nemet-Nejat, 1998), and bovine mastitis has probably been a problem ever since (Ruegg, 2017). Mastitis is an inflammatory response of the mammary gland tissue caused by physical injury or microbial infections (Ezzat Alnakip et al., 2014; Ruegg, 2017). It is currently the most prevalent disease affecting dairy cows and negatively impacts animal welfare and farm profitability (Cheng and Han, 2020). The mastitis process most often develops when immune cells are released into the mammary gland in reaction to the invasion of the teat canal and mammary tissue, typically by bacteria that inflame the udder (Sordillo, 2018a; Cheng and Han, 2020). Then, these pathogens proliferate and release toxins that harm the mammary gland's milk-secreting tissue and ducts. Consequently, there is an increase in white blood cells (i.e., somatic cells), also known as leukocytes, a reduction in milk production, and a change in the milk composition (Jones, 2009; Wellnitz and Bruckmaier, 2012). The somatic cell count (SCC), widely used to determine milk quality, represents the total count of immune cells in the milk and indicates the inflammatory response in the mammary gland, hence a proxy for measuring intramammary infections (IMI) (Wall et al., 2018; Halasa and Kirkeby, 2020).

Somatic cells (SC) can be defined as a mixture of milk-producing and immune cells that are usually present and secreted during milking. Most of these cells in normal milk are cells from the udder secretory tissue (epithelial cells), and some are leukocytes

(white blood cells such as polymorphonuclear neutrophils (PMN), lymphocytes, and macrophages).

Monitoring the SCC values at both the herd and cow levels regularly is important when managing mastitis. The evaluation of the SCC pattern for the herd is usually performed monthly and can help troubleshoot mastitis problems.

*Causes of mastitis in dairy cattle*

Mastitis can be caused by different factors, such as physical trauma, thermal and chemical stress, or microorganism infections (Zhao and Lacasse, 2008). Even though these factors can cause mastitis cases, the primary cause of mastitis in dairy cows is IMI by a bacterium (Haxhiaj et al., 2022), and many bacterial species have been associated with IMI (Adkins and Middleton, 2018).

The pathogens causing mastitis, depending on their main reservoir and transmission method, are classified as contagious or environmental (Azevedo et al., 2016; El-Sayed et al., 2017). Contagious pathogens are those for which udders of infected cows serve as the major reservoir (Abebe et al., 2016), and milker hands, towels, milking equipment, or udder, especially during milking, could be a vector (Fox and Gay, 1993). Mastitis pathogens in this category include *Staphylococcus aureus, Streptococcus agalactiae,* and *Corynebacterium bovis* (Fox and Gay, 1993; Dufour et al., 2019). On the other hand, environmental pathogens, as the name implies, are found in the cow's environment, such as bedding, soil, manure, and water, and thus are highly influenced by management practices (Smith and Hogan, 1993). The most common environmental pathogens causing mastitis are *Escherichia coli*, *Streptococcus uberis*, *Streptococcus*

*dysgalactiae*, and other Gram-positive and catalase-negative cocci (Larry Smith et al., 1985; Dufour et al., 2019).

*Types of mastitis based on level of inflammation*

Based on the level of inflammation and clinical signs, three different types of bovine mastitis have been studied: sub-clinical mastitis (SCM), clinical mastitis (CM), and chronic mastitis (Cheng and Han, 2020). SCM is characterized by moderated inflammation due to an imbalance between the infection agent (bacteria) and the host defense mechanisms (Boutet et al., 2004). In response to bacterial infections, SCM causes an increase in SCC even if it does not show indications of local or systemic inflammation (Gonçalves et al., 2018) without any visible abnormalities of the milk and udder. In the same way as SCM, CM is an inflammatory response to the infection with clinical disease signs in the infected quarter (i.e., redness, swelling, warming), visibly abnormal milk (Rees et al., 2017), and an increase in SCC (Halasa et al., 2007). CM can also be accompanied by fever and decreased dry matter intake (DMI). Finally, chronic mastitis is known for presenting a moderate inflammatory process that lasts for several months, with fluctuating clinical outbreaks at irregular intervals and persistent increase in SCC (Boutet et al., 2007; Cheng and Han, 2020).

The high SCC in milk is an indicator of low milk quality (Li et al., 2014), poor ability of the milk to coagulate (Ikonen et al., 2004; Stocco et al., 2019), and reduced cheese yield and recovery of milk nutrients in the curd (Leitner et al., 2004; Bobbo et al., 2016).

## Mastitis diagnostic tools

### *SCC*

Diagnosis of mastitis is generally based on measuring the inflammatory response by measuring inflammation indicators, often used as an indirect method to identify cows with an IMI (Adkins and Middleton, 2018). One of the most common methods to monitor udder health and diagnose SCM is the SCC, log-transformed SCC, or somatic cell count linear score (SCS) (Adkins and Middleton, 2018). Nonetheless, setting the cow-level threshold of SCC is important to differentiate non-affected from SCM-affected cow's milk. An SCC of 100,000 cells/mL in cow's milk is considered low and indicates good udder health. However, it is important to note that SCC can fluctuate over time and may be affected by various factors such as cow's age, stage of lactation, breed, and environmental conditions.

In a healthy gland, milk with 100,000 cells/mL will have 80% of the cells as macrophages and 20% or less as neutrophils. However, during the inflammatory process, leukocytes migrated significantly to the area of inflammation, making it possible to use the cellularity of the milk to monitor the health of the mammary gland. An arbitrary value of 200,000 cells/mL is usually the cut-off point to distinguish SCM and a healthy mammary gland and often leads to decreased milk production (Petzer et al., 2017; Kirsanova et al., 2019). Thus, when there is inflammation by an infectious process, the number of neutrophils will represent up to 90% of the somatic cell count due to their phagocytic action at the infection site (Alhussien et al., 2016).

It is generally accepted that cows recovered from mastitis should have less than 200,000 cells/mL (Cobirka et al., 2020). Cows with counts over 400,000 cells/mL should be considered as having an IMI (Cobirka et al., 2020). On the other hand, a large number

of cows with chronically elevated SCC in a herd could be indicative of the presence of pathogens that are transmitted in a contagious manner (da Costa, 2014).

On the other hand, bulk tank somatic cell count is the measure used to test milk quality for a herd. A few cows with high individual SCC can skew the entire bulk tank high. Dairy farmers are financially rewarded for low herd SCCs and penalized for high ones because cell counts reflect the quality of the milk produced and how mastitis can affect its constituent parts, having implications for its keeping abilities, its taste, and how well it can be made into other dairy products such as yogurt or cheese. Processing plant contracts often define several SCC *thresholds* and any respective bonus for attaining them. The U.S. maximum SCC level is 750,000 cells per milliliter per farm for domestic sales and 400,000 cells per milliliter for exports since it is deemed unfit for human consumption by the European Union.

Another standard indirect method for diagnosing SCC in cows is the qualitative measurement known as California Mastitis Test (CMT). This method uses a reagent that causes lysis of cell membranes and precipitation of the cell DNA and proteins resulting in a change in the viscosity of the reagent when added to milk. Also, the main advantages of CMT are that it is a quick, cheap, simple test, and it can be performed on-site (Persson and Olofsson, 2011; Adkins and Middleton, 2018). Unfortunately, the difficulty in quickly identifying and diagnosing animals with SCM causes a delay in treatment and infection management, increasing the risk of the disease spreading to other cows and quarters that are already unaffected (Thomas et al., 2015). In contrast, CM can be quickly identified via fore-stripping, the visual examination of a stream of milk taken just before routine milking (Adkins and Middleton, 2018) for visual milk abnormalities.

*Electrical conductivity*

The concentration of anions and cations in milk determines the electrical conductivity (EC). If the cow suffers from an inflammatory process, the milk's $Na^+$ and $Cl^-$ concentration increases, leading to increased milk EC from the infected quarter (Kitchen, 1981). Changes in EC are often associated with mastitis, although they can also be due to other health problems, genetic factors, lactation, and changes in milk composition. Most automatic milking systems have EC sensors incorporated for measuring EC during milking (in-line) and examining the composition of each cow's milk (Norberg et al., 2004)

*Thermal cameras*

The advancements in precision farming technology require adopting a new data-driven approach to farming management and monitoring animal health (i.e., IMI). Thermal cameras mounted in a milking parlor have been used to detect temperature changes on udder skin associated with clinical mastitis or other diseases in a dairy herd (Hovinen et al., 2008; Castro-Costa et al., 2014).

**The economic impact of mastitis on the dairy industry**

Mastitis is the most common disease in dairy cows, with well-known negative consequences for animal welfare and dairy farm revenue. Many studies have evaluated the economic impact of mastitis and its effects on dairy farms worldwide is a concern (Hogeveen et al., 2011). It is estimated that the cost of mastitis in the U.S. dairy industry could reach $2 billion annually, or 11% of the total value of U.S. milk production, with an average of ca. $170/cow annually (Jones, 2009). A more recent study concluded that

the cost of CM in the first 30 days of lactation is $128 in direct costs, and $316 in indirect costs, totaling $444 per case (Rollin et al., 2015). Indeed, CM results in high costs to dairy producers related to the costs of diagnosis, treatment, additional labor, ongoing production losses, milk disposal, reduced milk quality, increased probability of deaths, and premature culling and death of cows (Mcdougall et al., 2007). In addition, mastitis raises concerns about public health, animal welfare, and the dairy industry.

As has been shown previously (Gianneechini et al., 2002; Seegers et al., 2003; Cobirka et al., 2020), SCM occurs 15 to 40 times more often than the clinical form, and its duration is more prolonged (Mbindyo et al., 2020; Girma and Tamir, 2022). SCM is, therefore, more difficult to detect, and infection serves as a reservoir of pathogens that spreads the udder infection among animals within the herd (Cobirka et al., 2020). Consequently, the economic impact of subclinical infections is more difficult to quantify and predict across herds since herd-level screening intensity and case definition can vary (Rollin et al., 2015). It is known that SCM causes three times more production losses than CM; thus, it is responsible for significant losses of 60-70% of the total economic losses associated with all mastitis due to infections (De Vliegher et al., 2012; Sinha et al., 2014).

**The immune response of the mammary gland to intramammary infection**

Mammary gland immunity uses a complex network of physical, cellular, and soluble factors that can be classified as *innate* or *adaptive* immune responses (Sordillo, 2018a). Innate immunity is considered a nonspecific response, and it has a broad scope with the capacity to respond to tissue injury or neutralize various potential pathogens. In

contrast, the adaptive immune response is very specific, and its response can be increased by repeated exposure to the same pathogen (Vivier and Malissen, 2005; Sordillo, 2018a).

*Innate immunity*

The innate immune system includes physical, chemical, and biological barriers. Innate immunity commonly refers to its components that provide immediate host protection, such as neutrophils, monocytes, macrophages, cytokines, and acute-phase proteins (APPs) (Parkin and Cohen, 2001) (Figure 1.1). In dairy cows, the teat end and canal are considered the first line of localized physical barrier or defense against an IMI. The physical barrier is present due to the sphincter muscles surrounding the teat canal and the waxy material called keratin that seals the canal. The keratin is derived from the teat canal epithelial lining since the accumulation of it will provide a physical obstruction to bacteria invasion (Sordillo, 2018a). Furthermore, it has been shown that the antibacterial fatty acid components of the keratin lipid components act as bacteriostatic and bactericidal (Sordillo, 2018a).

The start of the innate immune response, followed by pathogens successfully invading and breaking the mechanical barrier defenses, depends on the ability to detect the presence of bacteria inside the mammary gland. Pattern Recognition Receptors (PRR) present in both immune and nonimmune cells play a critical role in sensing the bacterial presence; and can identify particular microorganisms and/or microbial components (Sordillo, 2016; Vlasova and Saif, 2021). PRRs recognize highly conserved motifs that are expressed by numerous species of pathogens known as Pathogen-Associated Molecular Patterns (PAMP) or molecules released by damaged cells called Damage-

Associated Molecular Patterns (DAMPs) (Medzhitov, 2007; Amarante-Mendes et al., 2018).

Additionally, PRRs are divided into three different families, specifically the Toll-like (TLR), NOD-like, and RIG-1-like receptors (Porcherie et al., 2012). The best characterized among PPRs are TLRs, a family of transmembrane proteins expressed on endothelial cells, epithelial cells, and fibroblasts found throughout mammary tissues and leukocyte populations (Jungi et al., 2011; Vlasova and Saif, 2021). Within the TLR family, both TLR-2 and TLR-4 are of particular importance to mammary immune defense since these receptors identify PAMPs related to gram-positive (peptidoglycans) and gram-negative (lipopolysaccharide) mastitis-causing pathogens (i.e., *Staphylococcus aureus*, *Streptococcus uberis*, and *Escherichia coli* (Goldammer et al., 2004; Porcherie et al., 2012; Vlasova and Saif, 2021).

Upon PAMP and DAMP sensing, PRR signals the host infection's presence and triggers pro-inflammatory and antimicrobial responses by gene expression activation and synthesis of a series of molecules, for example, cell adhesion molecules, immunoreceptors, and intracellular signaling pathways activation such as nuclear factor (NF)-kB (Mogensen, 2009). The nuclear factor (NF)-kB regulates the expression of many soluble mediators that initiates the inflammatory cascade (Yan Liang, 2004; Kumar et al., 2011).

During inflammation, cytokines are one of the primary soluble mediators produced at all stages of the inflammatory response (Sordillo, 2018a). Cytokines are small secreted glycoproteins released by immune and nonimmune cells that specifically affect cell interactions and communications during the immune response, including

inflammation (Zhang and An, 2007; Sordillo, 2018a). Different types of cytokines have been discovered based on their structure, function, and origin, including chemokines, interferons, interleukins (IL), lymphokines, and tumor necrosis factor (TNF) (L. Ferreira et al., 2019). Cytokines have a broad range of biological functions, and they act in a nonspecific manner. For example, IL1, IL6, and TNF produce cytokines and chemokines that attract the migration of neutrophils and monocytes into the site of inflammation. These cytokines are expressed rapidly during the initial stages of inflammation and have potent pro-inflammatory functions (Kany et al., 2019). In contrast, IL4, IL10, and IL17 promote the resolution of the inflammatory cascade (Bannerman, 2009).

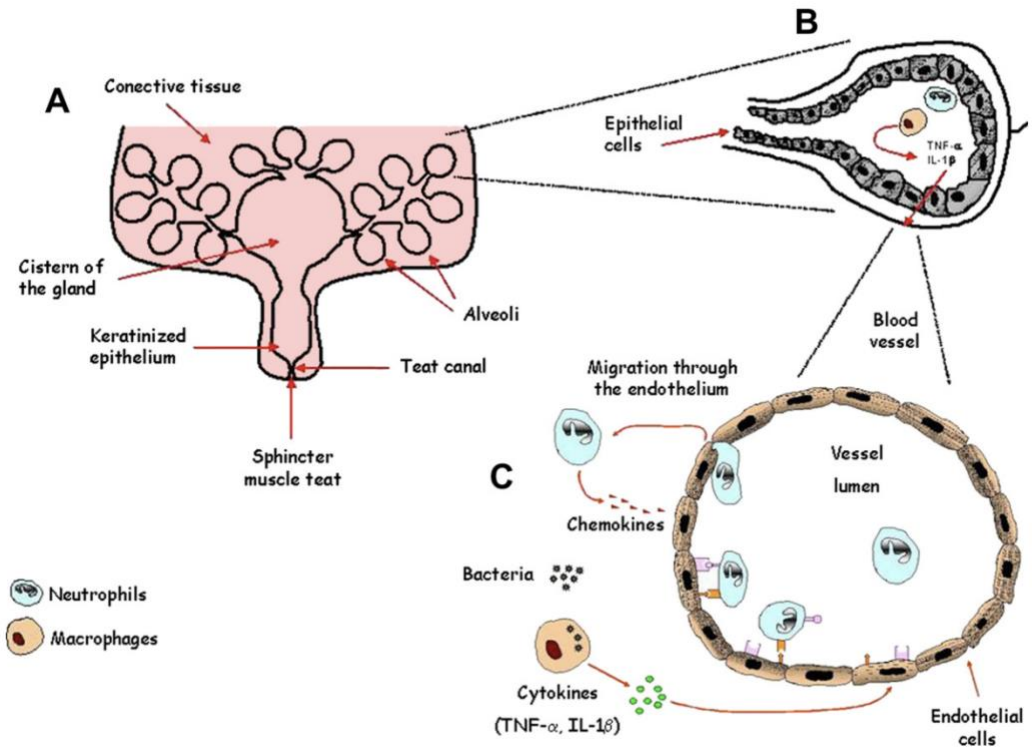


**Figure 1.1.** (A) The bovine mammary gland has defense mechanisms such as the teat sphincter muscle and keratinized epithelium. (B) Macrophages in the gland phagocytize bacteria and release cytokines, (C) while endothelial cells recruit neutrophils to eliminate invading bacteria. Source: Oviedo-Boyso et al. (2007)

Oxylipids are another important class of immune molecules derived from cellular lipids that regulate the inflammatory response's onset, magnitude, duration, and resolution (Vlasova and Saif, 2021). Oxylipids are synthesized from polyunsaturated fatty acids, which are oxidized non-enzymatically by reactive oxygen species (ROS) or enzymatically by different cyclooxygenases, lipoxygenases, and cytochrome P450 (Raphael and Sordillo, 2013). Even though numerous immune and nonimmune cell populations can produce oxylipids, macrophages and endothelial cells represent a significant cellular source in most tissues, including the mammary gland (Hirayama et al., 2017; Sordillo, 2018b).

The innate immune system also has cellular defenses, which are crucial for a successful response to infections in addition to the physical barriers and soluble components. To specify, neutrophils, a subclass of PMN among the cells' defense mechanisms (Sordillo, 2016), are the first cells attracted to the inflammatory site in response to local chemokine gradients. Once there, neutrophils are activated by binding their PRRs to DAMPs or PAMPs to contain the foreign entities by phagocytosis. When the containment is not possible, neutrophils attempt to destroy pathogens by releasing destructive contents of their granules extracellularly, in a process called degranulation (Pechous, 2017). Other cellular components include dendritic cells, monocytes, and macrophages, known as phagocytic immune cells, which can produce cytokines to regulate or promote inflammation (Mak et al., 2014).

*Acute-phase response*

The acute phase response is induced by pro-inflammatory cytokines (i.e., IL-1, IL-6, TNF-α) serving as intermediaries between the area of injury and the hepatocytes

responsible for synthesizing and secreting the APPs (Petersen et al., 2004; Jain et al., 2011). The innate immune system is also activated by APP synthesis, as these proteins mediate inflammation. In summary, pro-inflammatory cells are activated and produce cytokines that diffuse into the extracellular fluid and circulate in the bloodstream. In response, the liver upregulates or downregulates the synthesis of APPs, preceding the specific immune reaction within a few hours (Khalil and Al-Humadi, 2020). The systemic response to inflammation is significantly influenced by APPs, which also play important roles in opsonization, toxins removal, and control of various stages of the inflammatory process (Ceciliani et al., 2012).

Based on protein blood concentrations, APPs are classified as positive or negative in response to inflammation (Petersen et al., 2004; Khalil and Al-Humadi, 2020). If the APPs' expression level increases during an inflammatory response, these proteins will be classified as positive APPs in which serum amyloid A (SAA), haptoglobin, fibrinogen, ceruloplasmin, α1-acid glycoprotein, α-1, antitrypsin, lactoferrin, and C-reactive protein are included (Khalil and Al-Humadi, 2020). On the other hand, if the APPs concentrations in blood plasma are reduced, it is referred to as negative APPs. These negative APPs correspond to albumin, transferrin, transthyretin, transcortin, and retinol-binding protein (Jain et al., 2011). In addition to APPs being utilized for general health screening, they can also be used as quantitative biomarkers of disease diagnosis, prognosis, and tracking of the therapeutic response (Eckersall and Bell, 2010).

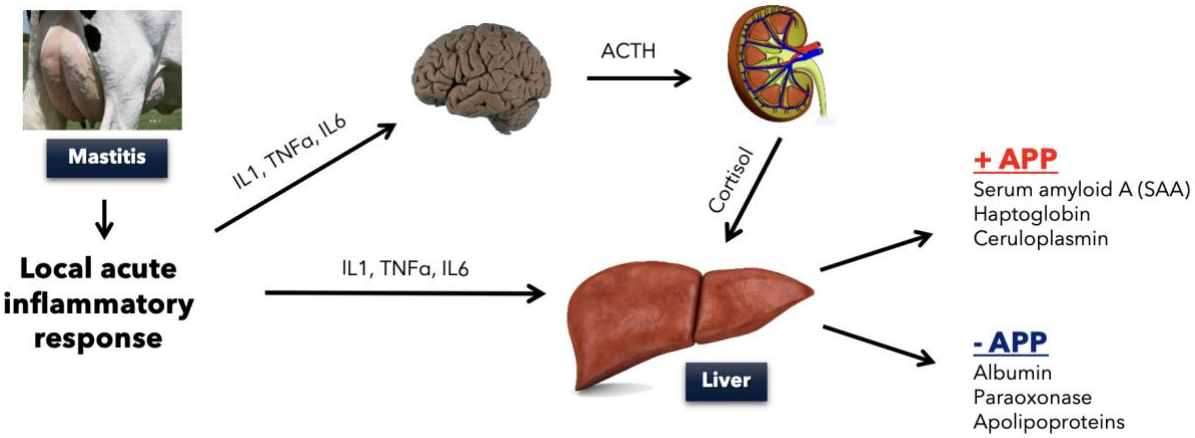


**Figure 1.2.** Immune cells release cytokines, such as IL-1, IL-6, and TNFα, that travel through the bloodstream and reach the liver. The stimulated hepatocytes respond by synthesizing and releasing or decreasing the expression levels of APP into the bloodstream.

**Oxidative Stress**

During the inflammatory response to infection, the mastitis-causing microorganisms produce metabolites and toxic compounds such as ROS. Besides ROS playing a key role in immune response (i.e., involved in destroying phagocyted pathogens), they are generally produced as byproducts of cellular metabolism, which is essential for physiological functions such as cell differentiation or proliferation. In addition, ROS has been proven to be involved in the expression of cell signaling molecules such as cytokines, eicosanoids, and other immunoregulatory substances that are indispensable for a satisfactory immune response against invading pathogens (Forman and Torres, 2002; Asehnoune et al., 2004; Abuelo et al., 2015b).

Oxidative stress refers to the excessive production of ROS in the tissue cells, and the antioxidant system cannot neutralize them (Hussain et al., 2016) due to an imbalance between oxidant and reductant (antioxidant) substances in a living organism (Laliotis et

al., 2020). The oxidants can reduce themselves and oxidize other molecules, whereas antioxidants will prevent cell damage against free radicals (Abuelo et al., 2015b). ROS are the most abundant oxidants; however, other species as reactive nitrogen species, in less proportion, also contribute to the overall oxidant pool (Mavangira and Sordillo, 2018). Commonly, reactive nitrogen species (e.g., nitroxide, peroxy-nitrite, and nitrogen dioxide) have been ascribed to ROS (Abuelo et al., 2015a). ROS is involved in immune system activation and has many other essential roles in the physiological process (Alfadda and Sallam, 2012). Although ROS help in the clearance of pathogens and foreign particles, it can harm the cells when produced in excess, leading to loss of cell function and tissue damage (Valko et al., 2007). Increased ROS production in dairy cows, especially during inflammation of the mammary gland, may affect milk quality resulting in technological problems, such as off-flavors and even risks to human health (Oliver and Calvinho, 1995; Paixão et al., 2017)

Furthermore, oxidative stress causes lipids and other macromolecules to be oxidatively damaged, which affects cell membranes and other cellular components (Toyokuni, 1999). It is important to add that other numerous pathological problems relating to animal productivity, reproduction, and welfare involve oxidative stress (Lykkesfeldt and Svendsen, 2007).

Luckily, the organism is equipped with several antioxidant substances that are capable of counteracting the overproduction of ROS and their accumulation (Abuelo et al., 2015b)

*Antioxidants*

Antioxidants can be synthesized in the body, derived from the diet (i.e., vitamins and minerals), or administered parentally (Abuelo et al., 2015b; Yang and Li, 2015). Antioxidants are usually divided based on their chemical and physical characteristics as enzymatic or nonenzymatic. The main enzymatic antioxidants are superoxide dismutase, catalase, and glutathione peroxidase. On the other hand, nonenzymatic antioxidants include glutathione, ascorbic acid (vitamin C), α-tocopherol (vitamin E), β-carotene (vitamin A), uric acid, and polyphenols (Sordillo and Raphael, 2013; Morillas-Ruiz and Hernández-Sánchez, 2015).

The antioxidants also have specific roles in the IMI of dairy cows; for example, vitamin A and β-carotene are crucial components of many important and diverse biological functions, including reproduction, immune functions, and health (Johansson et al., 2014). Vitamin C has an important physiologic role in numerous metabolic processes, including tissue growth and maintenance, amelioration of oxidative stress, and immune regulation (Matsui, 2012; Gordon et al., 2020). In addition, selenium and vitamin E provide antioxidant properties and functions essential for glutathione peroxidase, which reduces $H_2O_2$ and free $O_2$ to $H_2O$ (Yang and Li, 2015; Xiao et al., 2021).

*Glutathione*

The thiol tripeptide glutathione (GSH; γ-L-glutamyl-L-cysteinylglycine) is the most abundant endogenous antioxidant due to its marked ability to scavenge ROM and free radicals, which leads to being commonly used as a biomarker in oxidative stress-related diseases (Romeu et al., 2010; Vetrani et al., 2013). Glutathione is the major non-protein sulfhydryl compound in mammalian cells and is known to have numerous

biological functions (Lu, 2013), serving as an important hepatic antioxidant. Glutathione can also be exported into the bloodstream and aid in the control of systemic oxidative stress response (Osorio et al., 2014a). Glutathione combines three simple building blocks of protein or amino acids (AA): cysteine, glycine, and glutamine (Adeoye et al., 2018).

**Nutritional approaches to improve the immune system during an infectious process**

The ability of cows to resist the establishment of new IMI depends on the efficiency of the mammary gland's immune system (Sordillo, 2018a). Maintaining optimal immune function and health in dairy cows is commonly correlated with their nutritional status, which plays a pivotal role in the immune response (Ingvartsen and Moyes, 2013; Sordillo, 2016). Mammals rely on nutrients such as AA, fatty acids, vitamins, and minerals from ingested food to maintain an adequate nutritional status for regulating metabolic, physiological, and neuronal homeostasis and preventing diseases (Trumbo 2008; Stover et al. 2017).

Therefore, nutritional approaches have been used to boost dairy cows' immune systems (Sordillo, 2016; Martins et al., 2022). A balanced supply of dietary micronutrients associated with antioxidants mechanisms (i.e., vitamins A, C, E, Selenium, Copper, and Zinc) is widely recognized as essential in ensuring production efficiency and immune competence in early lactation dairy cows (Sordillo, 2016). Further, supplementation with vitamins and trace elements minimizes the harmful consequences of excessive ROS production, improving animals' health status and reducing disease incidence (Abuelo et al., 2015b).

Additionally, glucose is known to be required by phagocytic cells such as macrophages and PMN for proliferation, survival, and differentiation (Pavlou et al., 2018). Glucose is the preferred metabolic fuel during inflammation for activated PMN, macrophages, and lymphocytes rather than fatty acids, AA, or ketone bodies (Sameer Barghouthi, 1995; Gamelli et al., 1996; Pithon-Curi et al., 2004). Furthermore, feeding phytonutrients (chemical compounds produced by plants) can be an alternative to boost immune function and antioxidant activity in livestock (e.g., capsaicin, tannins, curcumin, garlic) (Pavlou et al., 2018). These methods combined might help us better understand how dairy cows' immunometabolism is regulated.

**The important role of AAs and their supplementation to dairy cows**

AA are natural compounds involved in various important biological processes, such as metabolism, growth, and immunity (Martínez et al., 2017). AA requirements may increase as a direct consequence of metabolic changes associated with inflammation and infection, where its supplementation in dairy cows can also be a strategy to enhance the immune system (Le Floc'h et al., 2004).

Essential AAs promote metabolizable protein (MP) synthesis in dairy cows, and increasing its supply to the mammary gland is the basis of most dietary strategies to increase MP content (Zhou et al., 2021). Free essential AA can enhance MP synthesis through cell proliferation and activation of the mammalian target of the rapamycin (mTOR) pathway in bovine mammary epithelial cells (BMECs) (Dai et al., 2018; Qi et al., 2018).

The microorganisms in the rumen are known to degrade AA sources, such as lysine and methionine (Met) (Movaliya et al., 2013). The increased protein available in the rumen is broken into non-nutrients like ammonia (Mazinani et al., 2020); and consequently, only a small fraction of dietary protein consumed by the animals passes through the rumen. Therefore, supplementation with free essential AAs in ruminants' diets is not a good choice (Mazinani et al., 2020). A strategy to improve AA utilization in the cow's diet is to add AAs that will be protected from rumen microbial degradability to promote subsequent digestibility by the other stomach chambers (Mazinani et al., 2020). The pH-sensitive or lipid encapsulation method has been used to encapsulate lysine and Met in a polymer that is pH dependent (resiting rumen pH), allowing its breakdown in the abomasal pH (Socha et al., 2005). As a result, various rumen-protected (RP) AA products have been created to be delivered effectively.

**Methionine supplementation to dairy cattle**

Protein synthesis is believed to be initiated with the AA Met, which has a simple structural role in the hydrophobic core, similar to other hydrophobic AA like leucine, isoleucine, and valine (Aledo, 2019). Met is an essential AA, and its metabolism starts with converting into S-adenosyl methionine (SAM). This universal methyl donor, known as SAM is a key cofactor of the Met intermediate metabolism used for methylation reactions (Mcfadden et al., 2020; Junior et al., 2021). In addition, Met plays an essential role in polyamine biosynthesis, where decarboxylated SAM adds amino propane to the forming polyamines required for cell proliferation (Igarashi and Kashiwagi, 2000).

In ruminants, Met is well known for being the first limiting AA (NRC, 2001).

The effects of rumen-protected Met (RPM) on dairy cow's performance have been extensively evaluated, and it has been shown that increasing the Met supply by feeding diets supplemented with RPM can increase milk production, protein yield, and fat milk synthesis (Armentano, 1997; Osorio et al., 2013; Junior et al., 2021).

*In vitro* studies have demonstrated that Met significantly increases milk protein and fat synthesis, and cell proliferation by increasing the mTOR phosphorylation, protein expression of sterol regulatory element-binding protein (SREBP-1c), and Cyclin D1 (Qi et al., 2018). The mTOR is a central regulator of milk protein synthesis and cell growth, while SREBP-1c is a key transcription factor for fat milk synthesis, and Cyclin D1 is a protein required for the proliferation of BMECs (Zhang et al., 2018).

mTOR is also known to be a central regulator of immune responses (Powell et al., 2012) (Figure 1.3). mTOR regulates translation, cytokine reactions, antigen presentation, macrophage polarization, and cell migration and has been linked to reorganizing the cellular metabolism (Weichhart et al., 2015). mTOR activity can be regulated on multiple levels by different factors depending on environmental conditions (González and Hall, 2017).

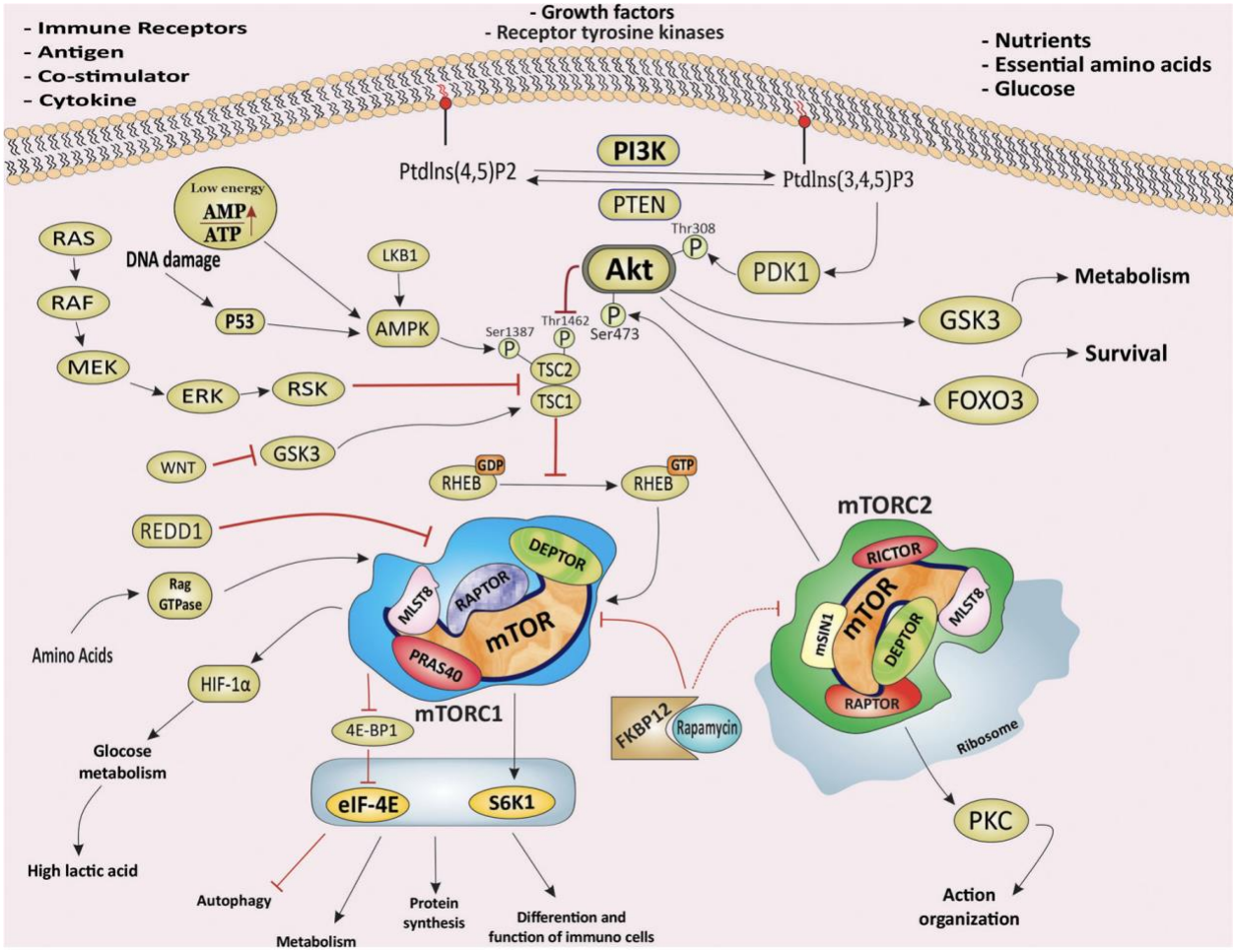


**Figure 1.3.** Schematic illustration of the molecular components and signaling events related to the mTOR signaling pathway. Source: Mafi et al. (2021)

*Methionine modulation of the immune response during infection models*

Research studies have demonstrated that Met positively impacts cow's performance and plays an important role in regulating numerous cellular processes affecting the immune system and enhancing the host's ability to regulate inflammation (Batistel et al., 2018; Machado et al., 2018). During an infection process, inflammatory mediators and ROS release occur as a response; hence, increased ROS production and decreased antioxidant protection can originate from oxidative stress (Turk, 2017). Numerous studies support the idea that Met serves as an important cellular antioxidant by

increasing glutathione levels in the liver of transition dairy cows (Osorio et al., 2014a; Zhou et al., 2016; Batistel et al., 2018). Therefore, Met has been associated with *de novo* glutathione synthesis pathway (Halsted, 2013). This concept illustrates Met's important role in reducing ROS during inflammatory processes by protecting cells from oxidative stress through glutathione metabolism. For example, Osorio et al. (2014b) observed that during the peripartal period, RPM-supplemented cows showed lower peripartal ceruloplasmin and SAA concentrations while the albumin concentration increased. These results suggested decreased inflammatory response while promoting liver function (Osorio et al., 2014b).

Phagocytosis is a key function of PMN, which is involved in host defense. During mastitis, bacteria release toxins that cause macrophages and mammary epithelial cells to secrete cytokines that attract PMN to the infection site, where they can act as phagocytes (Zhou et al., 2015). Cows supplemented with RPM are observed to decrease SCC (Li et al., 2016), which could indicate an enhancement of the immune system. Moreover, in the same study, an increased expression of genes that facilitate migration into inflammatory sites (*SELL* and *ITGAM*), enzymes essential for reducing reactive oxygen metabolites (*SOD1* and *SOD2*), and an increase in transcription factors required for controlling PMN development (*RXRA*) were observed in cows supplemented with RPM (Li et al., 2016). A summary of recent studies supplementing RPM to dairy cows and their main findings are shown in Table 1.1.

**Table 1.1.** Summary of studies investigating the influence of supplementing Met in dairy cows.

| Subject | Supplement | Dose | Effect | Reference |
|---|---|---|---|---|
| 76 multiparous cows | Smartamine M (SM) | 0.9% of DM | Improved milk yield and milk components. | (Junior et al., 2021) |
| 37 multiparous cows | MetaSmart (MS) and Smartamine M (SM) | 0.9% of DM | Increased phagocytosis activity. | (Osorio et al., 2013) |
| 28 multiparous cows | Smartamine M (SM) | 0.07% of DM | Increased cell receptors in neutrophils. | (Li et al., 2016) |
| 48 multiparous cows | Smartamine M (SM) | 0.08% of DM | Improves liver function, inflammation status, and immune response. | (Zhou et al., 2016) |
| 60 multiparous cows | Ethyl- cellulose RPM RPM | 0.09% and 0.10% of DM | Alleviated inflammation and oxidative stress. Enhanced liver and neutrophil function. | (Batistel et al., 2017; Batistel et al., 2018; Han et al., 2018) |

# RATIONALE AND OBJECTIVES

The overall goal of this project was to explore the effects of rumen-protected methionine during a subclinical mastitis challenge in dairy cows. Therefore, the objectives of this study were to evaluate the response of RPM-supplemented cows on:

a) Lactation performance
b) Blood biomarkers parameters
c) Oxidative stress
d) Blood and milk immune cells' protein synthesis

We speculate that RPM will positively affect milk quality, lower the risk of oxidative stress, and it may control inflammation while potentially increasing immune cell capabilities through enhancing cellular protein synthesis.

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# CHAPTER 2. RUMEN-PROTECTED METHIONINE SUPPLEMENTATION IMPROVES LACTATION, OXIDATIVE STATUS, AND IMMUNE RESPONSE DURING A SUBCLINICAL MASTITIS CHALLENGE IN LACTATING DAIRY COWS


## Abstract

This study aimed to evaluate the effects of rumen-protected methionine on lactation performance, inflammation, immune response, and liver glutathione of lactating dairy cows during a subclinical mastitis challenge (SMC). Thirty-two Holstein dairy cows (145 ± 51 DIM) were enrolled in a randomized complete block design. At -21 d relative to the challenge, cows were assigned to dietary treatments, and data were collected from 0 to 3 d relative to SMC. Cows were blocked according to parity, DIM, and milk yield and received a basal diet (17.4% CP; Lys 7.01% MP, and Met 2.14% MP) supplemented with 100 g/d of ground corn (CON; n = 16) or Smartamine M (SM, 0.09% DM; n = 16). The mammary gland's rear right quarter was infused with 500,000 CFU of *Streptococcus uberis* (O140J) at 0 h. Milk yield was recorded twice daily from 0 until 3 d relative to SMC. Milk samples were collected from 0 to 3 d relative to SMC, while blood samples were collected at 0, 6, 12, 24, 48, and 72 h relative to SMC. Blood samples were also collected at 0, 12, and 24 h, and milk samples at 24 h relative to SMC to study mTOR pathway activation by calculating the phosphorylated ratio to total AKT, S6RP, and 4EBP1. Liver biopsies were performed at -10 d and 1 d relative to SMC to measure glutathione. Data were analyzed using the MIXED procedure of SAS for repeated measurements. There was a trend for greater milk yield (+ 0.9 kg) after SMC in SM cows than in CON. Reactive oxygen metabolites were lower in SM cows than in CON. The

milk somatic cell linear score was not affected by treatment; however, a score > 4 at 24 h confirmed SCM on both SM and CON cows. SM cows had greater milk fat content at 24 and 36 h post-SMC, resulting in overall higher milk fat. Milk protein content tended to be greater in SM cows than in CON. We observed higher concentrations of liver glutathione in SM cows than in CON. Inflammation biomarkers showed lower ceruloplasmin levels for SM than CON cows. A greater p4EBP1:4EBP1 ratio in the blood PMN of SM cows than CON was observed. pAKT: AKT was greater in milk neutrophils from SM cows than CON. SM cows had a greater pS6RP: S6RP ratio in milk neutrophils and monocytes. Overall, methionine supplementation during an SMC may positively affect milk performance and lower the risk of oxidative stress by increasing liver glutathione in lactating dairy cows. In addition, methionine supplementation may attenuate inflammation while potentially enhancing protein synthesis through the mTOR pathway. Furthermore, the effects on inflammation need further confirmation.

**Keywords:** mastitis, methionine, immunity, dairy cows.

# INTRODUCTION

Mastitis is a well-known disease that affects the dairy industry and results in significant financial losses, an increase in the usage of antibiotics, and reduced animal welfare (Carlén et al., 2006). Mastitis is an inflammation in the mammary gland tissue due to physical trauma or microorganism infections. In dairy cows, it is mainly caused by pathogenic bacteria invading and multiplying in the mammary gland tissue (Ruegg, 2017).

Two different kinds of mastitis have been well-recognized in dairy cattle. The clinical inflammatory response to infection during mastitis (CM) is well characterized by visible abnormal gland secretions (i.e., watery appearance, flakes, clots, or pus) and udder signs such as redness, swelling, heat, harness, or pain; and the Other signs may include elevated body temperature and inappetence (Yang and Li, 2015). On the other hand, subclinical mastitis (SCM) is characterized by inflammation with a normal-appearing mammary gland and visibly normal milk (Bobbo et al., 2017; Gonçalves et al., 2018). However, it is accompanied by an elevation of somatic cell count (SCC) in the milk (Yang and Li, 2015; Adkins and Middleton, 2018). Additionally, SCM lasts longer and occurs 15 to 40 times more frequently than CM (Seegers et al., 2003). Therefore, SCM is more difficult to detect, and infection serves as a reservoir of pathogens that spreads the udder infection among animals within the herd (Cobirka et al., 2020).

The tremendous impact of mastitis on the dairy industry has been mainly reflected in economic losses. Jones (2009) estimated that the cost of mastitis in the US dairy industry by 2009 was ca. $2 billion annually, or 11% of the total value of US milk production, with an average of ca. $170/cow annually. These economic losses are mainly

caused by the decrease in milk production, the increase in treatments, and cow culling (Gonçalves et al., 2018).

Although the losses related to SCM are difficult to quantify, studies agree that SCM is responsible for most of the economic losses (60-70%) related to mastitis, with the reduction of milk production being the main factor (Zhao and Lacasse, 2008; Azooz et al., 2020; Cheng and Han, 2020).

Mastitis can also be classified based on epidemiology, namely contagious and environmental (Azevedo et al., 2016). Contagious bacteria are spread from cow to cow, from infected udder to healthy (Ruegg, 2017). On the other hand, environmental bacteria, as the name implies, come from the cow's environment, such as bedding, soil, and manure (Hogan and Smith, 2012b).

Mastitis occurs when bacteria invade the mammary gland via the teat canal (Sordillo et al., 1997; Cheng and Han, 2020). In the early phases of pathogenesis, the actions of resident and newly recruited leukocytes are crucial in developing intramammary infections (IMI) (Sordillo et al., 1997). Neutrophils are terminally differentiated cellular components of the innate immune system, constituting about 35–75% of peripheral leukocytes (Alhussien et al., 2016). The ability of neutrophils to migrate into infected tissues depends upon the recognition of inflammatory mediators by cytokines, chemokines, and complement receptors (Rambeaud and Pighetti, 2005).

Cytokines, particularly interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNFα) initiate the inflammatory response, which induces the acute phase response (APR) by decreasing or increasing the production of acute phase proteins (APPs) (Jain et al., 2011) produced in the liver and released into the bloodstream (Suojala

et al., 2008; Thomas et al., 2015; Sordillo, 2018). During an APR, levels of positive APPs (e.g., haptoglobin, SAA, and ceruloplasmin) increase, while negative APPs (e.g., albumin) decrease (Petersen et al., 2004; Bertoni et al., 2008). Once at the site of infection, neutrophils phagocytose and kill bacterial pathogens (Sordillo et al., 1997; Segal, 2005).

The early stages of inflammation attract activated phagocytic immune cells that produce countless oxidative compounds, such as the hydroxyl radical, reactive nitrogen species, and oxygen radicals or reactive oxygen species (ROS) that can engulf the pathogens (Labro, 2000; Andrés et al., 2022). In particular, activated neutrophils and macrophages are an essential source of molecular oxygen and superoxide anion formed enzymatically by NADPH oxidase during inflammatory reactions (Sordillo and Aitken, 2009).

Physiological levels of ROS are beneficial in regulating processes involving the maintenance of homeostasis and a wide variety of cellular functions (Bhattacharyya et al., 2014; Checa and Aran, 2020). However, high concentrations of ROS can cause oxidative stress and damage the cell's basic building blocks, including DNA, protein, and lipids (Shields et al., 2021). As a result, the immune defense may also act as a source of oxidative stress, which occurs when an organism has elevated levels of ROS but low levels of antioxidants (Schieber and Chandel, 2014). Consequently, oxidative stress in dairy cows occurs when there are low concentrations of antioxidants such as glutathione (GSH), superoxide dismutase (SOD), and vitamins A, C, and E (Bernabucci et al., 2005; Osorio et al., 2014; Yang and Li, 2015). Therefore, antioxidants protect the body from

free radicals, maintaining optimum animal health by improving immune function, response, and productivity (Yang and Li, 2015; Alhussien et al., 2021).

The effectiveness of the immune system of the mammary gland determines resistance to IMI (Sordillo, 2018). Hence, optimizing mammary gland defenses can help prevent the development of IMI (Ezzat Alnakip et al., 2014). In the last few decades, there has been an increased interest in studying alternative nutritional approaches, such as feed additives with antioxidant and anti-inflammatory properties, to improve immunity in dairy cattle (Sordillo, 2016). Recent studies indicated that using amino acids (AA) such as Methionine (Met) supplementation improves dairy cows' immunological response and lactation performance (Rulquin et al., 2006; Chen et al., 2011). Therefore, in transition dairy cows, an increased phagocytosis activity (Osorio et al., 2013) and cell receptors (Li et al., 2016) in neutrophils were observed, allowing these specialized immune cells to be more effective in facing bacterial infection. Total liver glutathione has also been increased in transition cows while supplementing Met (Osorio et al., 2014; Zhou et al., 2016a; Batistel et al., 2017).

Met is widely known as one of the most limiting AA for milk protein synthesis in lactating dairy cows fed diets (NRC, 2001). Recent studies have shown that Met can control mammals' innate immune system, digestive function, and metabolic activities (Martínez et al., 2017). In addition to its role in protein synthesis, Met is a precursor of S-adenosyl methionine (SAM), a major mammalian methyl donor (Roje, 2006; Gao et al., 2018). Its availability may also impact the level of oxidative stress since homocysteine produced during the Met cycle can act as a substrate for glutathione production (Martinov et al., 2010).

It has been demonstrated that anti-inflammatory-related pathways, such as the mechanistic target of rapamycin (mTOR), are regulated by AA supplementation (He et al., 2018). The mTOR pathway is a crucial regulator of immune response (Powell et al., 2012). Rumen-protected limiting AA, including Met supplementation, regulate mTOR signaling, which controls protein and nucleotide synthesis (Khan et al., 2022).

Even though studies supplement rumen-protected Met (RPM) during the transition period, Met supplementation effects during an SCM model in lactating dairy cows have yet to be evaluated. We hypothesized that cows supplemented with RPM would maintain a consistent milk yield and components (i.e., fat and protein) during a subclinical mastitis challenge. We also hypothesized an improved oxidative status and enhanced blood and milk immune cells protein synthesis for cows fed with the RPM. Therefore, this study aimed to evaluate the effects of RPM on lactation performance, inflammation and immune response, and liver glutathione of lactating dairy cows during a subclinical mastitis challenge (SMC).

## MATERIALS AND METHODS

All the procedures for this study were conducted in accordance with the protocol approved by the Institutional Animal Care and Use Committee at the South Dakota State University (Protocol no. 2002-014A). This study comprised an initial mastitis test, a titration experiment, and a final subclinical mastitis experiment.

### Preliminary subclinical mastitis test

A preliminary experiment was conducted to determine the feasibility of using 5,000 colony-forming units (CFU), as seen by other authors (Moyes et al., 2009), to

induce subclinical mastitis in dairy cows. The rear right quarter of eight cows (negative bacteriological culture and milk SCC <200 000 cells/mL at quarter level) was infused with either 2 ml of sterile saline as negative control (n = 8) or 5,000 CFU in a 2-mL volume (2,500 CFU/mL) of *Streptococcus uberis* strain O140J (n = 8). Milk samples were collected before infusion (0 h) and at different time intervals (12, 24, 36, 48, 60, and 72 h) after treatment to check the variation in somatic cell count (SCC). All mammary quarters infused with *Streptococcus uberis* presented SCC below 200,000 cells/mL (Figure 2.1), indicating that 5,000 CFU were insufficient to induce SCM in these cows. Since a significamce difference was detected at 0 h, this time point was used as a covariate. Based on these results, a titration experiment was necessary to establish an optimal concentration of *Streptococcus uberis* for inducing SCM.

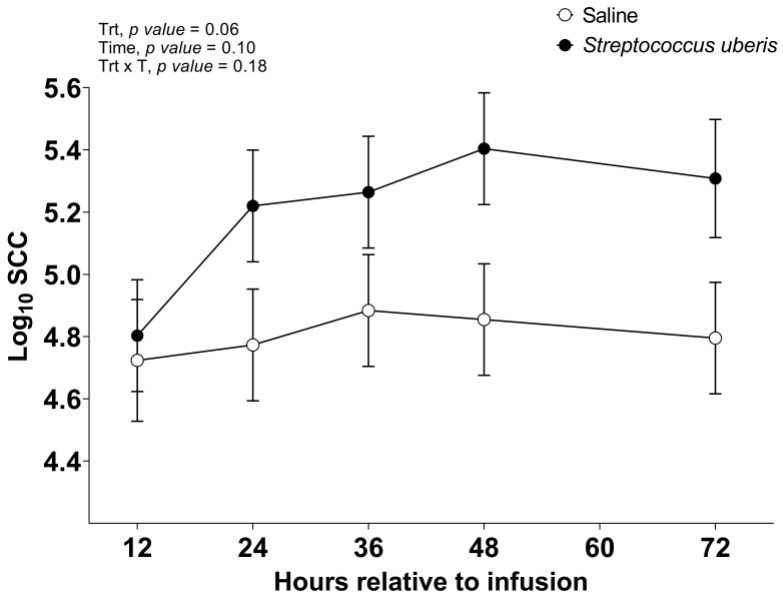


**Figure 2.1**.Somatic Cell Count (SCC) from the saline group and *Streptococcus uberis*-infused cows (5,000 CFU).

**Titration experiment**

A titration experiment was limited to six multiparous Holstein cows to determine the appropriate population density of bacteria (CFU/mL) to induce SCM. Cows were divided into three groups (G1, G2, and G3) of two animals each. Foremilk samples were collected before the SMC to confirm that the cows were bacteriologically negative. The mammary gland's rear right quarter from 6 cows was infused with 100,000, 1,000,000, and 10,000,000 CFU *Streptococcus uberis* strain O140J for G1, G2, and G3, respectively. Milk samples from the infected quarter were collected before infusion (0 h) and at different time intervals (12, 24, 36, 48, 60, and 72 h) relative to infusion to evaluate the variation in SCC. Based on the results obtained from the titration experiment (Figure 2.2), 500,000 CFU of *Streptococcus uberis* was considered suitable to induce SCM in the experiment.

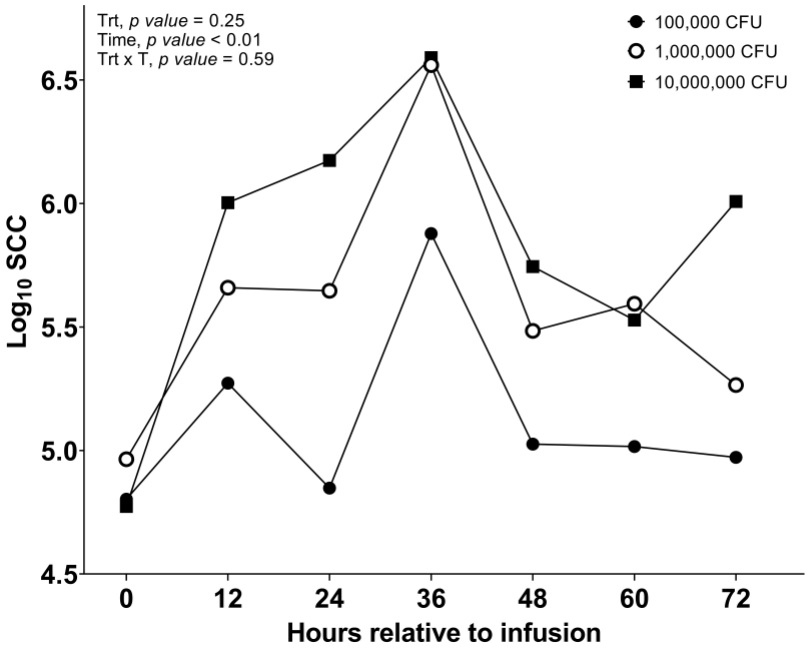


**Figure 2.2.** Somatic Cell Count (SCC) from the three groups of cows infused with *Streptococcus uberis* with 100,000, 1,000,000, and 10,000,000 CFU.

**Animal management**

The experiment was conducted from May to June 2021 at the South Dakota State University Dairy Research and Training Facility (Brookings, SD). Hobo Pro series Temp probes (Onset Computer Corp., Pocasset, MA) were used to record the air temperature and relative humidity in the cow's pen during the experimental period. Cows were housed in a ventilated enclosed barn with access to mattress-free stalls and fed using an individual gate system (American Calan, Northwood, NH, USA). Individual orts were collected once a day before feeding to determine daily feed intake. The feed offered was adjusted daily to achieve 5 to 10% refusal. The dry matter content of feed ingredients was determined once a week throughout the experiment, and diets were adjusted accordingly to maintain formulated DM ratios.

**Experimental design and treatments**

Thirty-two mid-lactation Holstein cows from 50 to 145 days in milk (145±51 DIM; mean ± SD), including 28 multiparous and four primiparous cows, were used in a randomized complete block design. Cows were blocked according to parity, DIM, and milk yield. Two multiparous cows were removed from the trial because of strongly altered eating behavior. A total of 30 Holstein cows were fed a control diet (CON) plus 100 g of ground corn (n = 15) and a treatment diet similar to CON, except that Smartamine M (SM; n =15) at a rate of 0.09% was added.

The ingredients and chemical composition of the experimental diets are shown in Table 2.1. DM content of individual ingredients was determined weekly (100˚C for 24 h),

and diets were adjusted to maintain the DM ratio of ingredients in total mixed ration (TMR).

**Table 2.1.** Ingredients and chemical composition of the experimental diets.

| Item | Control | Smartamine M |
|---|---|---|
| **Diet (DM as lbs/day)** | | |
| Corn silage | 22.3 | 22.3 |
| Alfalfa hay | 6 | 6 |
| Cottonseed | 4 | 4 |
| QLF 60 38 | 2.6 | 2.6 |
| Grain mix | 20.2 | 20.2 |
| Smartamine M | --- | 0.09 |
| **Diet specifications (AMTS)[1]** | | |
| CP, % DM | 16.7 | 16.76 |
| RDP, % DM | 10.15 | 10.15 |
| RUP, % DM | 6.56 | 6.61 |
| MP supply (g/d) | 2888.1 | 2902.4 |
| Lys:Met | 3.23 | 2.65 |

AMTS= Agricultural Modeling and Training Systems.

**Subclinical mastitis challenge**

*Streptococcus uberis* strain O140J was prepared by RTI, LCC (Brookings, SD) following the protocol described by Moyes et al. (2009). Briefly, a 10-µL loopful of colonies was incubated in 100 mL of Todd-Hewitt broth for six hours at 37°C. After incubation, the broth culture was diluted in sterile Mammalian Ringer's Solution (Electron Microscopy Sciences, Hatfield, PA) to yield approximately 500,000 CFU in a 5-mL volume. The actual titration of each preparation was performed on the day of challenge preparation.

A foremilk milk sample was collected 4 d before the SMC to verify that all experimental animals did not present SCM and were bacteriology negative. After 21 d on dietary treatment, immediately after the morning milking on day 22, the mammary gland's rear right quarter of all cows was infused with 500,000 CFU of *Streptococcus*

*uberis* in 5 mL of inoculum administrated via a sterile disposable syringe fitted with a sterile teat cannula using the full insertion infusion method. Infusions were performed by block (four cows-block) each day. Before inoculation, teats were rigorously cleaned with cotton balls containing 70% isopropyl alcohol. The experiment timeline is illustrated in Figure 2.3.

Starting at 72 h post-SMC, after samples had been collected, infected quarters were aseptically infused with 125 mg of ceftiofur HCl (SpectraMast LC, Pfizer Animal Health, Kalamazoo, MI) twice daily for four consecutive days. During the first 3 d of antibiotic treatment, cows also were administered 30 mL of procaine penicillin G (300,000 IU/mL, IM; US Vet, Hanford Pharmaceuticals, Syracuse, NY) once a day. This treatment resulted in bacteriological cures confirmed by culturing of milk.

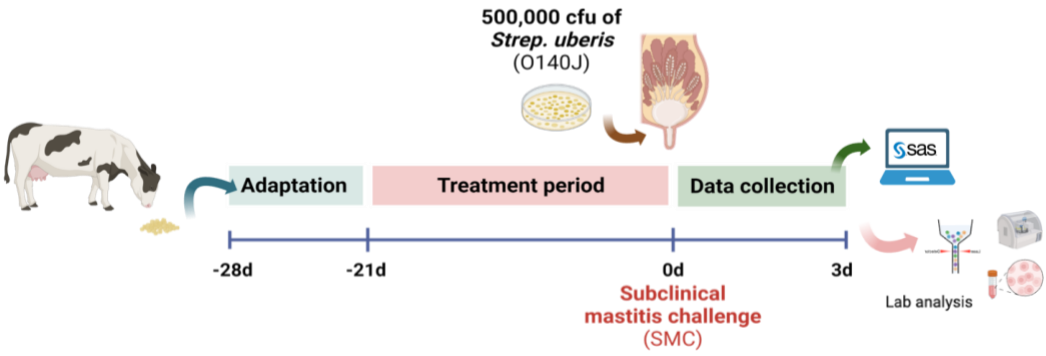


**Figure 2.3**. Experiment timeline.

**Health examinations**

Systemic and local inflammatory indicators were used to measure the health status of the cows and any clinical response to intramammary infusion with *Streptococcus uberis*. These physiological indicators included rectal temperature using a digital thermometer, respiration rate (counting inhalations and exhalations), rear quarters

temperature using a thermal camera (Flir Systems Inc.,Wilsonville, OR, USA), before infusion (0 h) and at different times intervals (4, 8, 12, 16, 20, 24, 32, 40, 48, 56, 64, and 72 h) post-challenge.

**Milk sample collection and analysis**

Cows were milked twice daily, and the milk yield was recorded at each milking during the adaptation and experimental periods. Consecutive morning (6:00 AM) and evening (6:00 PM) milk samples were collected from 0 to 72 h relative to the SMC. Composite milk samples were performed in proportion to milk yield at each milking, preserved with bronopol and natamycin (Broad Spectrum Microtabs II, Advanced Instruments), and analyzed for fat, protein, and SCC using Fourier-transform infrared spectroscopy technology by a commercial laboratory (Dairy One; Ithaca, NY, USA).

**Blood collection and analysis**

Blood samples were collected from the coccygeal vein using a 20-gauge vacutainer needle (Becton Dickinson, Franklin Lakes, NJ) before infusion (0 h) and at different time intervals (6, 12, 24, 48, and 72 h) relative to SMC for blood biomarkers analysis. For biomarkers analysis, blood samples were taken before infusion (0 h), 12 and 24 h relative to SMC for flow cytometry analysis (Attune™ NxT Flow Cytometer, Thermo Fisher Scientific, Waltham, USA). Blood samples were collected into evacuated tubes (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ) containing either serum clot activator or lithium heparin. After collection, lithium heparin tubes were placed on ice while others with serum clot activator were kept at 21°C until centrifugation in the

lab. Serum and plasma were obtained by centrifugation at 1,300 × *g* for 15 min at 21°C and 4°C, respectively. The aliquots were stored at −80°C until further analysis.

Plasma samples were analyzed for biomarkers related to energy metabolism [i.e., glucose, free fatty acids, β-hydroxybutyric acid (BHB)], inflammation [i.e., ceruloplasmin, lactic acid D], liver function [i.e., albumin, cholesterol, globulin, and paraoxonase (PON)], oxidative stress [i.e., myeloperoxidase (MPO), reactive oxygen metabolites (ROM), and ferric reducing antioxidant power (FRAP)], and calcium. Albumin, cholesterol, and glucose were analyzed using the IL Test purchased from Instrumentation Laboratory Spa (Werfen Co., Milan, Italy) in the ILAB 600 clinical auto-analyzer (Instrumentation Laboratory, Lexington, MA), following the procedures described previously by (Trevisi et al., 2012; Batistel et al., 2016; Jacometo et al., 2016). Ceruloplasmin in plasma was determined based on Sunderman and Nomoto (1970), with modifications described by (Jacometo et al., 2015). Antioxidant potential in plasma was assessed as FRAP using a colorimetric method (Benzie and Strain, 1996). ROM in plasma was analyzed with the d-ROMs-test (cod. MC002), purchased from Diacron (Grosseto, Italy). PON in plasma was analyzed according to methods described by Trevisi et al. (2013). MPO was determined via colorimetry based on the reaction of MPO contained in the plasma sample with hydrogen peroxide, which forms $H_2O$ and O−; the O− dianisidine dihydrochloride, and electron donor, reacts with the O−, releasing $H_2O$ and a colored compound, which is then measured (Bionaz et al., 2007; Jacometo et al., 2015). Free fatty acids and BHB were measured in plasma using kits from Wako (Chemicals GmbH, Neuss, Germany) and Randox (Randox Laboratories Ltd., Crumlin,

UK), respectively, following the procedures described previously by (Bionaz et al., 2007; Trevisi et al., 2012; Osorio et al., 2013).

**Liver biopsies**

The liver biopsies were sampled as described by Dann et al., 2006). Briefly, liver samples were taken via biopsy puncture from cows under local anesthesia at -10 d and 1 d relative to SMC. The biopsy fields were shaved and cleaned with iodine and isopropanol. Local anesthesia was achieved by subcutaneous and intramuscular injections of lidocaine around the biopsy site. Liver samples were collected using a stainless steel liver puncture biopsy tool on the animal's right side through the intercostal space on an approximate line from the hooks to the elbow. The liver was frozen immediately in liquid nitrogen and stored at – 80 ºC until further analysis. Total and oxidized glutathione (GSH) in liver tissue were measured using a commercial kit (Cat. No. NWH-GSH01; Northwest Life Science Specialties LLC, Vancouver, WA). Reduced GSH was calculated as reduced GSH = total GSH – oxidized GSH.

**Milk cells isolation**

Milk cell isolation was done following the protocols previously described by (Litherland et al., 2011; Boutinaud et al., 2013; Boutinaud et al., 2015). Milk sample from the infected quarters was taken 24 h after the SMC. Briefly, 950 ml of milk sample were centrifuged at 1,500 × *g*, 15 min at 4°C, and the pellet was resuspended in 50 mL of phosphate-buffered saline (PBS) and again centrifuged for 10 min at 1,000 × *g* and 4°C. Pellet was then resuspended in 10 mL of PBS. The sample was passed through a 40μm

cell strainer and centrifuged again for 10 min at 1,000 × *g* and 4°C. Washed cell pellets were resuspended at a final 2 mL of PBS containing 1% Bovine Serum Albumin (BSA). In the end, milk cell concentration was determined using the cell counter slide in the automated Countess II FL (Life Technologies Inc., Carlsbad, CA, United States). The milk cell suspension was kept on ice for further flow cytometry procedure.

**Flow Cytometry**

***Lysing, fixing, and permeabilizing.*** Milk (24 h) and blood samples (0, 12, and 24 h) were lysed, fixed, and permeabilized using the BD Phosflow Lyse/Fix buffer I (5X concentration) and BD Perm buffer III (Cat. No. 558049 and Cat, No. 558050 respectively, BD Biosciences, San Jose, CA) following protocols previously described by Sipka et al. (2020), with modifications. Briefly, 1.9 mL of whole blood collected at 24 h after SCM and $1x10^7$ milk cells (suspended in PBS 1% BSA) were lysed and fixed in 38 mL of 1:5 BD Lyse/Fix buffer I for 10 min at 37°C. Subsequently, samples were centrifuged for 8 min at 500 × *g*, and the supernatant was discarded. Samples were again washed with 38 mL of PBS and centrifuged at 500 × *g* for 10 min; the supernatant was removed, and cell pellets were placed on ice. Samples were permeabilized by adding 1.5 mL ice-cold BD Perm buffer III and incubating for 30 min on ice. After incubation, cells were washed with 850 µL of PBS, centrifuged at 250 × *g*, and frozen at -80°C until antibody labeling.

***Antibody labeling*.** Cell pellets from all cows and time points (24 h for milk and 0, 12, and 2h for blood) were thawed on ice, centrifuged for 10 min at 500 × *g* and 4°C,

and the supernatant was carefully discarded. Samples were then washed with 1 mL of PBS, separated into 3 aliquots ($1 \times 10^6$ cells each), and centrifuged for 10 min at $500 \times g$ and 4°C. The supernatant was discarded, leaving about 100 µL of PBS and cells. The 3 aliquots were labeled with different surface and intracellular antibodies, as illustrated in Figure 2.4.

The used surface antibodies were to detect monocytes and macrophages (APC anti-human CD14, Cat. No. 301807, BioLegend, Inc, San Diego, CA, USA), and neutrophils (anti-bovine CH138A IgM, Cat. No. BOV2067, Monoclonal Antibody Center, Washington State University) with its corresponding secondary antibody (Goat Anti-Mouse IgM, Human ads-PE/CY7, Cat. No. 1020-17, Southern Biotech, Birmingham, AL, USA), as detailed in Table 1. In addition, each corresponding aliquot was labeled with an antibody pair to detect intracellular expression of phosphorylated (p) AKT and total AKT, pS6RP, and S6RP, or p4EBP1 and 4EBP1 (Cell Signaling Technology, Danvers, MA, USA). The antibodies used in the current experiment cross-react in bovine species, as documented by the manufacturer and previous studies (Mann et al., 2018; Sipka et al., 2020).

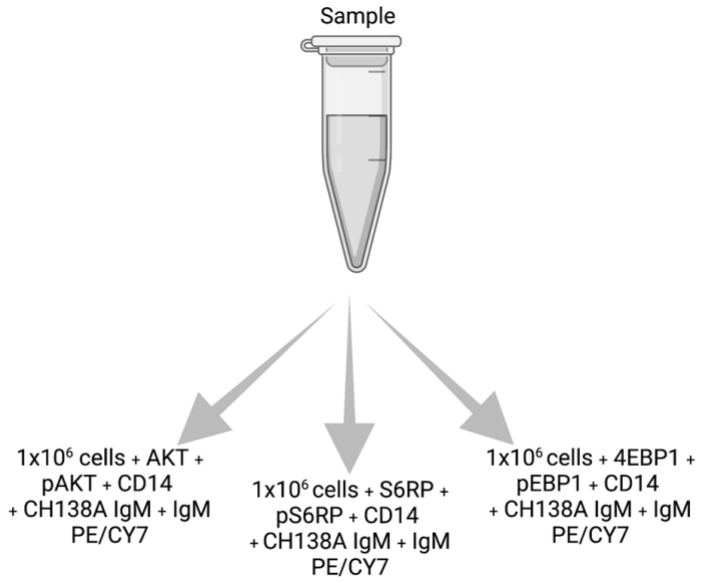


**Figure 2.4.** Schematic illustration of how samples were divided for flow cytometry analysis and the antibodies used for each aliquot.

Each aliquot was incubated with its corresponding antibodies (Figure 2.4) for 1 h at room temperature and protected from light. Samples were then washed two times with PBS to remove unbound antibodies. Following washings, the CH138A secondary antibody was added to 100 µL of PBS and cells and then incubated on ice for 15 min. Samples were again washed twice, and cells were resuspended in 350 µL of PBS. Finally, cells were fixed with 150 µL of 4% paraformaldehyde (Cat. No. BP531-25, Fisher Scientific) and protected from light at 4°C until further analysis. Samples were measured in an Attune Acoustic Focusing Flow Cytometer (Thermo Fisher Scientific, Waltham, MA, USA) and analyzed using FlowJo V10 software (BD Biosciences, San Jose, CA, USA). Controls included unstained cells and cells with single stains for each antibody.

**Table 2.2.** Antibodies used for surface and intracellular labeling of bovine blood and milk leukocytes.

| Antibody target | Source | Phosphorylation site | Conjugation/Secondary antibody | Quantity/100 µL cell suspension |
|---|---|---|---|---|
| CD14 (Cat. No. 301807) | Mouse monoclonal IgG2a | — | APC | 1 µg |
| CH138A (Cat. No. BOV2067) | Monoclonal IgM | — | Goat Anti-Mouse IgM PE/CY7 (Cat. No. 1020-17) | Primary: 0.75 µg Secondary: 0.05 µg |
| pAKT (Cat. No. 4071S) | Rabbit monoclonal IgG | Ser473 | ALEXA488 | 0.1 µg |
| AKT (Cat. No. 8790S) | Rabbit monoclonal IgG | — | Phycoerythrin (PE) | 0.1 µg |
| pS6RP (Cat. No. 4803S) | Rabbit monoclonal IgG | Ser235/236 | ALEXA488 | 0.1 µg |
| S6RP (Cat. No. 55594S) | Mouse monoclonal IgG1 | — | Phycoerythrin (PE) | 0.1 µg |
| p4EBP1 (Cat. No. 2846S) | Rabbit monoclonal IgG | Thr37/46 | ALEXA488 | 0.1 µg |
| 4EBP1 (Cat. No. 34470S) | Rabbit monoclonal IgG | — | Phycoerythrin (PE) | 0.1 µg |

## Statistical analyses

Performance data (health checks, milk composition, and milk yield) and blood biomarkers were analyzed by repeated measures using the MIXED procedure of SAS 9.4 (SAS Institute Cary NC, USA). The statistical model contained the effects of treatment, time (day or hours relative to SMC), and their interactions as fixed effects, while the cow within block and treatment was considered as a random effect. Data collected at a single time point were analyzed following the same model without the time effect.

Blood biomarkers data (0, 6, 12, 24, 48, and 72 h relative to SMC) were unequally spaced; therefore, the Spatial Power covariance structure was used for this analysis. For the equally spaced measures, the covariance structure was chosen between first-order autoregressive and heterogeneous first-order autoregressive based on goodness of fit (smaller Akaike information criteria). Blood biomarkers were log-scale transformed if needed to comply with a normal distribution of residuals.

Statistical significance was declared at $P < 0.05$, and tendencies at $P < 0.10$. Observations were considered outliers when Cook's distance was > 0.50 and consequently excluded from the analysis. For flow cytometry data, changes in the ratio of MFI for phosphorylated to total protein were analyzed. The difference from 0 was statistically analyzed as the null hypothesis.

## RESULTS AND DISCUSSION

Mastitis has been identified as the most common and problematic IMI in dairy cattle (Ruegg, 2017). The interest in preventing mastitis more than treating it has increased in recent decades to save on associated labor and treatment costs and potentially reduce overall antibiotic use. One of the most used preventive methods is using nutritional approaches to enhance the cows' immune system and allow them to prevent microbial invasion of the body and eliminate existing infections (Hogan and Smith, 2012a; da Costa et al., 2016; El-Sayed and Kamel, 2021). In this experiment, we used RPM in the form of Smartamine Mexperiment, RPM in the form of Smartamine M while inducing subclinical mastitis to study its effects on lactation performance, inflammation, antioxidant activity, and pathways highly related to the immune system.

**Indicators of clinical disease**

Results from health examinations post-SMC are shown in Figure 2.5. No differences ($P < 0.05$) were observed within treatments for systemic and local inflammatory indicators. The interaction treatment × time (Trt × T) was not significant ($P > 0.10$) for any of the health parameters. Cows presented an increment in rectal temperature after the challenge (8 and 12h), which might be related to the inflammatory response after the SMC (Figure 2.5A). Even though there is a significant increase in rectal temperature during the first hours after SMC, body temperature in our cows did not exceed 39.7 °C, a threshold value to distinguish healthy cows from those with an infectious disease (Wagner et al., 2008). The increase in body temperature was followed by an increase in respiratory rate during the first 12 h after the SMC. In addition, the infected quarter temperature also increased due to the SMC, but it fluctuated within the healthy range (Figure 2.5B). The increase in the respiration rate during the first hours after the SMC could be attributed to different activities, including sampling, to which the cows are generally not exposed (Figure 2.5C). Lastly, there was no udder swelling or detectable milk abnormalities to indicate the presence of CM.

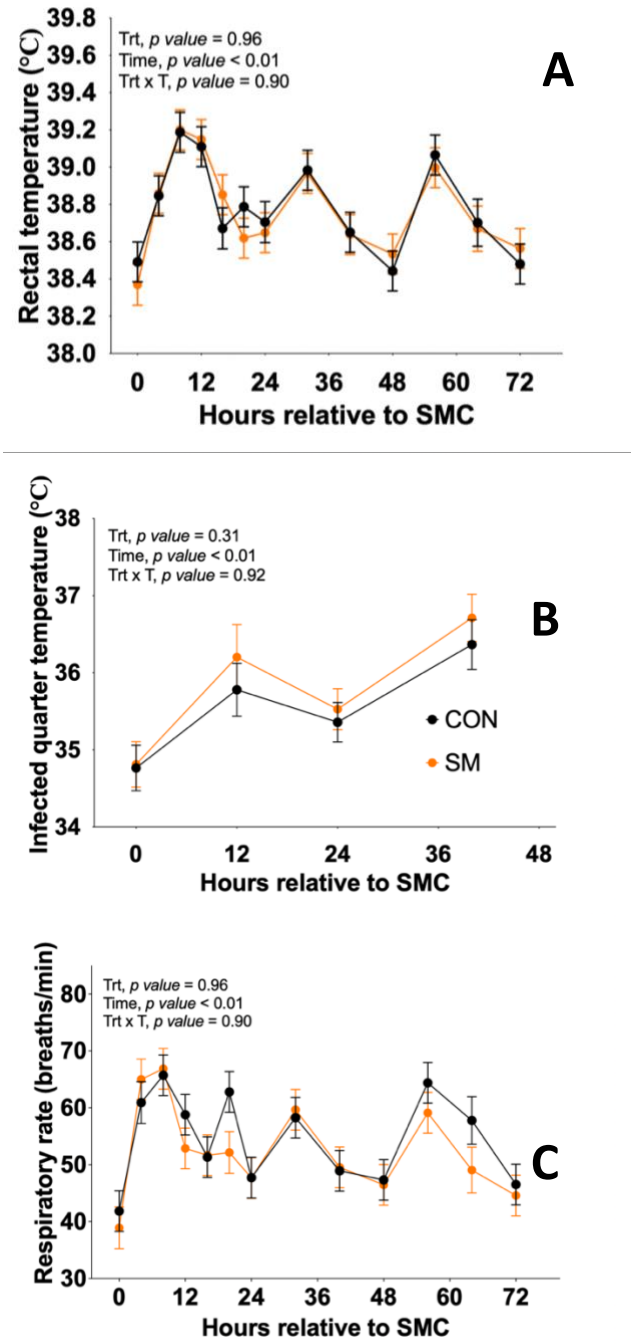


**Figure 2.5.** Systemic and local inflammatory indicators were measured during the first 72 h after the SMC. Rectal temperature (A), infected quarter temperature (B), and respiratory rate (C) from Smartamine M (SM; Adisseo Inc.) supplemented cows and control (CON) cows. Values are means, with standard errors represented by vertical bars. Time points are relative to subclinical mastitis challenge with *Streptococcus uberis*.

**Milk production and composition**

The main effects of diet, time, and interactions on milk yield and milk composition are presented in Figure 2.6. A trend ($P < 0.10$) for greater milk yield (Figure 2.6A) was observed in SM cows in comparison to CON. Additionally, SM cows produced milk with greater milk fat content ($P < 0.05$; Figure 2.6C), fat yield ($P = 0.05$; Figure 2.6D), protein content ($P = 0.07$; Figure 2.6E), and protein yield ($P < 0.05$; Figure 2.6F) than CON. The SM cows produced ca. 0.88 kg more milk per milking session than the CON group. No differences were observed between treatment groups for milk SCCLS (Figure 2.6B)

The negative effects of SCM on reducing milk production, decreasing milk quality, and suppressing reproductive performance are well described (Ruegg, 2017; Fernandes et al., 2021). Consequently, we observed a decrease in milk yield at 36 h after SMC in both CON and SM cows (Figure 2.6A). As expected, our results showed a trend for higher milk production from SM cows under an SMC compared with the CON group. Several studies have reported increases in milk yield when supplementing RPM to periparturient cows (Osorio et al., 2013, Sun et al., 2016, Zhou et al., 2016). The higher milk production when supplementing RPM may result from the mammary gland using the other absorbed proteinogenic AA more effectively to synthesize milk components (Junior et al., 2021).

Previous studies with dairy cows have not found any effect of supplementing Met on milk fat percentage in dairy cows (Zhou et al., 2016b; Batistel et al., 2017). In contrast, other researchers observed increased milk fat content when supplementing RPM to dairy cows (Chen et al., 2011). Similarly, other studies have not found the effects of

RPM on milk SCC or fat content (Malek Dos Reis et al., 2013; Cinar et al., 2015). It is worth mentioning that SM cows in our study had greater milk fat at 24 and 36 h post-SMC, resulting in higher milk fat content than CON cows. Compared to the basal values at 0 h, milk fat content, and yield spiked at 24 h in SM cows but not in CON cows, resulting in a significant ($P < 0.05$) treatment by time interaction.

In addition, milk protein content and yield were positively affected by Met supplementation, increasing by ca. 0.19 percentage units and 0.06 kg/day, respectively. Our results were consistent with the findings of Junior et al. (2021) and King et al. (2021). Qi et al. (2018) developed an *in vitro* experiment using bovine mammary epithelial cells (BMEC), where the effects of different Met concentrations on fat and protein synthesis were evaluated. These authors demonstrated that Met significantly increased milk protein, fat synthesis, and cell proliferation by increasing the mechanistic target of rapamycin (mTOR) phosphorylation and protein expression of sterol regulatory element-binding protein (SREBP-1c) and Cyclin D1. One of the functions of mTOR is that it is a central regulator of milk protein synthesis and cell growth; SREBP-1c is a key transcription factor for milk fat synthesis, and Cyclin D1 is a protein required for the proliferation of the mammary cells (Zhang et al., 2018).

**Inflammation and antioxidant biomarkers**

Immunometabolism effects of SM were evaluated from 0 to 72 h relative to SMC and are shown in Table 2 and Figure 2.7. Lower ($P < 0.01$) ceruloplasmin (Figure 2.7A) and ROM (Figure 2.7B) were observed in SM cows in comparison to the CON group.

Ceruloplasmin is a well-recognized positive acute-phase protein with increased levels in response to infection and inflammation (Kaya et al., 2016). An objective assessment of the health status of dairy cows might be using ceruloplasmin measurements, which could also serve as indicators of the welfare and health of animals (Skinner, 2001). Interestingly, this pattern was observed before and after the SMC in our study. The reasons can be attributed to Met reducing the basal inflammatory conditions. In line with our study, other authors (Osorio et al., 2014) found lower ceruloplasmin in Met-supplemented cows after calving, indicating reduced proinflammatory signaling within the liver. Among blood biomarkers, we observed a treatment-by-time interaction (Figures 2.7C and 2.7D) for calcium ($P < 0.05$) and cholesterol ($P = 0.06$). In the case of calcium, supplemented cows tended to have lower ($P = 0.08$) levels at 12 h after SMC compared with the CON group.

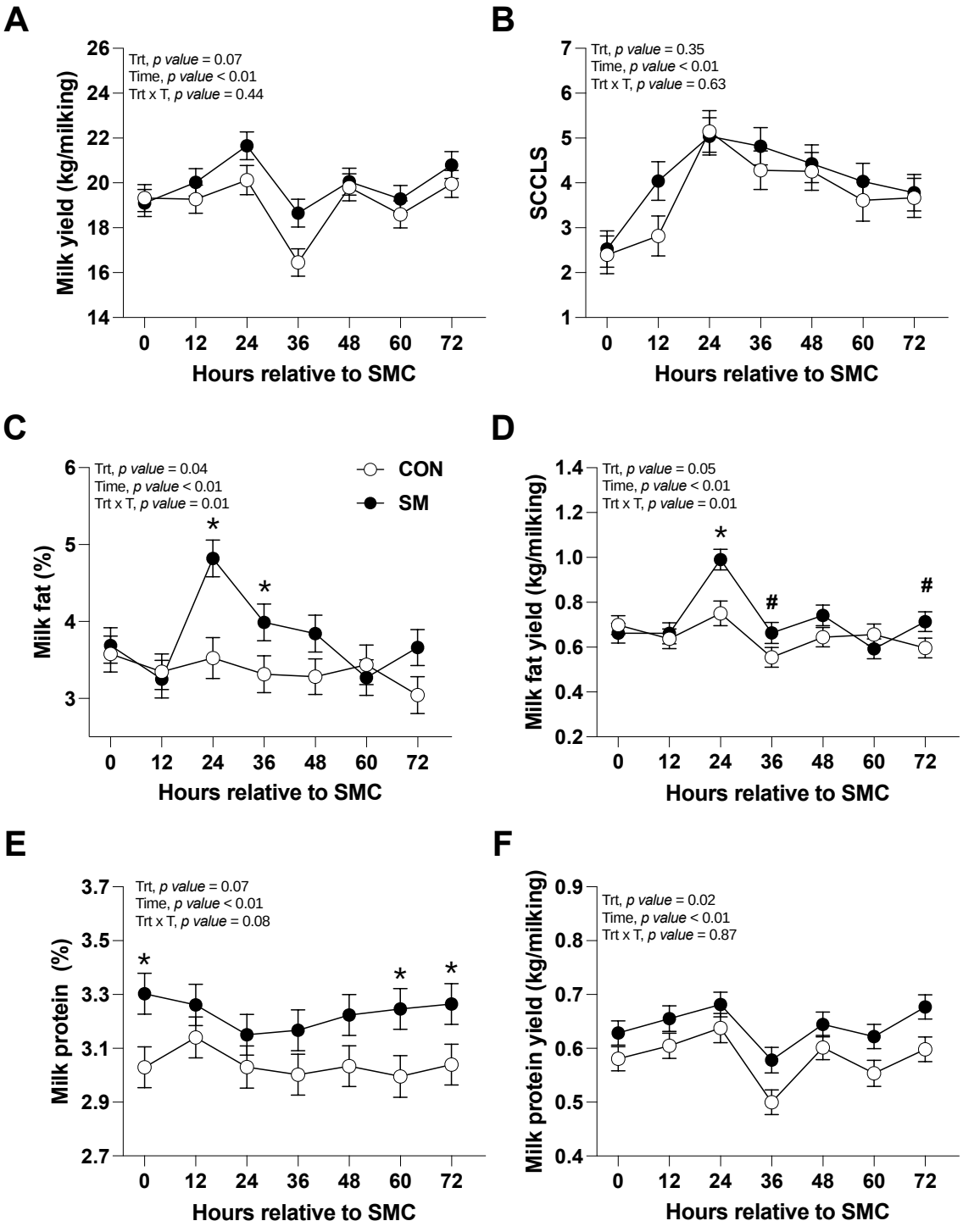


**Figure 2.6.** Effects of supplemental Smartamine M (SM; Adisseo Inc.) on milk yield (A), Somatic Cell Count Linear Score (SCCLS) (B), fat milk content (C), fat milk yield (D), milk protein content (E), milk protein yield (F) for cows from 0 to 72 h after the SMC. SMC = Subclinical mastitis challenge. CON = control. Values are means, with standard errors represented by vertical bars. Time points are relative to subclinical mastitis challenge with *Streptococcus uberis*.

**Table 2.3.** Effects of supplemental Smartamine M (SM; Adisseo Inc.) on blood biomarkers related to energy metabolism, inflammation, liver function, and oxidative stress in dairy cows from 0 to 72 h relative to SMC.

| | Treatment[3] | | | P-value | | |
|---|---|---|---|---|---|---|
| **Parameter** | **CON** | **SM** | **SEM[1]** | **Trt** | **Time** | **Trt × T** |
| *Energy metabolites* | | | | | | |
| Glucose, mmol/L | 4.55 | 4.60 | 0.04 | 0.33 | 0.00 | 0.79 |
| BHB, mmol/L | 0.50 | 0.51 | 0.02 | 0.86 | 0.01 | 0.48 |
| NEFA, mmol/L | -3.52 | -3.52 | 0.11 | 0.98 | <0.01 | 0.85 |
| *Inflammation* | | | | | | |
| Ceruloplasmin, µmol/L | 2.78 | 2.14 | 0.13 | <0.01 | <0.01 | 0.38 |
| D-Lactic acid | 3.07 | 3.09 | 0.06 | 0.81 | 0.08 | 0.83 |
| *Liver function* | | | | | | |
| Albumin, g/L | 36.6 | 36.4 | 0.32 | 0.73 | <0.01 | 0.37 |
| Cholesterol, mmol/L | 6.14 | 6.17 | 0.16 | 0.89 | <0.01 | 0.06 |
| Paraoxonase, U/mL | 102.9 | 95.9 | 4.03 | 0.24 | <0.01 | 0.64 |
| Globulin g/l | 43.6 | 43.6 | 0.76 | 1.00 | <0.01 | 0.71 |
| *Oxidative stress* | | | | | | |
| FRAP [2], µmol/L | 167.1 | 174.6 | 3.3 | 0.13 | <0.01 | 0.62 |
| Myeloperoxidase, U/L | 499.8 | 483.3 | 8.40 | 0.18 | 0.32 | 0.66 |
| ROM, mg $H_2O_2$/100mL | 15.37 | 13.01 | 0.56 | 0.01 | <0.01 | 0.40 |
| *Minerals* | | | | | | |
| Calcium mmol/L | 2.59 | 2.54 | 0.03 | 0.28 | 0.20 | 0.05 |
| *Glutathione activity* | | | | | | |
| Total GSH -10 d, mM | 0.01 | 0.02 | 0.00 | 0.05 | - | - |
| Reduced GSH -10 d, mM | 0.01 | 0.02 | 0.00 | 0.18 | - | - |
| Total GSH 24 h, mM | 0.23 | 0.35 | 0.04 | 0.07 | - | - |
| Reduced GSH 24 h, mM | 0.19 | 0.41 | 0.04 | <0.01 | - | - |

[1] Largest standard error of the mean is shown.
[2] Data were log-transformed before statistics. The standard errors of the means associated with log-transformed data are in the log scale.
[3]CON = control; SM = CON + SM (0.09% of DMI).

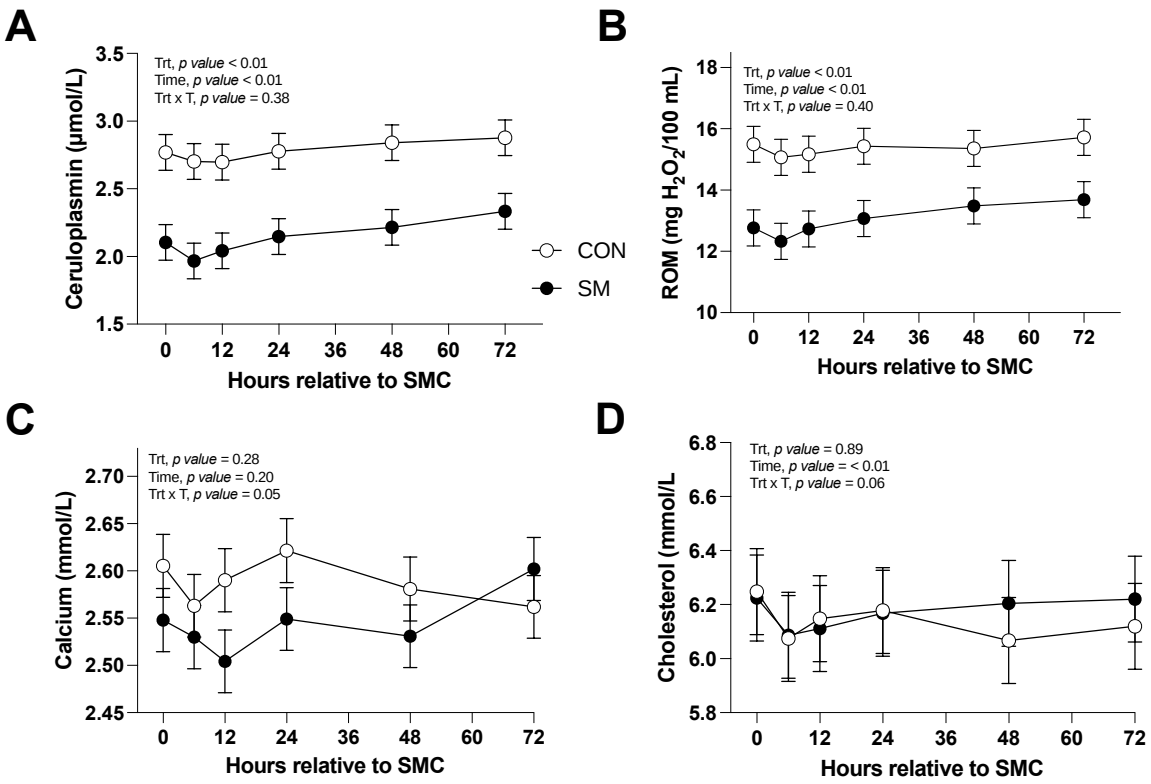


**Figure 2.7.** Influence of supplemental Smartamine M (SM; Adisseo Inc.) on ceruloplasmin (A), Reactive Oxygen Metabolites (ROM) (B), Calcium (C), and Cholesterol (D) from 0 to 72 h after the SMC. CON = control. Values are means, with standard errors represented by vertical bars. Time points are relative to subclinical mastitis challenge with *Streptococcus uberis.*

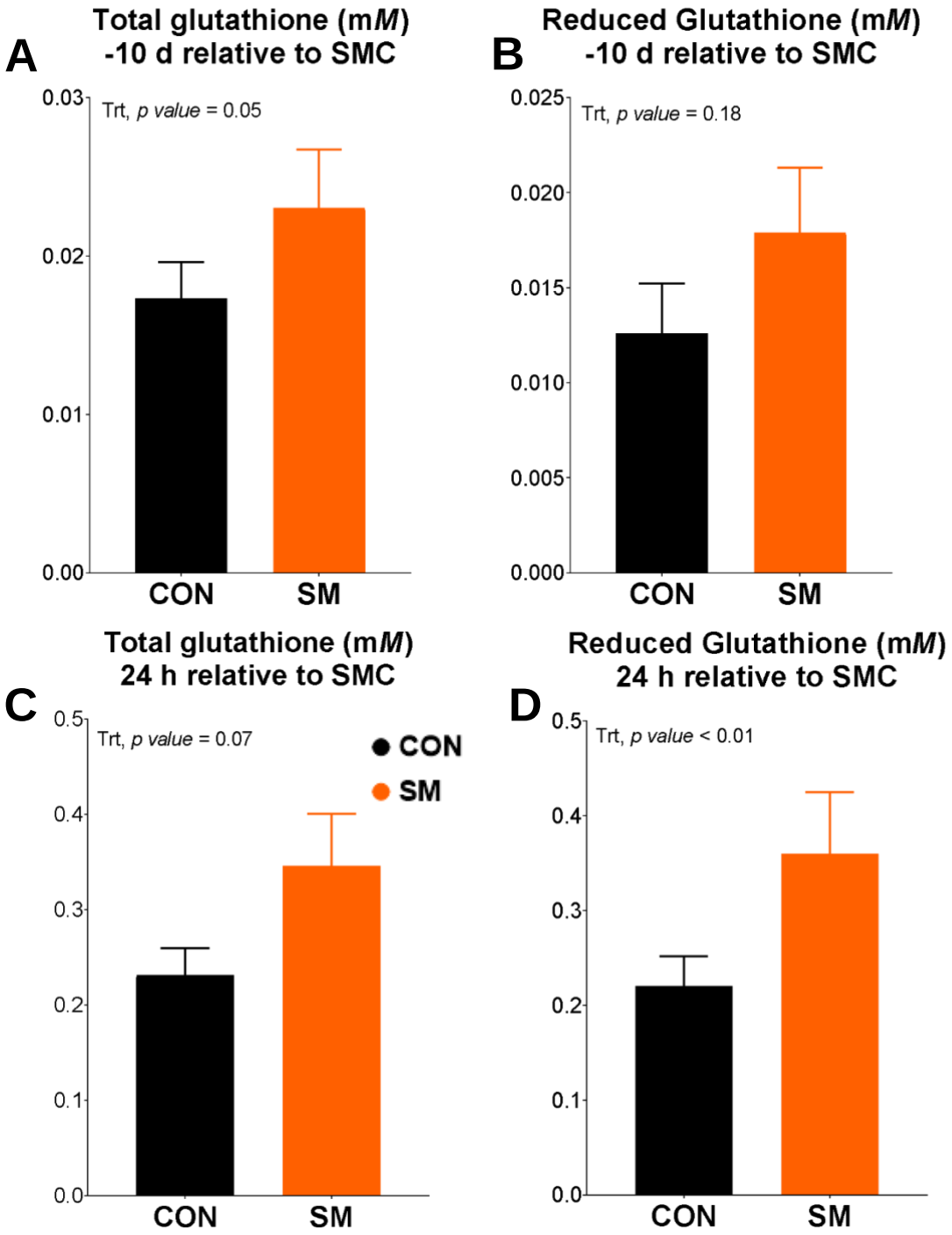


**Figure 2.8.** Effects of supplemental Smartamine M (SM; Adisseo Inc.) total liver glutathione (A and C) and reduced liver glutathione (B and D) in cows at -10 d and 24 h relative to SMC with *Streptococcus uberis*. CON = control. Values are means, with standard errors represented by vertical bars.

Figure 2.8 shows the effects of supplemental Smartamine M on total liver glutathione and reduced liver glutathione. Total liver glutathione at -10 d relative to SMC was greater ($P < 0.05$) in SM cows compared to the CON group (Figure 2.8A). No treatment difference was observed between SM and CON cows in reduced glutathione – 10 d before the SCM (Figure 2.8 B). In addition, at 24 h after the SMC, total liver glutathione tended ($P = 0.07$) to be greater in SM cows than in the CON group. Furthermore, at 24 h, reduced glutathione was greater ($P < 0.01$) in SM than in CON cows. It is important to highlight that glutathione is the most-important redox regulator that controls inflammatory processes (Bains and Bains, 2015) as it serves as a defense mechanism against oxidative stress (Lu, 2013).

Met is a precursor for de *novo* glutathione synthesis, which allows it to be directly linked to the liver glutathione concentration (Halsted, 2013; Osorio et al., 2014). In the reduced form, GSH plays a vital role in cellular metabolism and protects against free-radical-induced oxidant injury (Lobo et al., 2010). Interestingly, we observed greater total liver glutathione and reduced glutathione levels in SM cows after the SMC. Previous studies have shown an increased hepatic expression of genes associated with liver glutathione (Osorio et al., 2014; Jacometo et al., 2017; Batistel et al., 2018) while supplementing Met. The antioxidant role of greater liver glutathione concentration in our study was demonstrated by lower ($P < 0.01$) ROM in SM cows, indicating a less pronounced oxidative stress (Zheng et al., 2021). Thus, the tendency for higher glutathione concentration and lower ROM due to the inclusion of SM during the SMC suggests that supplemental Met might have influenced the reduction of oxidative stress. Additionally, glutathione in dairy cows can supply AA, such as Cys, to the mammary

gland for milk synthesis (Pocius et al., 1981). Moreover, previous studies have associated an increase in oxidative stress markers with a decrease in milk production (Tanaka et al., 2008; Pedernera et al., 2010), which may also explain the greater milk performance of SM-supplemented cows during the SMC when comparing with the CON group.

**mTOR pathway**

The ratio of phosphorylated (p) to total protein mean fluorescence intensity for AKT protein kinase, eIF4E-binding protein 1 (4EBP1), and S6 ribosomal protein (S6RP) in isolated immune milk and blood cells are presented in Table 2.4. Compared to the CON group, a greater (P = 0.04) pAKT:AKT ratio in milk neutrophils was detected at 24 h in SM cows (Table 2.4 and Figure 2.9A). Similarly, pS6RP:S6RP ratio was greater in milk neutrophils ($P = 0.04$) and macrophages ($P = 0.01$) from SM cows than in the CON group (Table 2.3 and Figure 2.9B and C)). Regarding the blood cells, the only p:total protein ratio affected by the treatment was the p4EBP1:4EBP1 ratio in neutrophils, which was greater ($P = 0.02$) in SM cows than in CON (Table 2.3 and Figure 2.10).

Besides being a central regulator of protein synthesis at the cellular level, the mTOR pathway is also recognized to have a pivotal role in regulating immune response (Powell et al., 2012; Khan et al., 2022). It is a vital link between cellular immune function and metabolism (Powell et al., 2012). mTOR regulates translation, cytokine reactions, antigen presentation, macrophage polarization, and cell migration (Weichhart et al., 2015). The mTOR pathway forms two multiprotein complexes: mTORC1, which is sensitive to nutrients, and its primary function is to regulate protein synthesis and cell

growth; and mTORC2 which is regulated via PI3K and growth factor signaling (Jhanwar-Uniyal et al., 2019).

(Salama et al., 2019) documented that Met treatment regulates most of the genes that are associated with transcription and translation (*MAPK1, MTOR*, *SREBF1*, *RPS6KB1*, and *JAK2*), AA transport, and cell proliferation in BMEC in vitro. Additionally, it has been found that Met-treated mammary gland cells show increased expression of *MTOR*, which is positively linked to the milk protein synthesis (Nan et al., 2014).

In our study, we evaluated the phosphorylation level of AKT 4EBP1, and S6RP, which interact with the mTOR pathway (Sipka et al., 2020). For instance, AKT is involved in the PI3K-AKT-mTOR pathway (Zhang et al., 2013). In our study, pAKT:AKT was greater in milk neutrophils from SM cows than in CON cows. The kinase AKT has been widely recognized to play an essential role in activating mTOR by sensing signals related to cell differentiation, proliferation, and apoptosis (Laplante and Sabatini, 2012). Furthermore, it also plays an important role in immune metabolism by regulating the development and functions of innate immune cells such as neutrophils, macrophages, and dendritic cells (Zhang et al., 2013). For example, AKT promotes inflammatory neutrophil recruitment and extends neutrophil survival during inflammation and macrophage cytokine expression survival and death (Zhang et al., 2013), which is key in the resolution of inflammation.

Furthermore, essential substrates of mTORC1 include ribosomal protein S6RP and 4EBP1 (Salmond et al., 2009). In our study, pS6RP:S6RP was greater in milk neutrophils and macrophages from SM cows than in CON cows. In addition, the p4EBP1:4EBP1 ratio in blood neutrophils was greater in SM cows than in the CON

group. 4E-BP1 is a translation repressor downstream of the mTORC1 pathway and plays a pivotal role in the adaptive immune response (Li et al., 2021). Its phosphorylation is generally considered positive and a consequence of the mTORC1 activation (Chi, 2012).

The mTOR pathway controls basic cellular processes such as translation and protein synthesis through its substrates (4EBP1, S6RP) and usually prevents exaggerated innate immune response (Weichhart et al., 2008; Powell et al., 2012). For example, the depression of the mTOR signaling in immune cells is responsible for increasing pro-inflammatory cytokines, including IL–12, TNF-a, and IL-6, while decreasing anti-inflammatory cytokines essential for the resolution of inflammation, such as IL-10 (Powell et al., 2012). TORC1 activity can be regulated on multiple levels by different factors depending on environmental conditions (González and Hall, 2017). Met boosts the synthesis of the methyl donor S-adenosylmethionine (SAM). SAM inhibits autophagy and promotes growth through the action of the methyltransferase Ppm1p, which modifies the catalytic subunit of PP2A in tune with SAM levels (Sutter et al., 2013). Methylated PP2A promotes the dephosphorylation of Npr2p to prevent the assembly of the complex and eventually activate TORC1. Thus, Met could modulate TORC1 activation through regulating SAM and the methylation status of the PP2A (Sutter et al., 2013; González and Hall, 2017). Overall, our results indicate that Met may enhance both blood and milk immune cells' protein synthesis by activating the mTOR pathway, consequently helping the cows to boost the immune system in the resolution of infection.

**Table 2.4.** Ratio of phosphorylated (p) to total protein mean fluorescence intensity for AKT, 4EBP1, and S6RP in mammary gland cells at 24 h, and blood cells at 0, 12, and 24 h relative to SMC with *Streptococcus uberis.*

| | Treatment[3] | | | P-value | | |
|---|---|---|---|---|---|---|
| **Parameter** | **CON** | **SM** | **SEM[1]** | **Trt** | **Time** | **Trt × T** |
| ***Mammary gland cells*** | | | | | | |
| pAKT:AKT | | | | | | |
| Neutrophils | -1.36 | 0.71 | 0.21 | 0.04 | - | - |
| Macrophages | -0.39 | -0.25 | 0.17 | 0.54 | - | - |
| p4EBP1:4EBP1 | | | | | | |
| Neutrophils | -0.72 | -1.04 | 0.24 | 0.35 | - | - |
| Macrophages | 0.52 | 0.39 | 0.14 | 0.51 | - | - |
| pS6RP:S6RP | | | | | | |
| Neutrophils | -0.14 | 0 .17 | 0.11 | 0.04 | - | - |
| Macrophages | 0.33 | 0.67 | 0.08 | 0.01 | - | - |
| ***Blood cells*** | | | | | | |
| pAKT:AKT | | | | | | |
| Neutrophils | 0.8 | 0.71 | 0.09 | 0.51 | 0.83 | 0.55 |
| Monocytes | 1.34 | 1.27 | 0.07 | 0.51 | 0.77 | 0.42 |
| p4EBP1:4EBP1 | | | | | | |
| Neutrophils | 0.54 | 0.72 | 0.05 | 0.02 | 0.67 | 0.64 |
| Monocytes | 0.5 | 0.63 | 0.06 | 0.13 | 0.76 | 0.47 |
| pS6RP:S6RP | | | | | | |
| Neutrophils | 0.38 | 0.40 | 0.03 | 0.56 | 0.73 | 0.52 |
| Monocytes | -0.61 | -0.46 | 0.09 | 0.25 | 0.51 | 0.39 |

[1]Largest standard error of the mean is shown.

[3]CON = control; SM = CON + SM (0.09% of DMI).

S6RP = S6 ribosomal protein. AKT = Protein kinase. 4EBP1 = Eukaryotic Translation Initiation Factor 4E Binding Protein 1.

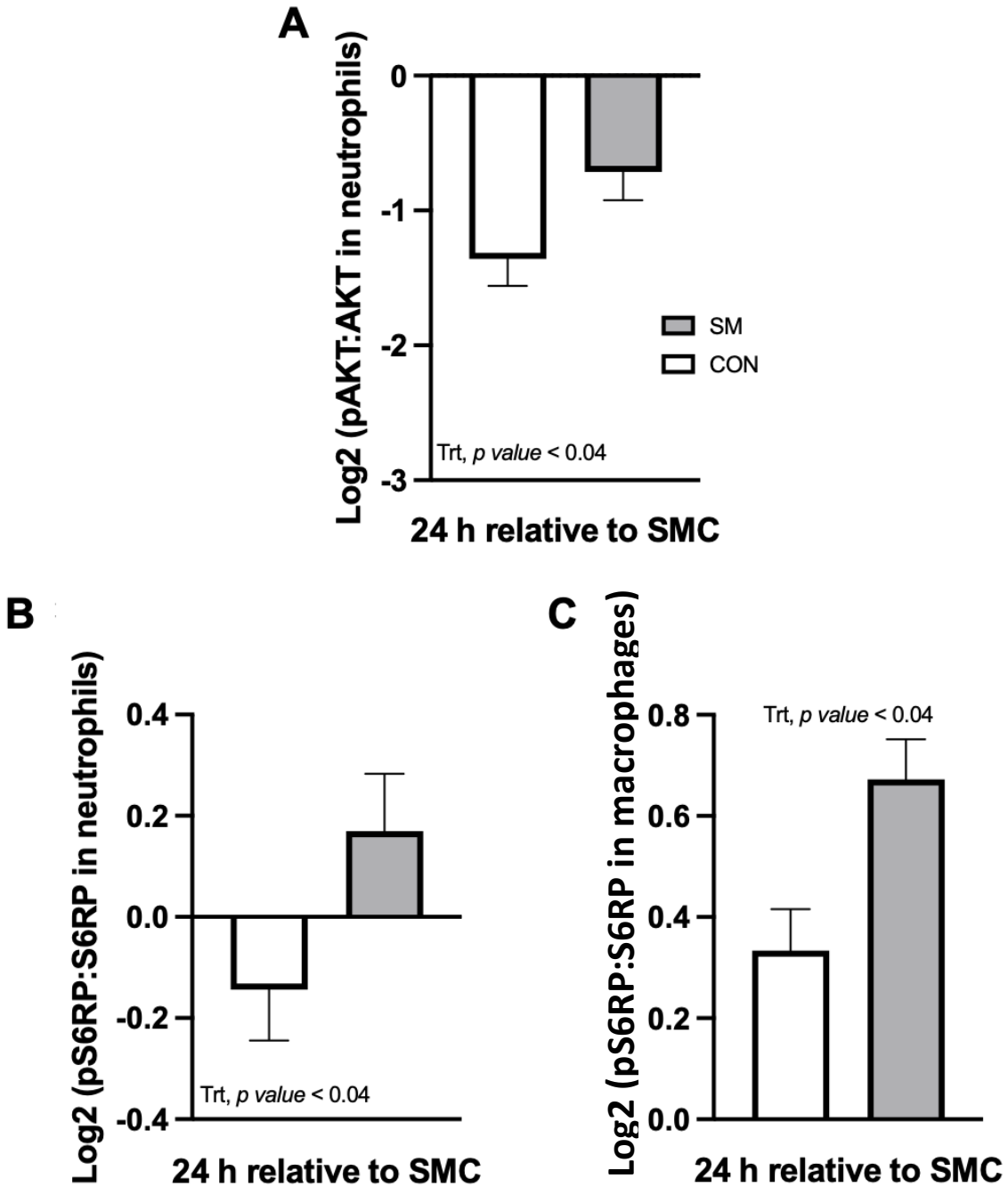


**Figure 2.9.** The ratio of phosphorylated (p) to total protein mean fluorescence intensity for AKT in neutrophils (A) and S6RP in neutrophils and macrophages (B and C) from mammary gland cells at 24 h after the SMC. S6RP = S6 ribosomal protein. AKT = Protein kinase. Values are means with standard errors represented by vertical bars.

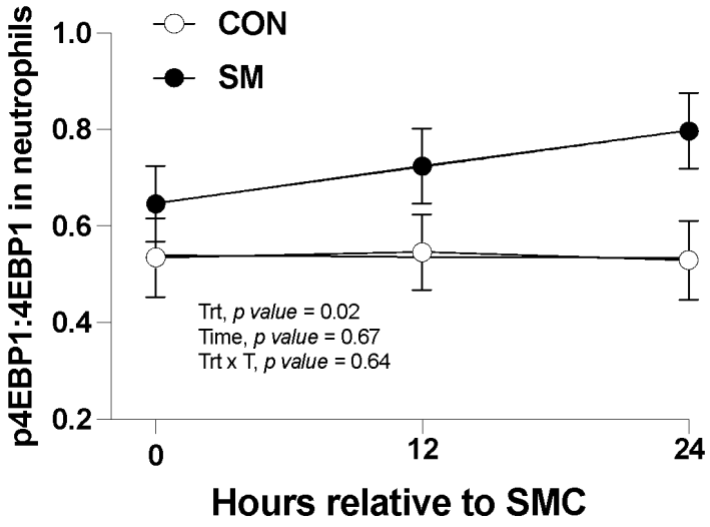


**Figure 2.10.** Ratio of phosphorylated (p) to total protein mean fluorescence intensity for 4EBP1 in PMN from blood at 0, 12, and 24 h relative to SMC. 4EBP1 = Eukaryotic Translation Initiation Factor 4E Binding Protein 1. Values are means, with standard errors represented by vertical bars. Time points are relative to subclinical mastitis challenge with *Streptococcus uberis.*

## CONCLUSIONS

The current study showed that supplementing Smartamine M to mid-lactation Holstein dairy cows during a subclinical mastitis challenge maintains greater milk yield while improving milk components. Regarding oxidative stress, SM cows had lower blood ROM concentration and greater liver glutathione. In addition, Smartamine M supplementation might attenuate inflammation and enhance blood and milk immune cells' protein synthesis by activating the mTOR pathway. Further research is needed to understand the detailed effects of Met on immune cells in terms of the expression of cytokines and transcription factors. A better understanding of the linkages between nutrition and immunity could facilitate the design of effective nutritional strategies to reduce disease susceptibility in dairy cows.

# CHAPTER 3. OVERALL CONCLUSIONS

Supplementing dairy cows with RPM during lactation can significantly benefit milk production and the immune system. Met is an essential AA required for protein synthesis and numerous metabolic processes. It plays a crucial role in milk production and the proper functioning of the immune system.

Met is a limiting AA for milk protein synthesis. By supplementing dairy cows with RPM, the availability of this essential AA is increased, which can improve milk protein yield, quality, and overall production.

During lactation, dairy cows experience increased metabolic demands, and optimizing their nutrition is essential to support their health and productivity. Met supplementation can contribute to the overall metabolic balance of cows by ensuring sufficient Met availability for protein synthesis and metabolic pathways.

The immune system plays a crucial role in maintaining the health and productivity of dairy cows. Met is also involved in immune cell function and antioxidant defense mechanisms. Therefore, supplementing dairy cows with RPM can enhance the cow's immune response, making them more resistant to infections. Improved immune function can also reduce the incidence of mastitis, metritis, and other illnesses, improving overall cow health and treatment costs.

In conclusion, supplementing dairy cows with RPM during lactation benefits milk production, metabolic health, and immune system function. RPM supplementation can enhance cow productivity, health, and farm profitability by providing an essential amino acid critical for protein synthesis and immune response.

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