MANAGING INFLAMMATION THROUGH NUTRITION: AN INVESTIGATION OF NIACIN, CANNABINOIDS, AND CARBOHYDRATES FOR LACTATING DAIRY CATTLE

By

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ABSTRACT

Improving animal health, resilience, and efficiency through diet formulation is a critical area of investigation for livestock. Improving health and resilience through nutrition will reduce the need for pharmaceuticals, such as antibiotics, while also improving the health and longevity of dairy cattle. Specific interest lies in investigating nutrients or feed additives and how they may improve resilience as well as diet formulations of macronutrients, such as starch.

We investigated whether rumen protected niacin (RPN) altered inflammation, improved animal health, or improved milk production. In late-lactation cows, RPN reduced milk SCS prior to intramammary challenges and reduced or blunted the inflammatory response after the intramammary challenges. These data also indicate that some epigenetic programming may be occurring as the inflammatory response was blunted well after RPN supplementation had ceased. When we translated this approach of supplementing RPN to dairy cows on a farm in Michigan we observed that RPN did not alter inflammation or health. The incidence of mastitis or culling was not different across the treatments. We observed an increase in peak milk yield and more persistent lactation for cows supplemented with RPN. Primiparous and 2nd parity cows supplemented with RPN had 639 kg and 712 kg more milk over the course of their lactation. This, again, indicates some kind of programming affect as the vast majority of the increase in milk yield occurred after RPN supplementation had ended.

Additionally, we demonstrated that β-caryophyllene (BCP) has promise as an anti-inflammatory feed additive for dairy cattle. Our results suggest that BCP reduced cytokine secretion and alters immune cell migration capacity, but in vivo investigation is necessary. We also investigated the role of dietary starch in inflammation. Upon review of the data, we observed that dietary starch alone does not consistently modulate inflammation. Under challenge

scenarios, where starch is abruptly added to the diet, then inflammation occurs. The data suggest that diet adaptation and feeding management play a role in diet-derived inflammation. We fed increasing levels of starch and different corn silage varieties to lactating dairy cattle and found that inflammatory biomarkers were similar for each treatment group. Furthermore, additional starch increased feed efficiency, energy-corrected milk yield, and milk protein yield. The α-amylase enhanced silage increased digestibility and reduced milk urea nitrogen but it did not affect milk production. In summation, nutrition can modulate the inflammatory status of dairy cattle and it should continue to be investigated across a variety of life cycle stages and under a variety of stressor scenarios.

For Gigi & Grandpa –
You watched me leave the starting line and I know you're in heaven watching me cross it. You both did the hard work for our families – your children, grandchildren, and great-grandchildren stand on your shoulders. You both demonstrated the grit, determination, toughness, and goodness that we all should strive to emulate. I miss you both every day.

"A grandparent is a little bit parent, a little bit teacher, and a little bit best friend."

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CHAPTER 1: INTRODUCTION

Research across species has demonstrated that inflammation and metabolism are interlinked and implicated in various diseases and chronic health problems. Additionally, in the context of dairy cattle, biomarkers of inflammation and metabolism are associated with reduced performance, poorer reproduction, and reduced longevity. Therefore, modulating inflammation and metabolism of dairy cows, especially through their transition from gestation to lactation or during periods of stress, may improve their health, welfare, and efficiency.

Pharmaceuticals and antibiotics have been used to control disease, inflammation, or other conditions in dairy cattle, but consumer sentiment and microbial antibiotic resistance has been driving research into how nutrients, feed additives, or nutritional strategies may improve health and resilience of livestock, dairy cows specifically. Such feed ingredients and additives have been dubbed “nutraceuticals”. Of particular interest are nutraceuticals that reduce inflammation and improve metabolism during the transition period leading to improved milk production. Also, nutraceuticals that reduce incidence of disease and culling would be especially impactful on the dairy industry.

One candidate for improving dairy cow health through the transition period is niacin, also known as vitamin B_3. It reduces lipolysis in transition dairy cows when supplemented from 3 weeks prior to 3 weeks after calving. Another marker of lipid metabolism and health is beta-hydroxy butyrate (BHB), which is also reduced by niacin supplementation during the peripartum period. Reducing lipolysis may be beneficial as epidemiological research associates increased circulating free fatty acids and BHB with disease, poor fertility, and culling. In addition to its lipid mediating effects, niacin has potential to reduce inflammation. Research conducted in mice, swine, and humans demonstrates that niacin has anti-inflammatory and anti-oxidative properties

which have not yet been investigated in dairy cows. Excess lipolysis and dysregulated inflammation are often implicated as the root of problems cows experience during lactation – niacin may help remedy both of those potential provenances.

Also, many experiments with dairy cattle evaluate feeding strategies or feed ingredients, like niacin, and their effects on biomarkers of metabolism or inflammation. These studies are excellent at demonstrating whether these ingredients nudge inflammation or metabolism, but do they demonstrate improved animal health? Maybe. Do they demonstrate more resilience to challenges or disease? Maybe. Do they demonstrate reduced disease and improved reproduction? No. To truly demonstrate improved health, resilience, and reproduction as a result of a nutraceutical supplementation, we must enroll thousands of cows across herds to observe whether actual disease incidence, culling, or reproduction is affected; our goal was to conduct such research.

Additional candidates for reducing inflammation and improving health of dairy cattle are phytochemicals like terpenes. One specific terpene that interacts with the cannabinoid system, a regulator of inflammation and metabolism, is β-caryophyllene (BCP). BCP selectively binds to the CB2 receptor which is highly expressed on various immune cell populations and results in anti-inflammatory downstream effects but research investigating BCP supplementation in dairy cattle has not yet been done.

Our objective was to determine whether different feeding strategies or nutraceuticals alter the inflammation, metabolism, disease, culling, or reproduction of dairy cattle with the ultimate goal being to increase longevity of dairy cattle. We wanted to combine bench-top *in vitro* experiments, intensive *in vivo* inflammation and metabolism experiments, and large randomized controlled field experiments to clearly understand how the biology of the cow was affected and

demonstrate whether any changes to metabolism or inflammation translated to whole animal outcomes relevant to dairy farmers like disease, culling, and reproduction.

CHAPTER 2: LITERATURE REVIEW

Dairy cow welfare and longevity are growing concerns for consumers of dairy products. This presents the industry with opportunities as well as ethical, animal welfare, and sustainability challenges. As research into dairy cattle health has progressed, we have learned that dairy cows experiencing greater inflammation are poorer performers, have greater levels of disease, and are less fertile (Bionaz et al., 2007; Bertoni et al., 2008; Bradford and Swartz, 2020; Kerwin et al., 2022). Excess inflammation reduces the cow's efficiency, welfare and longevity. In the last two decades, genetic selection for health traits and reproduction has improved the fertility and health of dairy cows (CDCB, 2022) but we're also learning that feed supplements and certain feeding strategies can modulate inflammation and improve health in substantial ways (Yuan et al., 2015; Michelotti et al., 2021). This literature review will synthesize previous research into how different nutritional strategies may relate to or affect systemic inflammation in dairy cattle with particular focus on the lipid metabolism, niacin, and the endocannabinoid system. Furthermore, it will review how potential nutraceuticals may be tools to improve health, production, and resilience of lactating dairy cows.

INFLAMMATION, LIPOLYSIS, AND THE TRANSITION DAIRY COW

The transition period of dairy cattle, demarcated as 3 weeks before calving to 3 weeks after calving, is characterized by tremendous metabolic and physiological changes. As the cow moves from gestation to lactation, she experiences hypoglycemia, hypocalcemia (Wilkens et al., 2020), hyperketonemia, and hyperlipidemia (Figure 2.1). She will also experience increases in circulating levels of glucocorticoids and somatotropin while insulin, progesterone, and estrogen are reduced (Ingvartsen and Andersen, 2000; Ingvartsen and Moyes, 2013). The biological changes that cows undergo is accompanied by the majority of disease that cows experience

(Ingvartsen, 2006) throughout lactation. Defining and neutralizing the root cause of these diseases is critical to improving animal health and welfare but challenging due to the breadth of systemic changes occurring during this timeframe.

One risk factor for challenges during the transition period is the mass of adipose tissue, or body condition score (BCS). Cows with greater BCS have lower DMI at calving (Al Ibrahim et al., 2010). The relationship between BCS and milk yield is more nuanced; It has been observed to be curvilinear with maximum lactation milk yield occurring at BCS between 3 and 3.5 (Roche et al., 2009). For cows in the United States specifically, milk yield increased as BCS increased from 1 to 3.5 at which point it slowly declined (Waltner et al., 1993). For production variables like DMI and milk yield, a moderate BCS is most preferable to emaciated or excessively fat cows.

As it relates to reproduction, BCS loss after calving is more often associated with reproductive performance than BCS at calving (Roche et al., 2009). Increased BCS at calving was associated with an increase in fertility while increased BCS loss was associated with reduced likelihood of pregnancy (Roche et al., 2007). Additionally, increased BCS during lactation is generally favorable for reproductive outcomes (Roche et al., 2009). These results suggest that energy balance plays a significant role in reproductive efficiency. Further demonstrating this, one experiment observed that cows who gained BCS during the transition period had a 3-fold increase in conception rate compared to cows who lost BCS (Barletta et al., 2017). Managing energy balance and BCS loss may aid in improving reproductive outcomes for dairy cows. The BCS and BW loss experienced may be difficult to manage though as it seems to be similar for cows across lactations (Zachut and Moallem, 2017). Another factor to consider is inflammation; cows with greater BW loss have greater inflammation (Zachut et al., 2018) so cows with greater

BW loss may be experiencing additional inflammation that may also be impairing their health and fertility.

BCS and BCS loss have been associated with increased disease incidence, but the results are not consistent. Barletta et al. (2017) observed that cows who lost BCS were 1.8 times more likely to develop mastitis than cows who gained BCS. Contrary to this, BCS loss was not related to clinical mastitis and other BCS associations "…lacked biological significance within the ranges of BCS and BW generally observed on-farm." (Berry et al., 2007). However, increased BCS loss is associated with increased ketosis incidence (Barletta et al., 2017) and ketosis may leave cows more susceptible to mastitis by promoting a pathogen tolerance in the mammary gland (Swartz et al., 2021). Again, data suggest that maintaining moderate BCS prior to calving and minimizing BCS loss, or even gaining BCS, in the early lactation period may improve health outcomes in dairy cattle. Whether that is a result of improved energy balance or reduced inflammatory state is ambiguous.

The biomarkers associated with increased BCS and increased BCS loss also demonstrate an association with disease or fertility. Epidemiological studies have associated excessive free fatty acids and BHB during the transition period to diseases such as displaced abomasum, metritis (Ospina et al., 2010b), and mastitis (Melendez et al., 2009). Relationships between disease and these circulating metabolites have been repeated by different investigators and were reviewed by McArt et al. (2013); these consistent results suggest there may be a connection between adipose tissue mobilization, metabolism, and disease. Whether the relationship is causative has been hotly debated. Recently, researchers have been reevaluating these relationships and the long-held dogma that elevated free fatty acids and BHB are problematic in transition dairy cattle or causative of later disease occurrence. Horst et al. (2021) concluded that

the associations between biomarkers of metabolism, like free fatty acids and BHB, and disease is a result of preceding subclinical inflammation and concurring hypophagia that probably occurred at or around calving as a result of the inflammation. Otherwise, elevated free fatty acids and BHB resulting from lipolysis are healthy, homeorhetic responses to the commencement of lactation. Elevated free fatty acids and BHB are certainly part of a healthy adjustment to lactation, but increased BHB in particular may leave cows more susceptible to bacterial diseases like mastitis by reducing immune cell migration to the mammary system (Zarrin et al., 2014; Swartz et al., 2021). When managing lipolysis and ketosis, it's a matter of risk; increased BHB or NEFA does not guarantee disease occurrence and poor fertility, but they may increase the risk of such problems by leaving the cow in a more 'tolerant' or 'suppressed' immune state which may result in increased disease incidence, lost milk production, and increased risk of leaving the herd.

Whether inflammation is associated with lipolysis and BCS loss during the transition period is less clear. Some research has demonstrated that cows experiencing greater BCS loss have greater plasma Hp along with increased transcript abundance of inflammation-related genes like TNF-α, IL-6, and IL-1β (Dirandeh et al., 2020). Further supporting this relationship, Zachut et al. (2018) demonstrated the cows with greater bodyweight lost postpartum had greater Hp in adipose tissue at both 4 and 30 DIM. Additionally, there is some indication that cows experiencing greater BCS loss throughout the transition period have greater macrophage infiltration into adipose tissue (De Koster et al., 2018) but the phenotype of these macrophages is unknown. Whether the macrophages exhibit a more pro- or anti-inflammatory phenotype (Bajgar et al., 2021) would dictate the inflammatory cascade that follows their infiltration. Characterizing

the macrophage phenotype in adipose tissue during the transition period may be an area of future research worthy of more thorough investigation.

BCS, BCS loss, and inflammatory conditions like metritis are often associated, but determining which may have preceded the other is challenging. One retrospective experiment observed that cows with lower BCS (BCS of 2-2.5) had elevated concentrations of IL-1, IL-6 and TNF-α compared to cows with higher BCS (>3). Additionally, cows with persistent uterine inflammation had lower BCS than healthy cows (Kasimanickam et al., 2013). BCS loss and inflammation were associated but whether the inflammation resulting from metritis, or some other inflammatory condition may have increased cytokines and suppressed appetite which resulted in lower BCS or whether the excessive BCS loss promoted inflammation is not clear. Contrary to these results, cows with greater BCS (>3.75) had increased expression of TLR4 and TLR9 while also having reduced expression of antioxidant related genes indicating an increased inflammatory state in cows with greater BCS than cows with lower BCS (<3.25); cows with more BCS also lost more body condition than cows with lower BCS (Alharthi et al., 2018). Also, cows with greater BCS at mid-lactation had greater concentrations of TNF-a in plasma with reduced antioxidant defenses than cows with lower BCS indicating that excessive BCS may exhaust antioxidant defenses and leave those cows more susceptible to oxidative stress and resulting inflammation (O'Boyle et al., 2006). These results align with Bernabucci et al. (2005) who observed cows with greater BCS loss had greater free fatty acids, greater reactive oxygen metabolites (ROM), and greater thiobarbituric acid-reactive substances (TBARS) during the postpartum period indicating greater oxidative stress and possibly increased risk of inflammation and disease. Clearly, moderate BCS (~3) is preferred as both high and low BCS which may be problematic. Excess BCS prepartum and excess BCS loss may reduce available antioxidant

defenses and leave those cows more vulnerable to oxidative stress, inflammation, and disease during the early lactation period. Managing BCS and lipolysis may improve inflammatory tone, oxidative defenses, and health of cattle but the relationship between lipolysis and inflammation is not clear; whether the inflammation or lipolysis comes first is up for debate.

The research investigating these adaptations to lactation, with a greater focus on the inflammatory status, has increased in the last two decades. We know that during the transition period, dairy cattle experience some degree of inflammation (Bertoni et al., 2008; Bradford et al., 2015; Bradford and Swartz, 2020). We also know that cows with greater inflammatory tone during the periparturient period produce less milk throughout their entire lactation; cows with greater Hp produced 500 kg less milk over their course of their lactation (Bertoni et al., 2008; Cattaneo et al., 2021; Kerwin et al., 2022). Cows with greater levels of pro-inflammatory cytokines also experience reduce DMI and milk yield after calving (Trevisi et al., 2015). Controlling inflammation is especially important considering the hypophagic effects of various cytokines (Brown and Bradford, 2021). Whether controlling and managing lipolysis also controls inflammation is much less clear. Regardless of the ambiguity of the relationship between lipolysis and inflammation, reducing or improving the regulation of inflammation through the transition period or during bouts of other physiological stressful periods by nutritional interventions may be an opportunity to improve health, production, reproduction, and welfare of dairy cows.

Role of Niacin in Metabolism and Inflammation

Niacin, also known as vitamin B_3 or nicotinic acid, is involved in metabolism as a precursor for the coenzymes NAD(H) and NADP(H) (NASEM, 2001). Generally, dairy cow B-vitamin requirements are assumed to be met by ruminal synthesis (NASEM, 2021). Dietary

composition and nutrient intake influence net ruminal B-vitamin synthesis; niacin synthesis increases with increasing non-fiber carbohydrates (Schwab et al., 2006; Niehoff et al., 2013; Castagnino et al., 2017; Seck et al., 2017). Research has demonstrated that 2-4 g of niacin exit the rumen each day (Schwab et al., 2006; Seck et al., 2017). Interestingly, only 8% of supplemental rumen degradable niacin reaches the duodenum (Niehoff et al., 2013). Feeding additional niacin is of interest because niacin has anti-lipolytic properties working through the HCA2 receptor (Carlson, 2005; Chen et al., 2019) which may help reduce excess lipolysis and ketosis in dairy cattle. In fact, niacin was first evaluated as a treatment for ketosis in dairy cattle 44 years ago (Fronk and Schultz, 1979). It may be that reducing lipolysis and ketosis by supplementing niacin may be a nutritional avenue that improves the health and disease occurrence on dairy farms.

The HCA2 receptor, niacin and butyrate's receptor, is expressed in various tissues of dairy cattle. Titgemeyer et al. (2011) observed that HCA2 mRNA and protein were present in tail head fat, back fat, perirenal fat, longissimus muscle, liver, and brain of dairy cattle. The HCA2 mRNA was also detected in bovine mammary tissue (Swartz et al., 2021). It has also been expressed at the mRNA and protein level in bovine neutrophils (Carretta et al., 2020) indicating that niacin may possibly have immune modulating effects in dairy cattle.

Supplementing niacin in human medicine is practiced because it reduces LDL, VLDL, and triglycerides while increasing HDL (Taylor et al., 2009; Villines et al., 2012). Favorable effects on cholesterol and triglycerides were confirmed in the AIM-HIGH study (2011). Supplementation of niacin to ruminants has been thoroughly investigated (Table 2.1) but it is complicated by whether or not the niacin is protected or encapsulated, as the vast majority of niacin is degraded in the rumen if not encapsulated or protected (NASEM, 2001; Niehoff et al.,

2013). NASEM (2001) concluded that additional niacin from non-encapsulated forms had little to no benefits on milk production of dairy cows which my literature review agrees with. There is some evidence indicating that provision of niacin may increase milk yield in high producing cows (Jaster et al., 1983; Muller et al., 1986) but these were retrospective analyses and more vigorous testing of such a hypothesis may be prudent. Niacin also may be beneficial in low protein diets as it increased organic matter and NDF digestibility in such diets (Aschemann et al., 2012). Although results from supplementing rumen-available niacin to dairy cattle have been neutral it must be revisited as rumen-protected niacin has become available and has been shown to have anti-lipolytic properties in cattle similar to those observed in other species (Yuan et al., 2012). Additionally, emerging evidence demonstrates that niacin has lipid-independent anti-inflammatory properties (Wu Ben et al., 2010; Graff et al., 2016) which may aid resolution of troublesome chronic transition cow inflammation (Bradford et al., 2015; Contreras et al., 2018; Bradford and Swartz, 2020). Since transition dairy cattle experience lipolysis and inflammation, niacin may improve dairy cow health and lactation performance by attenuating both challenges. Although the body of research on niacin is expansive, we may have been asking the wrong questions. Investigating the inflammatory status and potential health benefits of niacin for dairy cattle would fill a gap in the current literature.

Interestingly, niacin affects many different cell and tissue types (Kamanna and Kashyap, 2008; Graff et al., 2016). In adipose tissue, niacin binding to the HCA2 receptor inhibits lipolysis while also increasing adiponectin release (Plaisance et al., 2009). Adiponectin may be important in dairy cattle because adiponectin is associated with increased insulin sensitivity and may have anti-inflammatory effects, which both may improve the transition of dairy cows from gestation to lactation. Serum adiponectin concentration is inversely related to BCS of dairy cattle (De Koster

et al., 2017) which echoes data indicating the cows with greater BCS experience more challenging transition periods. Interestingly, mice fed high fat diets with supplemental niacin increased adiponectin secretion and reduced adipose gene expression of pro-inflammatory markers, MCP-1 and IL-1β (Wanders et al., 2013). Further supporting the notion that niacin promotes an anti-inflammatory tone in adipose tissue, Heemskerk et al. (2014) observed that niacin supplementation increased docosahexaenoic acid (DHA) synthesis, an anti-inflammatory ω-3 polyunsaturated fatty acid. This may be a point of future investigation for dairy cattle as polyunsaturated fatty acids and oxylipids have been useful markers of inflammation in dairy cattle (Putman et al., 2022). These data demonstrate that niacin may reduce adipose tissue inflammation and may be particularly useful for over-conditioned dairy cows which is a common challenge faced in the dairy industry.

In immune cells, results suggest that niacin is anti-inflammatory. The previously mentioned mouse study (Wanders et al., 2013) observed that niacin reduces expression of pro-inflammatory markers in macrophages. This phenomenon was also observed in patients with Parkinson's Disease; patients given 250 mg of niacin per day had a shift from M1 to M2 macrophage polarization (Wakade et al., 2018). This is compelling because M1 macrophages may infiltrate adipose tissue and promote chronic inflammation and excessive lipolysis during the transition period (Contreras et al., 2018). It may be that reducing more inflammatory macrophage phenotype may reduce inflammation in adipose tissue. This might result in reduced lipolysis and reduced free fatty acid concentration which may promote a smoother transition from gestation to lactation for high producing dairy cattle. Furthermore, supplementation with niacin reduced secretion of pro-inflammatory cytokines (IL-1, IL-6, and TNF-α) from macrophages of both lean and obese mice *in vitro*; *in vivo* results echoed those observations

(Lipszyc et al., 2013). Using human monocytes and mature macrophages, Montserrat-de la Paz et al. (2017) demonstrated that niacin reduced reactive oxygen species (ROS), reduced cytokine secretion, and encouraged an M2 phenotype of macrophages challenged with LPS. Niacin's effect on bovine monocytes has not been investigated yet but considering the presence of the HCA2 receptor, it is reasonable to infer that it may increase the population of pro-resolving M2 macrophages which may aid in resolving inflammation in dairy cattle.

Other tissues and cell populations have also been evaluated in response to niacin and echo that niacin has anti-inflammatory properties. In the colon, niacin modulated inflammation in colitis induced mice (Salem and Wadie, 2017). Niacin has attenuated inflammatory responses in mammary epithelial cells by increasing translocation of NRF2, a transcription factor related to antioxidant defenses (Guo et al., 2020). Evidence also suggests that niacin inhibited *S. aureus* induced NF-kB translocation in bovine mammary cells (Wei et al., 2014). Unfortunately, it also appears that niacin may reduce fatty acid synthase expression and fat production in mammary epithelial cells (Wang et al., 2020) but this has not been investigated *in vivo*. Niacin supplementation in dairy cows has reduced milk fat concentration (Yuan et al., 2012) although that may be more related to reduced circulating free fatty acids and preformed fatty acid incorporation into milk fat than reduced de novo fatty acid synthesis. Direct investigation of the effects of niacin on milk fatty acid profile and mammary gland metabolism may be warranted. Niacin has also reduced palmitic acid-induced inflammation and oxidative stress in human hepatocytes (Ganji et al., 2015). In vitro cell culture models have demonstrated the potential of niacin across many cell and tissue types. These anti-inflammatory responses would be favorable in dairy cattle.

An additional benefit of niacin is that it may reduce heat stress by increasing heat dissipation and evaporative cooling in dairy cattle under heat stress (Di Costanzo et al., 1997; Zimbelman et al., 2010; Zimbelman et al., 2013; Pineda et al., 2016). The alterations in body temperature are small and observations as to whether it improves milk production during heat stress are mixed. Milk production during heat stress has increased (Lohölter et al., 2013; Zimbelman et al., 2013; Pineda et al., 2016) or been unchanged (Di Costanzo et al., 1997; Zimbelman et al., 2010; Rungruang et al., 2014) as a result of niacin supplementation. Interestingly, heat stress is associated with heightened inflammatory state (Al-Qaisi et al., 2020; Kra et al., 2022a) which niacin may attenuate. Investigation of whether niacin reduces heat stress associated inflammation is needed. Also, investigation of possible seasonal benefits to niacin supplementation may provide more context about when niacin supplementation would be most beneficial for dairy cow health and welfare.

Evaluation of rumen-protected niacin supplementation to dairy cattle is scarce but what data is present demonstrates the well-known anti-lipolytic effects of niacin (Morey et al., 2011; Yuan et al., 2012). An underappreciated observation when supplementing rumen-protected niacin is that it has reduced SCC in dairy cattle (Morey et al., 2011; Yuan et al., 2012) which is one demonstration of immune modulation resulting from niacin supplementation. It may be that niacin promotes greater resilience to mastitis or reduces excess immune cell infiltration into the mammary gland. Unfortunately, there is no experiment using a mastitis challenge model to investigate whether niacin alters immune response or inflammation in response to mastitis. Such a study would demonstrate whether niacin improves a cow's ability to battle an infection or improve resolution of such an infection. Also, *in vivo* anti-inflammatory effects of rumen-protected niacin have been only sparsely measured. The only measure of inflammation when

feeding rumen-protected niacin was Hp and they observed a treatment × time interaction across the transition period but within each timepoint treatment was insignificant (Morey et al., 2011). Yuan et al. (2012) evaluated the oxidative stress of transition cows and observed that niacin did not affect plasma concentrations of superoxide dismutase which is one way for measuring oxidative stress. The authors concluded that "More bovine-specific oxidative stress markers need to be assessed to test the effects of RPN on the oxidative status of transition dairy cows.". A more comprehensive evaluation of niacin's effects in dairy cattle may uncover benefits not observed or measured by previous investigators. Specifically, determining whether rumen-protected niacin may reduce inflammation and oxidative stress during the periparturient period is warranted.

Current research investigating niacin in cattle has neglected the potential role it may play in modulating inflammation; research investigating whether niacin may reduce inflammation in bovines is warranted. There is evidence it may alter the mammary gland inflammatory status but effects on systemic inflammation have not been investigated. Additionally, research into its effects on disease incidence, fertility, or risk of leaving the herd is also needed.

Potential secondary effects of Niacin by reducing Free Fatty Acids

As previously mentioned, niacin is antilipolytic, which is especially relevant in the high-producing transition dairy cow. Two weeks prior to calving, free fatty acids begin to rise and peak within the first week postpartum where they may reach 1 mmol/L or greater (Contreras and Sordillo, 2011). The consistent relationship between elevated free fatty acids and disease occurrence (Dyk, 1995; McArt et al., 2013) has prompted further investigation into whole animal, tissue, and cellular responses to free fatty acids. The goal being to delineate the mechanism by which free fatty acids leads to immune incompetence or suppression during the

transition period. Various models such as feed restriction (Moyes et al., 2009) and administration of pro-inflammatory cytokines (Kushibiki et al., 2003) have been used to replicate the increased circulating free fatty acid concentrations observed during the transition period. There have also been cell culture experiments to evaluate responses of bovine cells to different levels of free fatty acids (Scalia et al., 2006; Li et al., 2020). None of the models alone provide a complete picture of how free fatty acids affect the cow during the transition period but together they provide a roadmap of where to continue such investigation.

In vitro studies demonstrate that free fatty acids influence the metabolism, inflammation, and activity of different cell types and in various species. Beginning with immune cells, increasing free fatty acids concentration reduced IgM and INF-γ secretions while also reducing DNA synthesis of peripheral blood mononuclear cells (PBMC) from dairy heifers (Lacetera et al., 2004). In a similar study with leukocytes, Scalia et al. (2006) observed that increasing the concentration of free fatty acids up to 2 mmol/L increased their oxidative burst activity which may increase or contribute to excess inflammation and oxidative stress during the transition from gestation to lactation. Further evidence that free fatty acid concentration affects immune cell activity was observed by Ster et al. (2012). They observed that serum with greater free fatty acids reduced PBMC proliferation and reduced release of INF-γ. Contrary to observations from Scalia et al. (2006), oxidative burst was reduced by increasing free fatty acid concentrations (Ster et al., 2012). Consistent reduction of INF-γ is compelling because mice with a disrupted INF-γ receptor gene are much more susceptible to infectious diseases (Huang et al., 1993) indicating the importance of INF- γ signaling in immunity. Stemming the increase in free fatty acids through niacin supplementation may promote immune competence through improved INF- γ signaling. Effects on other immune functions like oxidative burst or phagocytosis are less certain.

To investigate the effect of ketosis and elevated free fatty acids together, investigators harvested neutrophils from healthy cows and cows with ketosis (plasma BHB >2.6mM) and incubated them with increasing concentrations of free fatty acids. Increasing free fatty acids increased expression of pro-inflammatory cytokines IL-1β, IL-6, TNF-α. They also found that including an inhibitor to NF-kB suppressed the synthesis of these cytokines. They concluded that free fatty acids activate inflammation and cytokine production through toll like receptor 4 (TLR-4) binding and NF-kB signaling (Zhang et al., 2018). There is also evidence that ceramide synthesis may play a role in the free fatty acid initiated inflammatory response (Mamedova et al., 2013). This evidence taken together with evidence showing that niacin reduces lipolysis and NF-kB expression suggest that niacin may be a useful tool during the dairy cow transition period as it may both reduce free fatty acids and inflammation independently which may lead to improved immune responses and health of dairy cattle.

Effects of free fatty acids have also been investigated in hepatocytes. The liver is the metabolic hub and it's vital for transition dairy cows as it processes the excess free fatty acids that are mobilized during the transition period. Ruminants are unique as their liver is less inclined to export triglycerides and lipoproteins than other species (Chilliard, 1999) which makes increased circulating free fatty acids a risk factor of developing fatty liver in dairy cows. Increasing free fatty acid concentrations increased reactive oxygen species (ROS) production and apoptosis in dairy cow hepatocytes. Investigators also observed that free fatty acids inhibited activation of NRF2. Interestingly, niacin may promote this same transcription factor (Guo et al., 2020). The researchers concluded that increased free fatty acids lead to oxidative stress and possibly liver damage in dairy cows (Li et al., 2020). These results are supported by Gao et al. (2018) and Shi et al. (2015) who observed oxidative stress and decreased activity of antioxidant

defenses from increasing free fatty acid concentrations when evaluating bovine hepatocytes. In both immune cells and hepatocytes, free fatty acids can cause stress and inflammation. These *in vitro* studies suggest that reducing excessive free fatty acid mobilization, possibly through niacin supplementation, may lead to improved health through reduced oxidative stress and inflammation. Furthermore, niacin may promote NRF2 translocation which would help alleviate oxidative stress through increased transcription of antioxidant related genes. Investigation of liver function and health during the transition period when cattle are supplemented with niacin may provide valuable insights into improving transition dairy cow health and liver function.

The effects of free fatty acids on oocytes are also of interest because increased circulating NEFA is associated with poorer dairy cow reproductive efficiency (Ospina et al., 2010a). In vitro experiments have demonstrated that increasing free fatty acids alters oocyte quality and metabolism. Authors noted that follicular fluid (FF) has 40% lower free fatty acid concentrations than blood plasma and that increasing stearic and palmitic acid levels in FF reduced cleavage rates and blastocyst yields. Fertilization rate was also reduced in the presence of stearic and palmitic acid (Leroy et al., 2005). In support of these results, Van Hoeck et al. (2011) found that increasing free fatty acid concentrations during oocyte maturation resulted in blastocysts with lower cell number (less cleavage reactions) and increased the fraction of apoptotic cells. They also observed altered metabolism, such that oocytes exposed to elevated free fatty acid concentrations had reduced oxygen, glucose, and pyruvate consumption while also exhibiting greater lactate consumption. Microarray mRNA analysis supports the notion that increased free fatty acid concentrations modulate metabolism, especially lipid metabolism of oocytes (Van Hoeck et al., 2015). In addition to poor oocyte maturation and blastocyst formation, follicle formation has been affected as diameter of follicles was reduced with high stearic acid treatments

(Valckx et al., 2014). Clearly, elevated free fatty acid concentrations during oocyte maturation reduce oocyte quality which may provide a link between elevated free fatty acids and reduced fertility in lactating dairy cattle. Supplementing niacin may attenuate the problems resulting from excess free fatty acids. The effect that niacin may have on bovine fertility and reproduction has not been investigated in dairy cattle.

As mentioned previously, free fatty acids can reach up to 1.0 mmol/L or more (Yuan et al., 2012) but levels greater than 0.7 mmol/L has been associated with greater risk of a displaced abomasum, increased time to pregnancy, and reduced milk yield (Ospina et al., 2010b; McArt et al., 2013). *In vivo* studies attempting to model the transition period show little effect of free fatty acids on the immune system or cow's response to a disease challenge (Moyes et al., 2009). The limitation of these experiments is that they do not mimic the transition cow physiology. Tissue sensitivity to catecholamines, insulin, other hormones differ between early lactation and mid to late-lactation cows which may be why there are differences between the degree of inflammation and immune response in experiments attempting to model the transition period (Ingvartsen and Andersen, 2000). Supplementing rumen-protected niacin to transition cows has reduced peak circulating free fatty acids by ~50% (Morey et al., 2011; Yuan et al., 2012) but the literature lacks data to elucidate whether this would improve health outcomes of dairy cows. One field study demonstrated little effect of niacin on production or health, but the study was not properly replicated so the hypothesis deserves revisiting (Havlin et al., 2017); additionally, farm-to-farm variation in response to different strategies may exist as well. A large-scale, long term study supplementing rumen-protected niacin to dairy cows, instead of pens of cows, will provide an opportunity to rigorously investigate whether niacin supplementation would improve health and reproductive outcomes of dairy cows.

Characterizing the effects of niacin on systemic inflammation as well as collecting health and reproductive outcomes from dairy cattle supplemented with niacin will fill a gap in the current body of literature. Such data would demonstrate whether rumen-protected niacin supplementation is a viable strategy for dairy farmers and nutritionists to employ to improve dairy cow health and welfare.

THE ENDOCANNABINOID SYSTEM AND β-CARYOPHYLLENE

The endocannabinoid system (ECS) is a regulator of metabolism and inflammation in mammals (Myers et al., 2021). The system is composed of multiple receptors, multiple enzymes, and various endogenous and exogenous compounds. The two main receptors, cannabinoid receptor 1 and 2 (CB1; CB2) are present in different parts of the body. The CB1 receptor is present mainly in neural tissue (Tsou et al., 1998; Kuhla et al., 2020) and has lower levels of expression in adipose tissue (Roche et al., 2006; Daddam et al., 2021), liver tissue (Teixeira-Clerc et al., 2006), and other peripheral tissues (Liu et al., 2005; Juan-Picó et al., 2006; Walker et al., 2022). The CB2 receptor is highly expressed in immune cells (Montecucco et al., 2008; Turcotte et al., 2016). It is also present in adipose tissue, liver tissue (Deveaux et al., 2009), the brain (Gong et al., 2006), and the colon (Wright et al., 2005). Within these tissues it is likely that CB2 expression may stem from the presence of local resident immune cells. Both receptors are G-protein coupled receptors that inhibit adenylyl cyclase (Klein et al., 2003). The CB1 receptor is the main cause of psychotropic effects of cannabinoids, and it also plays a role in regulating metabolism and feed intake (Myers et al., 2021; Kuhla and van Ackern, 2022), but the following discussion will focus on the anti-inflammatory and immune modulating effects of the CB2 receptor.

Activating the CB2 receptor has been shown to have effects on immune cell phenotypes and activity. Specifically, it biases the T-cell response toward humoral immunity and may affect immune cell migration (Klein et al., 2003). The CB2 receptor is critical for the development of memory CD4+ and CD8+ T-cells along with NK cells in the large intestine which are cells with important regulatory and homeostatic roles (Ziring et al., 2006). These data indicates that CB2 mediated signaling may have important immune regulating implications worth investigating in dairy cattle.

In various cell and disease models, activating CB2 with either synthetic, exogenous, or endogenous ligands seems to promote anti-inflammatory results by suppressing cytokine release, immune cell migration, or cell functions. In an ischemia/reperfusion injury model, mice receiving a CB2 agonist had reduced infarct size, reduced IL-1β, and reduced myeloperoxidase compared to controls. A CB2 antagonist removed these anti-inflammatory effects indicating that the effects were mediated through CB2 signaling. Immune cell infiltration was similar across groups indicating that immune cell function may have been altered more than cell migration (Di Filippo et al., 2004). It may be that activation of CB2 promotes more regulatory immune cell phenotypes that promote healing and resolution to inflammation. This may be a promising approach to aid in resolving chronic inflammation that dairy cows experience either during the transition period, heat stress, or disease.

Research has demonstrated that activating the CB2 receptor may affect cell migration but results are mixed (Miller and Stella, 2008; Figure 2.2). In mice treated with *Mycobacterium bovis* BCG, supplementation of a CB2 agonist (β-caryophyllene; BCP) reduced leukocyte invasion; specifically, neutrophils were dramatically reduced (Andrade-Silva et al., 2016). The BCP supplementation also reduced integrin expression on immune cells indicating that it may

affect migration by downregulating cell adhesion molecules. Rajesh et al. (2007) demonstrated similar effects on migration and adhesion in human endothelial cells and monocytes. Adhesion molecules ICAM-1 and VCAM-1 were dramatically reduced in endothelial cells by CB2 provision. They also demonstrated a reduced expression of monocyte chemoattractant protein-1 and reduced monocyte adhesion to the endothelial cells. These data further demonstrate the multi-faceted effects that CB2 activation may have on cell migration and cytokine signaling. They also demonstrated that the mechanism of this anti-inflammatory effect was by inhibiting RhoA activation and NF-kB translocation (Rajesh et al., 2007). Montecucco et al. (2008) demonstrated the activation of CB2 desensitized human monocytes to chemokine ligand 2 and 3 (CCL2; CCL3; Figure 2.2) giving further evidence that CB2 ligands affect cell migration. They also demonstrated that untreated cells migrated toward increasing concentration of CB2 ligands, meaning CB2 ligands may have chemoattractant properties themself. Montecucco et al. (2008) observed that the mechanism of this reduced migration was due to increased phosphorylation of ERK1/2 and PI3K which in turn reduce the expression of the chemokine receptors 2 and 3 (CCR2; CCR3). Reducing cell migration may be beneficial by reducing unnecessary inflammation but it may also leave a cow more vulnerable to infection or pathogen pressure. The role CB2 activation plays in animal health and resilience requires further investigation.

Additionally, these anti-inflammatory effects and their mechanism may be dependent on the physiological context or the cell type (Gertsch, 2008). For example, ERK1/2 is increased by CB2 activation under non-stressed conditions (Montecucco et al., 2008) but in the presence of TNF-α CB2 ligands reduced the phosphorylation of ERK1/2 in vascular endothelial cells and smooth muscle cells (Rajesh et al., 2007; Rajesh et al., 2008). Gertsch et al. (2008) demonstrated this as well; BCP alone increased phosphorylation of ERK1/2 but in the presence of LPS, BCP

reduced ERK1/2 by half. Additionally, CB2 activation increased IL-6 in human retinol pigment epithelial cells rather than reducing it (Hytti et al., 2017). This experiment also doubled the CB2 agonist concentration when compared to Rajesh et al. (2008) which may have been why it appeared to have a proinflammatory response in this case.

Further complicating the role of CB2 activation in inflammation is that activation of CB2 may contribute to adipose inflammation and liver steatosis in obesity models. Activation of CB2 increased mRNA expression of inflammatory genes coding for TNF-α and CCL2. CB2 activation also increased insulin resistance in the high fat diet induced obesity model (Deveaux et al., 2009). Contrary to these results, CB2 activation reduced fibrosis in a non-obesity challenged models (carbon tetrachloride) indicating the CB2 activation may be hepatoprotective in specific contexts but detrimental in others (Julien et al., 2005). These contradictory experiments, both conducted in mice, indicate that the physiological state of the animal may play a role as to whether CB2 activation reduces or increases inflammation. If CB2 activation contributes to insulin resistance, it may increase milk yield in transition cows by diverting more glucose to the mammary gland, but it may also exacerbate negative energy balance and body weight loss experienced during the transition period. Whether CB2 ligands affect dairy cow performance or health has not been investigated and deserves close scrutiny given its regulatory effects on inflammation and metabolism.

Activation of CB2 may also benefit gut health and reduce gut derived inflammation, an area that is under-researched in dairy cattle nutrition. CB2 is expressed in resident sub-epithelial macrophages and slightly expressed on epithelial cells of the color (Wright et al., 2005). Bento et al. (2011) demonstrated that CB2 activation with BCP dramatically reduced neutrophil and monocyte infiltration, dramatically reduced proinflammatory cytokine secretion, and reduced

myeloperoxidase in the colon of dextran sulfate sodium treated mice. Their results suggest that BCP may reduce cytokine secretion and immune cell migration in severe colonic inflammation. Interestingly, they found it does this by activating PPARγ and reducing phosphorylation of ERK1/2. Increased concentrations of endogenous ligands for CB2 have also been observed during colonic inflammation in mice and humans. Increasing the endogenous CB2 ligand, anandamide, ameliorated colonic inflammation, further demonstrating the potential of the CB2 receptor and the ECS in gut-derived inflammation (D'Argenio et al., 2006). In addition to reducing inflammation, CB2 activation reduced the inflammation associated increase in gut transit time in a dose dependent manner (Mathison et al., 2004).

Understanding the role that cannabinoids and the ECS play in dairy cattle is just beginning. Modulation of the endocannabinoid system may be a promising target for improving dairy cow health and metabolism (Myers et al., 2021; Zachut et al., 2022). Endocannabinoid concentrations increase in the adipose tissue of postpartum dairy cattle. Cows experiencing greater bodyweight loss had greater levels of anandamide and 2-arachadonylglycerol in adipose tissue (Zachut et al., 2018). Further demonstrating the relationship between lipolysis and ECS, cows with increased plasma free fatty acid concentrations postpartum had elevated plasma anandamide concentrations and a reduction in the enzyme that degrades anandamide, fatty acid amide hydrolase (Kuhla et al., 2020). Additionally, evidence indicates that anandamide may increase feed intake in dairy cattle by 2 kg within 10 h of intracerebroventricular administration in dairy cattle (Kuhla and van Ackern, 2022) but milk production was decreased in this experiment. Intraperitoneal administration also increased feed intake by 30-40% within 6 h of administration of anandamide (van Ackern et al., 2021a) but there was no effect on milk yield. van Ackern et al. (2021b) observed a similar 30-50% increase of feed intake in the short term;

they also demonstrated that the cannabinoids reduced the stress-associated reduction in feed intake by half. Furthermore, once daily injections of anandamide over 5 d did not affect feed intake. The short-term nature of the responses observed thus far is likely due to the short half-life of anandamide. Kuhla and van Ackern (2022) acknowledged that longer term experiments continuously infusing cannabinoids like anandamide into dairy cows are needed to better understand its effects on metabolism and feed intake. Investigation into the role that the ECS may play in dairy cow inflammation has received less attention and may present an opportunity for improving dairy cow health and welfare.

An additional strategy for modulating the ECS and inflammation is through feeding omega-3 fatty acids. Feeding omega-3 (flaxseed meal) throughout the transition period reduced circulating anandamide by half and also reduced feed intake by 10%, further demonstrating the relationship between anandamide and feed intake (Kra et al., 2022b). Additionally, concentrations of adipose and liver anandamide did not differ by treatment but 2-arachadonylglycerol increased in the liver when supplemented with omega-3. Understanding how omega-3 feeding and the ECS interact may yield fruitful results in understanding how omega-3 fatty acids modulate inflammation and metabolism of high producing dairy cows through the ECS system.

CONCLUSIONS

Nutrition clearly plays a role in the inflammation and immune competence that a dairy cow will experience throughout her lactation. Using nutritional approaches to reduce unnecessary inflammation and improve immunity may have tremendous effects on the dairy industry by improving dairy cow health, welfare, longevity, and efficiency.

Niacin and its effects on lipid mobilization, metabolism, heat dissipation, and milk production has been investigated but the vast majority of the research used rumen available niacin. As rumen protection technology advanced, rumen-protected niacin has been investigated, but we have not been addressing the correct questions. Rumen-protected niacin research has demonstrated that it has strong anti-lipolytic effects during the transition period, but the experiments have been small and short term which means they are not able to determine any effects on health, fertility, or production. Given the effects that niacin has on lipolysis and the negative relationship between free fatty acids and dairy cow health and fertility, it is reasonable to hypothesize that niacin may have long term benefits for dairy cow health, fertility, and longevity. This leaves a clear gap in the literature on the possible whole-lactation effects of niacin on disease incidence, fertility, and production in dairy cows. Additionally, understanding whether niacin may be anti-inflammatory in dairy cattle should be further explored. Previous investigations demonstrated that rumen-protected niacin reduces SCC in milk but there is no data as to whether rumen-protected niacin may improve the resilience or recovery to mastitis or other disease. We will investigate whether rumen-protected niacin reduces inflammation or improves a dairy cow's response to an acute mastitis challenge. We will also demonstrate whether niacin alters circulating immune cells in dairy cattle experiencing acute mastitis. Additionally, we will investigate whether rumen-protected niacin has benefits for dairy cattle in a commercial dairy herd in Michigan.

The ECS is a promising target for modulation of metabolism and inflammation in dairy cattle. Research into the ECS in dairy cattle is just beginning but effects on metabolism and feed intake have been demonstrated but effects on inflammation and health have not been demonstrated. β-caryophyllene (BCP) is one potential compound to use in feeding dairy cattle

that may modulate inflammation, immune phenotype, and immune cell function in dairy cattle. BCP has demonstrated that it reduces pro-inflammatory cytokine secretion, cell migration, and system inflammation in various models of disease. Since the CB2 receptor is present in various tissues of cattle and since BCP-activation of CB2 has anti-inflammatory effects, it may be beneficial for cattle experiencing stress or inflammation. Preliminary investigation demonstrating whether BCP is anti-inflammatory in bovine cells models may demonstrate its potential as a supplement for dairy cattle. Additionally, *in vivo* supplementation of BCP will demonstrate whether it alters circulating immune cells of dairy cattle experiencing inflammation.

The overall objective of this dissertation is to investigate nutritional strategies that may improve health and welfare of dairy cows. Specifically, we investigated the immune modulatory effects that rumen-protected niacin may have on dairy cows experiencing an acute intramammary mastitis challenge. We also investigated whether rumen-protected niacin reduced disease incidence and improved fertility of dairy cows on a commercial dairy farm in MI. We conducted preliminary investigations into targeting the ECS and how that may affect dairy cow blood-derived macrophages or *in vivo* bovine immune cells. Lastly, we reviewed whether high starch diets influence the inflammatory tone of dairy cattle. To further investigate how dietary starch may affect inflammation we conducted an experiment feeding different dietary starch concentrations and corn silage hybrids to cattle. The topics investigated herein were broad, but all contributed to our understanding of how dairy cow health and inflammation may be influenced through nutrition.

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APPENDIX

Table 2.1. Summary of experiments investigating the effects of niacin supplementation to lactating dairy cattle

Study	Stage of lactation[1]	Niacin dose	Rumen protected?	Highlights
Kung et al., 1980	Early or Mid-lactation	6 g	No	No differences due to niacin
Riddell et al., 1981	Early or Mid-lactation	3 or 6 g/d	No	↑Milk yield in early lactation ↑Milk protein % & yield in both early and mid-lactation
Dufva et al., 1983	Transition	3, 6, or 12 g/d	No	↓free fatty acids and BHB ↑Milk protein %
Jaster et al., 1983a	Transition	12 g/d	No	↔milk and component yield ↑ plasma nicotinic acid
Jaster et al., 1983b	Early lactation	6 g/d	No	↑ milk yield in high producing heifers ↔ milk yield in mature cows
Horner et al., 1986	Early lactation	6 g/d	No	↑Milk protein % & yield
Muller et al., 1986	Mid-lactation	6 g/d	No	↑Milk, fat, & protein yield in high producing cows
Horner et al., 1988	Early lactation	3, 6, 12 g/d	No	↔milk and component yield
Skaar et al., 1989	Transition	12 g/d	No	↔milk and component yield
Jaster and Ward, 1990	Transition	6 g/d	No	↑ milk yield
Erickson et al., 1990	Mid-lactation	12 g/d	No	↔milk and component yield
Erickson et al., 1992	Early lactation	12 g/d	No	↑ milk protein content and yield
Lanham et al., 1992	Late lactation	6 g/d	No	↔milk and component yield
Zimmreman et al., 1992	Early lactation	6 g/d	No	↔milk and component yield

Table 2.1. (cont'd)

Campbell et al., 1994	Late lactation	12 g/d	No	↔milk yield, component yield, total tract digestibility 17% of niacin passed through the rumen 38% increase in plasma nicotinic acid
Bernard et al., 1995	Transition	6 g/d	No	↔milk and component yield
Ottou et al., 1995	Mid-lactation	6 g/d	No (abomasal infusion)	5-fold increase in plasma niacin ↔milk and component yield ↑ 14-carbon milk fat ↓ 4-carbon milk fat
Christensen et al., 1996	Early lactation	12 g/d	No	↔milk and component yield
Wagner er al., 1997	Not reported	6 g/d	No (abomasal infusion)	↓ short chain (<18) carbon milk fat
Madison-Anderson et al., 1997	Early lactation	12 g/d	No	↔milk and component yield
Di Costanzo et al., 1997	Mid-lactation	12, 24, 36 g/d	No	↔milk and component yield ↓ reduced skin temperature
Minor et al., 1998	Transition	12 g/d	No	↔milk and component yield
Drackley et al., 1998	Early lactation	12 g/d	No	↑ Milk and component yield
Christensen et al., 1998	Not reported	12 g/d	No	↔milk and component yield
Ghorbani et al., 2008	Early lactation	6, 12 g/d	No	↔milk and component yield
Niehoff et al., 2009	Mid-lactation	6 g/d	No	↔milk and component yield
Zimbelman et al., 2010	Mid-lactation	7.8 g/d	Yes	↓ rectal and vaginal temperature ↑ evaporative heat loss ↔milk and component yield

Table 2.1. (cont'd)

Morey et al., 2011	Transition	9.6 g/d	Yes	↔milk and component yield ↓ free fatty acids and BHB ↓ SCC in multiparous cows
Aschemann et al., 2012	Early lactation	6 g/d	No	↑ OM and NDF digestibility ↔milk and component yield
Yuan et al., 2012	Transition	8 g/d	Yes	↓ Fat%, SCS, free fatty acids, BHB ↔milk yield
Zimbelman et al., 2013	Mid-lactation	8 g/d	Yes	↓ vaginal temperature ↑ milk yield during most severe heat stress
Lohölter et al., 2013	Mid-lactation	24 g/d	No	↑ milk and component yield ↔skin or rectal temperature
Rungruang et al., 2014	Mid-lactation	2.6, 5.2, 7.8 g/d	Yes	↔milk and component yield ↔skin temperature and heat dissapation
Aragona et al., 2016	Transition	48 g/d	No	↑ IgG and protein concentration of colostrum ↔ IgG yield and colostrum yield
Pires et al., 2016	Late lactation	50 g/d	No (abomasal infusion)	↓ milk and component yields ↓ Free fatty acids, BHB ↑ Insulin
Pineda et al., 2016	Late lactation	10.5 g/d	Yes	↓ vaginal temperature ↑ milk yield during heat stress
Havlin et al., 2017	Transition	0, 3.5, 7, 14 g/d	Yes	↓ ketosis (3.5 g/d) ↑ DMI (3.5 g/d) ↔milk and component yield
Bühler et al., 2018	Transition	24 g/d	No	↔ similar immune cell function and number
Zeitz et al., 2018	Transition	36 g/d	Yes	↓ milk fat %, yield, and BHB
Aragona et al., 2020	Prepartum	16, 32, 48 g/d	No	↓ DMI ↑ Colostrum IgG, Fat, Protein, Ash yield ↑ FE of calves from niacin fed dams

Table 2.1. (cont'd)

Gaowa et al., 2021	Transition	11 g/d	Yes	↑ DMI, milk yield, and component yield

[1]Transition = experiment began prepartum and continued into lactation; Early lactation = experiment began <90 days in milk, Mid-lactation = experiment began >90 days in milk and < 180 DIM; Late lactation = experiment began >180 days in milk

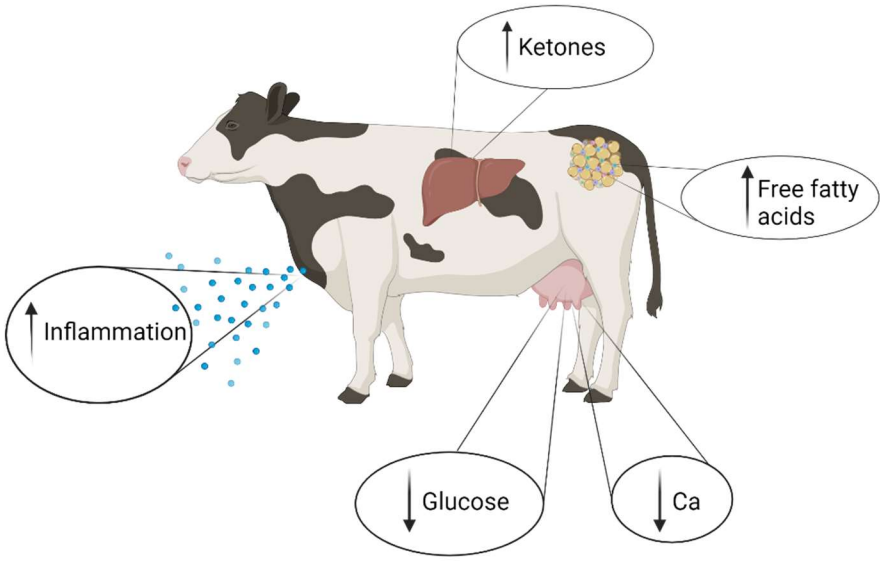

Figure 2.1. The transition dairy cow experiences tremendous metabolic and physiological changes including increased concentrations of inflammatory markers like Hp, increased free fatty acids, increased ketones, reduced blood glucose, and transient reductions in blood Ca.

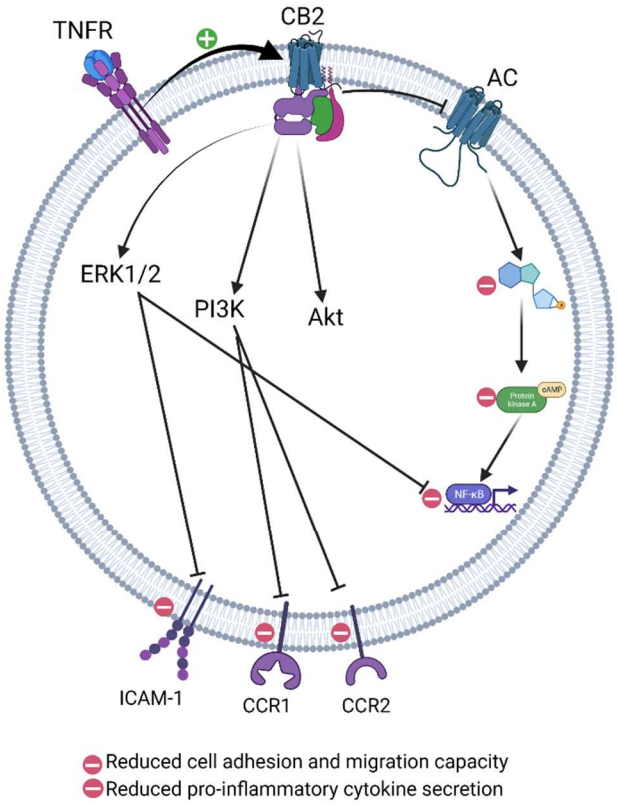

Figure 2.2. The possible roles of CB2 receptor and its activation on immune cells. Binding of TNF-α to TNFR increases the expression of CB2 (Rajesh et al., 2008). Furthermore, CB2 agonists reduce cAMP (Bayewitch et al., 1995), increase phosphorylation of ERK1/2 , PI3K , and Akt (Montecucco et al., 2008; Rajesh et al., 2008) but reduce LPS associated increases in phosphorylation of ERK1/2 (Gertsch et al., 2008). The downstream effects being reduced expression of adhesion molecules and chemokine receptors (Batkai et al., 2007; Bátkai et al., 2007; Rajesh et al., 2007a; Rajesh et al., 2007b; Montecucco et al., 2008). These cell mechanisms may result in anti-inflammatory effects. Specifically, the reduced adhesion and migration capacity of immune cells and reduced secretion of pro-inflammatory cytokines.

CHAPTER 3: EFFECTS OF RUMEN-PROTECTED NIACIN ON INFLAMMATORY RESPONSE TO REPEATED INTRAMAMMARY LIPOPOLYSACCHARIDE CHALLENGES.

ABSTRACT

Nutritional strategies that improve an animal's resilience to challenges may improve animal health and welfare. One nutrient is niacin, which has reduced inflammation in mice, humans, and swine; niacin's anti-inflammatory effects have not been investigated in lactating dairy cattle. Our objective was to determine whether rumen-protected niacin (RPN) alters lactating dairy cows' inflammatory response to a mastitis challenge. Twenty late-lactation Holstein cows (232 ± 65 days in milk; 39 ± 5.8 kg/d of milk) with no clinical mastitis within the previous 60 d were enrolled in a randomized complete block experiment which lasted 70 d. Cows received 26 g/d of RPN or no additional top-dress (CON) for the first 42 d of the experiment. On d 28 and 56, cows were challenged in their rear-right quarter (RR) with 100 µg of lipopolysaccharide (LPS) suspended in 5 ml of PBS. Milk yield, milk conductivity, and feed intake were measured daily. Milk composition was measured on d 14, 23, 24, 30, 37, 45, and 52. Blood samples were collected at 0, 8, 12, 24, 48, 72, 96, and 120 h after each LPS challenge. Quarter level (RR) milk samples were collected at 0, 8, 16, 24, 48, 72, 96, 120, 144, and 168 h after each LPS challenge. Body temperature was measured continuously during each challenge with vaginal thermometers. Milk production and milk conductivity were analyzed with linear mixed repeated measure models which included fixed effects of treatment, day, and their interaction, as well as the main effect of parity and its interaction with time. Cohort, block nested in cohort, and cow were random effects. Plasma biomarkers, body temperature, and SCS also included the effects of challenge (1^{st} vs. 2^{nd}) and its interactions with time and treatment. Model

hierarchy was always maintained. Prior to LPS challenge, RPN did not affect feed intake or milk production, but it reduced somatic cell score (SCS; 1.24 ± 0.41 vs 0.05 ± 0.45). After challenges, RPN did not affect feed intake, milk production, milk composition, SCS, body temperature, plasma glucose, or plasma insulin concentrations. During the 1st challenge, primiparous cows had reduced plasma glucose concentration at 12 h post-challenge compared to multiparous cows, but after the 2nd challenge, plasma glucose was not reduced in either parity group. RPN reduced peak Hp by 30% and the 2nd LPS challenge had reduced plasma Hp compared to the 1st LPS challenge. Peak plasma lipopolysaccharide binding protein concentration during the 1st challenge was reduced by 23% by supplementing RPN. The 2nd LPS challenge induced a greater peak SCS than the first LPS challenge. Our results suggest that repeated LPS challenges may promote a systemic tolerance but heightened local response to LPS-induced mastitis. Feeding RPN reduced SCS prior to challenge and reduced plasma acute phase proteins after challenge suggesting that RPN may reduce systemic inflammation without altering the local inflammatory responses.

Key words: mastitis, inflammation, immunity, HCA2

INTRODUCTION

Research is increasingly investigating management factors or nutritional strategies that modulate inflammation (Michelotti et al., 2021; Kerwin et al., 2022; Kerwin et al., 2023). Improving our understanding of strategies that reduce inflammation may have positive effects on dairy industry sustainability and animal welfare. Delivering nutrients with anti-inflammatory effects may reduce the need for pharmaceuticals such as non-steroidal anti-inflammatory drugs or antibiotics, which would be beneficial for dairy industry sustainability and social license.

Feeding rumen-protected niacin (RPN) reduces circulating fatty acids and β-hydroxybutyrate (BHB) during the postpartum period in dairy cattle (Morey et al., 2011; Yuan et al., 2012; Zeitz et al., 2018). In addition to potently inhibiting lipolysis, RPN may reduce inflammation and immune cell migration (Shi et al., 2017). One piece of evidence that RPN may improve health in lactating dairy cattle is that it reduces somatic cell score (SCS), especially in multiparous cows, by up to 40% (Morey et al., 2011; Yuan et al., 2012). Niacin's receptor, HCA2, is present on immune cells (Carretta et al., 2020) and in the mammary gland of cattle (Swartz et al., 2021), which suggests that niacin may modulate immune cell activity or improve mammry gland function (or both) to reduce local inflammation and immune function.

Both niacin and BHB activate the same HCA2 receptor (Graff et al., 2016) but niacin is a much more potent agonist of HCA2 than BHB (Taggart et al., 2005). Considering the association of plasma BHB with mastitis incidence later in lactaction (Raboisson et al., 2014) it may be that providing RPN would leave cows more susceptible to mastitis. Alternatively, the anti-inflammatory effects of activating HCA2 with niacin may avoid off-target effects of BHB that may alter immune responses and render cows more susceptible to mastitis. Whether feeding RPN improves a dairy cow's response to mastitis or reduces associated systemic inflammation deserves investigation.

In other species, niacin has demonstrated systemic anti-inflammatory effects that could be beneficial for cattle. In mice, supplementating niacin reduced monocyte chemoattractant protein 1 and the CD11c mRNA expression in adipose tissue (Wanders et al., 2013). Lipszyc et al. (2013) demonstrated that niacin supplementation, both *in vivo* and *in vitro*, reduced pro-inlammatory cytokines IL-1β, TNF-α, and IL-6. Additionally, both Wakade et al. (2018) and

Montserrat-de la Paz et al. (2017) observed that niacin altered monoctye phenotypes toward anti-inflmmatory or pro-resolving macrophages. They observed that niacin increased the proportion of IL-10 producing cells as well as reduced $CD14^{+}CD16^{-}$ monoctyes and increased $CD14^{+}CD16^{++}$ monocytes; these results suggest that niacin has immunomodulatory effects by altering monocyte phenotype and function. Considering that monoctye phenotype classifications seem to be conserved across species (Talker et al., 2022), investigating the expression of these markers (CD14, CD16) in dairy cattle may provide evidence as to whether niacin has similar immunomodulatory capabilites in the dairy cow.

Our objectives were to determine if RPN reduces SCS prior to and after repeated mastitis challenges in late lactation cows and determine if it alters the dairy cow's systemic response to repeated acute mastitis challenges. Additionally, we investigated whether supplementing RPN altered circulating immune cell phenotypes prior to or immediately after LPS challenges. We hypothesized that RPN would reduce SCS prior to mastitis, reduce SCS during mastitis challenge and improve resolution of mastitis. We also hypothesized that RPN would reduce markers of systemic inflammation and increase the pro-resolving $CD14^{+}CD16^{+}$ monocytes. Furthermore, our secondary objective was to determine the effects of repeated mastitis challenges on dairy cows' inflammatory and immune response to mastitis. We hypothesized that the cows would develop tolerance during the 2^{nd} mastitis challenge which would be expressed by reduced systemic positive acute phase proteins, reduced milk SCS, and reduced peak body temperature during the 2^{nd} challenge.

MATERIALS AND METHODS

Cows, treatments, and experimental design

This experiment was approved by the Michigan State University Institutional Animal Care and Use Committee. Twenty-four mid or late-lactation Holstein dairy cows (239 ± 60 DIM; 8 primiparous, 16 multiparous) averaging 38.5 ± 4.87 kg/d of milk yield, 94.8 ± 5.43 mOhm of milk conductivity, and no current clinical mastitis were enrolled in a randomized complete block experiment. Cows were housed in a tie-stall barn at the Michigan State Dairy Research and Teaching Center. Cows were milked three times daily (0500, 1300, and 2100 h) in a milking parlor and fed once per day (1000 h) for a 10% refusal rate. Cows had *ad libitum* access to water. The cows were enrolled in two cohorts of 12 cows each two weeks apart. Cows were blocked by parity and BLV status. Treatments were randomly assigned within each block using a random number generator (Microsoft Excel). Six cows receiving CON were positive for bovine leukemia virus (BLV) and 6 were BLV-negative while 7 cows receiving RPN were BLV-positive and 5 were BLV-negative. In the experiment, 12 cows were assigned to receive the control (CON) and 12 cows were assigned to receive 26 g/d of rumen protected niacin with the goal of providing 6 g of bioavailable niacin to the cow (RPN; Vetagro Inc). Rumen protection of the niacin was 93% and digestibility was 50% (personal communication, E. Grilli) which resulted in 46% bioavailability. These results were used to determine the needed RPN dose of 26 g/d. The treatment was top-dressed onto the ration after morning feeding from d 0 until d 42; cows were monitored until d 70. Prior to the first mastitis challenge, quarter-level milk samples from the rear right (RR) quarter were taken to screen for an active immune response in that quarter. The 5 cows with the lowest quarter-level SCC within each treatment group in each cohort were selected for mastitis challenge. Of the 10 CON cows that remained on the study, 5 were BLV-positive

and 5 were BLV-negative. Of the 10 RPN cows that remained, 7 were BLV-positive and 3 were BLV-negative.

Lipopolysaccharide challenges

Lipopolysaccharide (LPS; *E. coli* O11:B4, Millipore Sigma) challenges were conducted at the final milking (2100 h) on d 27 and d 55 of the experiment. The challenges were all conducted in the RR quarter of each cow. To conduct the challenge, cows were milked out according to farm protocols. After milking was completed the teat end was scrubbed with an alcohol swab to remove bacteria. Then, 5 mL of PBS containing 100 µg of LPS (20 µg LPS/mL PBS) were infused into the RR quarter. The LPS were of the same batch throughout the experiment. The solution was massaged upward into the mammary gland. The post-milking procedure was then completed according to farm protocols.

Data collection and sample analysis

Total mixed ration (TMR) samples were collected every other week throughout the experiment and composited by month prior to analysis. The TMR samples were analyzed by Cumberland Valley Analytical Services (CVAS; Waynesboro, PA) for DM (AOAC International, 2000), CP (Leco FP-528 N Combustion Analyzer, Leco Corp., St. Joseph, MO), starch (Hall, 2009), ethanol-soluble carbohydrates (Dubois et al., 1956), NDF (Van Soest et al., 1991), lignin (Goering and Van Soest, 1970), crude fat (2003.05; AOAC International, 2006), ash (AOAC International, 2000), and minerals (2003.05; AOAC International, 2006).

Bodyweight (BW) and body condition score (BCS) were measured once per week. The BCS was measured by 3 trained investigators on a 1-5 scale (Wildman et al., 1982). Milk yield and milk conductivity were measured at each milking in the milking parlor with an electronic monitoring system (AFIMILK). Milk components were collected at all three milkings on d 14,

23, 24, 30, 37, 38, 45, and 52. Samples were analyzed for fat, protein, and lactose by Central Star Cooperative using Fourier-transform infrared spectroscopy. Somatic cell count (SCC) was also measured by Central Star and was determined using flow cytometry (Bentley Combi-System; Bentley Instruments). Quarter-level milk samples were collected from the RR quarter on d 24 and 0, 8, 16, and 24 h after each challenge. Samples during the first 24 h after challenge were diluted 1:10 in PBS for flow cytometric analysis by Central Star. Quarter-level samples were then taken once daily for the next 6 d following each LPS challenge; these samples were not diluted in PBS.

Body temperature was collected every 10 min from 24 h prior to each LPS challenge until 72 h after each LPS challenge. The body temperature was collected using an iButton thermometer (iButton DS1921H; Embedded Data Systems) which was placed on a blank CIDR (Zoetis). Prior to insertion of the CIDR containing the iButton, the vulva of the cow was cleaned with water and disinfected with betadine solution. Then, a lubricant was applied to the CIDR and vulva and the CIDR containing the iButton was inserted into the vagina of each cow.

Blood samples were collected on d 28-34 and d 56-62 at 2130. Blood samples were also collected 8 h and 16 h after each LPS challenge. Blood was collected by coccygeal venipuncture with tubes containing K_2EDTA. Blood samples were immediately placed on ice and transported to the lab where they were centrifuged for 15 min at 1,500 × *g* to collect the plasma. Two aliquots of plasma were kept at -20°C for later analysis. Plasma was analyzed for glucose by enzymatic methods (kit #997-03001; Fujifilm), insulin using a bovine specific ELISA (no. 10–1201–01; Mercodia AB) haptoglobin using a bovine specific ELISA (Hp; kit # HAPT-11; Life Diagnostics), LPS binding protein by ELISA (LBP; cat # CKH113; Cell Sciences), and β-hydroxybutyrate (BHB; kit no. H7587-58; Pointe Scientific Inc.). The inter-assay coefficients of

variation (CV) were 5.23, 5.71, 5.81, 5.93, and 5.71 for glucose, insulin, Hp, LBP and BHB, respectively. Intra-assay CV were 4.36, 5.17, 6.50, 5.02, and 6.39 for glucose, insulin, Hp, LBP, and BHB, respectively.

One blood sample was collected on d 14 for analysis of plasma nicotinic acid and nicotinamide concentrations. Samples were analyzed by a LC/MS/MS method adapted from Lahély et al. (1999) and Schwieler et al. (2020). Samples were analyzed on a Waters Xevo TQ-S UPLC/MS/MS (Waters Corporation) at the MSU Mass Spectrometry and Metabolomics Core.

Additional blood samples were collected on d 22, on d 28 (8 h after LPS challenge), d 50, and d 56 (8 h after LPS challenge) for flow cytometric analysis of circulating immune cells and white blood cell differential counts (QScout BLD; Advanced Animal Diagnostics). Flow cytometric analysis was only conducted on cohort 1 (n = 5 per treatment) while white blood cell differential counts were conducted on both cohorts 1 and 2 (n = 10 per treatment). Immune cell isolation was based on Farschtschi et al. (2021) with some adjustments. Briefly, blood was collected by coccygeal venipuncture into tubes containing K_2EDTA and placed immediately on ice for transport back to the lab. Samples were then centrifuged for 15 min at 1500 × *g* at 4°C to remove the plasma. Then, 5 mL of cold $Ca^{++}Mg^{++}$ free DPBS were added, the contents were mixed, and the samples centrifuged for 10 min at 1500 × *g* at 4°C. The supernatant was removed, then 8 mL of ACK lysis buffer were added, the sample was inverted twice to mix, and then the sample was incubated at 4°C in the dark for 8 min to lyse red blood cells. Next, the sample was centrifuged for 10 min at 400 × *g* at 4°C. The supernatant was removed and the cell pellet was resuspended with 8 mL of cold $Ca^{++}Mg^{++}$ free DPBS and centrifuged for 5 min at 400 × *g* at 4°C. The supernatant was then discarded. This washing step was repeated once more. After final wash, the cell pellet was resuspended in 1 mL $Ca^{++}Mg^{++}$ free DPBS.

100 µL of the cell suspension was transferred into a 96-well plate along with 100 µL of $Ca^{++}Mg^{++}$ free DPBS. The plate was centrifuged for 5 min at 400 × *g* at 4°C and the supernatant was removed. Next, 100 µL of Live/Dead Blue (cat. # L34962; Invitrogen; 1:1000 dilution) was added and allowed to incubate in the dark on ice for 20 min. Then, 100 µL of FACS buffer (DPBS with 1% fetal bovine serum; Sigma Aldrich) was added to each well and the plate was centrifuged for 5 min at 400 × *g* at 4°C; the supernatant was removed. 50 µL of FACS was added and the plate was incubated in the dark on ice for 10 min. Then, 50 µL of the antibody mastermix cocktail (Supplemental Table 3.1) was added to each well and allowed to incubate on ice for 30 min in the dark. The antibodies included were CD3 (Bu-BOV2009; WSU Antibody Center) conjugated to CF405L (Biotium), CD4 (MCA1653A700; BioRad), CD8 (MCA837A647; BioRad), CD11b (BOV2024; WSU) conjugated to CF750 (Biotium), CD21 (MCA1424F; BioRad), CD14 (301836; BioLegend), and CD16 (MCA5665PE; BioRad).

The plate was centrifuged for 5 min at 400 × *g* at 4°C and the supernatant was removed. The final wash step was done by adding 200 µL of FACS buffer to resuspend the pellet; the plate was then centrifuged at 5 min at 400 × *g* at 4°C and the supernatant was removed. This wash step was repeated once more. The cells were then resuspended in FACS buffer and analyzed immediately by spectral flow cytometry using a Cytek Aurora Flow Cytometer (Cytek). The gating strategy is demonstrated in Figure 3.1.

Statistical analysis

All data were analyzed using linear mixed models (GLIMMIX; SAS 9.4, Cary NC). Dry matter intake (DMI), milk yield, milk composition, milk conductivity, and milk component yields were analyzed using repeated measures linear mixed models for pre-challenge, post-challenge 1, and post-challenge 2 data. Change in BW and BCS (ΔBW, ΔBCS) were evaluated

as repeated measures and included fixed effects of treatment, week, their interaction, and the main effect of parity. We evaluated compound symmetry, autoregressive, autoregressive heterogenous, and Toeplitz covariance structures for DMI, milk yield, milk conductivity, ΔBW, and ΔBCS; the covariance structure that yielded the lowest AIC was selected for each model. For milk composition, component yields, energy corrected milk yield (ECM), and feed efficiency (FE; ECM/DMI), the spatial power covariance structure was used due to unequal spacing of measurements. Cow, block nested within cohort, and cohort were included as random effects in these models. Normality of residuals was visually appraised. If the normality assumption was violated, the variable was log-transformed, and the model was re-fit. Observations with Studentized residual ≥ |4| were removed from the data set for each variable and the models were refit if observations were removed.

Quarter-level somatic cell score (SCS; [$\log_2$(somatic cell count/100,000) + 3]) was analyzed as a repeated measure using a compound symmetry covariance structure. The model included the fixed effects of treatment, h post-challenge, parity, and challenge (1^{st} vs. 2^{nd}). All two and threeway interactions were included in the initial model. Cow, block nested within cohort, and cohort were included as random effects in the model. Plasma markers were analyzed as repeated measures using linear mixed models. Glucose, insulin, and BHB were analyzed with a compound symmetry covariance structure while Hp and LBP were analyzed using compound symmetry, autoregressive, autoregressive heterogenous, or Toeplitz covariance structures depending on which yielded the lowest AIC. Analysis of the plasma nicotinic acid and nicotinamide included only the main effects of parity, BLV, treatment, and their interactions. Model assumptions were evaluated as described previously.

Body temperature was analyzed as an area under the curve (AUC; °C × Min). Baseline temperature was determined by averaging the body temperature for the 12 h prior to challenge for each cow. The model included treatment, challenge and the two way interaction of treatment and challenge. AUC was evaluated as a repeated measure (challenge 1 and challenge 2) using the compound symmetry covariance structure. Cow, block nested within cohort, and cohort were included as a random effects.

Flow cytometry data were analyzed as repeated measurements using the compound symmetry covariance structure. The model included effects of treatment, time, BLV, and the corresponding two way interactions. Cow and block were included as a random effects.

White blood cell differential counts (WBC) were analyzed in three phases; prior to challenge, change in baseline WBC before the 2nd LPS challenge, and change in WBC across each LPS challenge. The change in WBC prior to challenge was calculated by subtracting the WBC prior to the 1st challenge from WBC prior to the 2nd challenge. The change in WBC across the challenges were calcuated by substracting pre-challenge WBC from post-challenge WBC. The model included the fixed effects of treatment, BLV, parity, and challenge. We also tested the interactions of treatment × BLV, BLV × challenge, treatment × challenge. The random effects were block nested within cohort, cohort, and cow.

Statistical significance was declared at $P \leq 0.05$ and tendencies at $P \leq 0.10$

RESULTS

Plasma niacin status

Feeding RPN tended to increase plasma nicotinic acid ($P = 0.06$) and increased nicotinamide ($P < 0.01$) prior to the LPS challenges by 44% and 36%, respectively (Figure 3.2).

Feed intake, milk production, milk composition, and bodyweight

Prior to challenge, there was not evidence of a treatment difference for DMI but there was for parity (Table 3.1). Multiparous cows ate 3.5 ± 1.03 kg/d more feed than primiparous cows ($P < 0.01$). After each challenge, treatment did not affect feed intake ($P \geq 0.19$) but multiparous cows continued to consume 15% more DM that primiparous cows ($P \leq 0.02$). Milk yield (Figure 3.3) and energy corrected milk yield (ECM) were similar for each treatment group prior to challenge ($P \geq 0.86$). Multiparous cows tended to make more ECM (36.7 ± 2.04 vs. 40.8 ± 1.06; $P = 0.08$). There was a tendency for a treatment × day interaction for milk yield after the first challenge but there were no effects of treatment within day ($P \geq 0.15$). After the 1st challenge parity did not affect milk yield (33.0 ± 2.82 vs. 33.7 ± 1.54) or ECM (35.2 ± 0.82 vs. 35.2 ± 0.65; $P \geq 0.21$). Treatment did not affect concentrations of milk fat, milk protein, or their yields throughout the experiment ($P \geq 0.22$). Prior to challenge, protein yield was greater for multiparous cows (1.09 ± 0.078 vs. 1.29 ± 0.051; $P = 0.02$). Prior to the first challenge, FE tended to be affected by an interaction of treatment × day ($P = 0.09$). Within day, FE was greater for CON than RPN on d 14 (1.66 ± 0.054 vs. 1.51 ± 0.065), but did not differ on d 23 and 24 ($P \geq 0.18$). After the 1st challenge, FE was 14% greater for primiparous cows than multiparous cows (1.56 ± 0.043 vs. 1.40 ± 0.023; $P < 0.01$). Composite SCS was significantly reduced by RPN ($P = 0.01$; Table 3.1) prior to challenge, but after challenge there was not an effect of RPN ($P = 0.46$). After both challenges, milk conductivity was affected by parity ($P =$

0.04); multiparous cows had greater conductivity after challenge (1st Challenge: 90.3 ± 3.29 vs. 97.4 ± 2.31; 2nd Challenge: 90.5 ± 4.03 vs. 99.6 ± 2.90).

Quarter-level SCS of the RR quarter was not affected by treatment ($P = 0.71$; Figure 3.4). The quarter-level SCS was affected by challenge, being greater after the 2nd LPS challenge at 0, 8, 16, 48, and 72 h after challenge ($P \leq 0.04$). At 144 h after challenge, SCS was greater after the 1st challenge than 2nd challenge ($P = 0.03$).

The ΔBW and ΔBCS were not affected by treatment ($P \geq 0.21$; Table 3.2). Primiparous cows gained more BW throughout the experiment; Primiparous cows gained 0.74 ± 0.155 kg/d while multiparous gained 0.53 ± 0.142 kg/d.

Body temperature, plasma metabolites, and inflammatory markers

Neither treatment, BLV, nor challenge affected AUC for body temperature ($P \geq 0.41$). Treatment did not affect plasma glucose, insulin or BHB concentrations ($P \geq 0.14$; Figure 3.5). Plasma glucose was affected by a three-way interaction of challenge × parity × time ($P = 0.04$; Supplemental Figure 3.1). At both 8 and 12 h after challenge, first parity cows had lower plasma glucose after the first challenge than after the 2nd challenge ($P \leq 0.04$). Also, during the 1st challenge, primiparous cows had lower plasma glucose concentrations than multiparous cows at 12 h after challenge ($P < 0.01$). Insulin increased 8 h after LPS challenge from 0.89 ± 0.110 µg/L to 2.04 ± 0.110 µg/L ($P < 0.01$). By 12 h after the challenge, insulin was similar to 0 h ($P = 0.94$). There was a challenge × time effect on BHB ($P < 0.01$). At 0 h, mean BHB concentrations were 0.53 ± 0.0422 mmol/L during the 1st LPS challenge and 0.70 ± 0.0422 mmol/L during the 2nd ($P < 0.01$). At 8 h after the 1st LPS challenge, plasma BHB concentrations was 0.53 ± 0.0422 mmol/L, and 8 h after the 2nd LPS challenge it was 0.38 ± 0.0422 mmol/L ($P < 0.01$).

Supplementing RPN tended to reduce plasma Hp ($P = 0.08$; 92.9 ± 6.93 μg/mL vs. 73.76 ± 8.34 μg/mL). Plasma Hp was also affected by challenge × time interaction ($P < 0.01$). Plasma Hp was greater on d 1, 3 and 4 after the 1st LPS challenge than during the 2nd challenge ($P < 0.01$). There was a tendency for a challenge × parity interaction ($P = 0.09$); during the 1st challenge primiparous cows had greater Hp than multiparous cows ($P = 0.03$; 120.4 ± 13.38 μg/mL vs. 86.7 ± 7.51 μg/mL) but during the 2nd LPS challenge they were similar (P = 0.93; 62.2 ± 13.38 μg/mL vs. 63.6 ± 7.48 μg/mL). Plasma LBP was affected by a treatment × challenge × time intereaction ($P = 0.08$). During the first challenge, RPN cows tended to have reduced LBP 24 h after challenge relative to CON cows ($P = 0.08$).

CBC differential

Treatment did not affect WBC of any population prior to the 1st LPS challenge ($P \geq 0.48$; Table 3.3). BLV tended to increase total leukocytes, monocytes, and lymphocytes ($P \leq 0.06$). Treatment with RPN reduced the total leukocyte and lymphocyte populations prior to the second LPS challenge ($P \leq 0.03$). Supplementing RPN tended to result in a greater increase in immature neutrophils 8 h after challenge ($P = 0.08$). Additionally, during the 2nd challenge there was a greater increase in immature neutrophils than during the first challenge ($P \leq 0.01$; 137 ± 71.3 vs. 268 ± 67.9 cells/ μL).

Immune cell phenotype

$CD3^+$ immune cells tended to be affected by a treatment × time interaction ($P = 0.08$; Table 3.4; Figure 3.6). Prior to the 1st challenge, RPN tended to reduce the proportion of $CD3^+$ immune cells ($P = 0.06$; 7.8 ± 1.11 % of live cells vs. 5.3 ± 1.24 % of live cells). Prior to the 2nd challenge, RPN reduced proportions of $CD3^+$ cells ($P < 0.01$; 12.4 ± 1.21 % of live cells vs. 7.2 ± 1.36 % of live cells). $CD3^+CD4^+$ cells tended to be reduced for RPN ($P = 0.06$; 2.0 ± 0.31 % of

live cells vs. 1.1 ± 0.41 % of live cells). Prior to the 2nd challenge, RPN reduced $CD3^+CD8^+$ cells ($P < 0.01$; 5.4 ± 0.61 % of live cells vs. 2.8 ± 0.78 % of live cells) but at other timepoints $CD3^+CD8^+$ cells were similar ($P \geq 0.63$).

DISCUSSION

The primary objective of this experiment was to determine if RPN modulates a dairy cow's response to acute mastitis. Our experiment demonstrated that feeding RPN substantially increased the circulating concentrations of nicotinic acid and nicotinamide, which establishes the ability of RPN supplementation to alter niacin status. It also demonstrated that RPN has immunomodulation capabilities at the systemic level as inidicated by reduced positive acute phase proteins.

Research using rumen- available niacin or RPN both demonstrate very little effect of additional niacin on milk yield or milk component production (Schwab et al., 2005; Zeitz et al., 2018), consistent with our observations. Rumen microbial synthesis likely fills the vitamin requirement of dairy cows for niacin (NASEM, 2021); between 1 and 4 g/d of niacin flow out of a dairy cow's rumen each day (Schwab et al., 2006; Brisson et al., 2022). However, as cows increase milk yield or are in negative nutrient balance, they may require additional B-vitamins for optimal production (Brisson et al., 2022; Girard and Duplessis, 2022). Some data supports this suggestion, as retrospective analyses determined that supplemental niacin increased milk yield in higher-producing dairy cows (Jaster et al., 1983; Muller et al., 1986). We did not observe changes in milk fat and protein composition or yield as a result of RPN supplementation. Research with dairy cattle in early lactation demonstrates that feeding RPN results in a transient reduction in milk fat % and yield (Yuan et al., 2012; Zeitz et al., 2018), which likely stems from the reduction of lipolysis in these experiments. In vitro research also suggests that niacin may

reduce milk fat synthesis in bovine mammary cells (Wang et al., 2020). After the period of intense free fatty acid mobilization around calving, RPN does not seem to affect milk fat production. Our results also suggest that RPN has little effect on milk composition and yield of components in late lactation cows even with substantial increases in plasma niacin metabolites.

As we hypothesized, RPN reduced SCS prior to the LPS challenge. A reduction in SCS was also observed by Yuan et al. (2012) in all cows and by Morey et al. (2011) in multiparous cows. Rungruang et al. (2014), however, did not observe this reduction of SCS in response to RPN. The reduction we and others have observed in SCS as a result of RPN supplementation is likely related to immune cell migration and function. Niacin, *in vivo* and *in vitro*, reduces immune cell cytokine produciton (Lipszyc et al., 2013), reduces expression of integrins, reduces monocyte migration, and reduces neutrophil migration (Wu Ben et al., 2010; Shi et al., 2017), which would likely reduce immune cell recrutiment to the mammary gland. Since RPN supplementation has repeatedly reduced SCS in dairy cows, it may be a tool to improve milk quality in a variety of scenarios, including in both early and late lactation. These data confirm the potential of nutritional immunomodulation to improve milk quality.

Since RPN reduced SCS in lactating dairy cattle, we hypothesized that it may alter a dairy cow's local mammary immune response to mastitis challenge. Cows infused with BHB to induce hyperketonemia had reduced SCS after an LPS challenge (Zarrin et al., 2014) and considering that both BHB and niacin work through the same receptor (Gille et al., 2008), we expected a similar reduction in SCS during our LPS challenges. Within the challenge quarter, RPN did not affect SCS during the 168 h after the LPS challenges. The SCS of composite milk samples were also similar for both treatments after the LPS challenge. These data indicate that

RPN did not alter immune cell extravasation into the mammary gland during this challenge. Even though pre-challenge SCS was reduced, it does not appear that supplementing RPN affected the dairy cows local response to acute mastitis. Cell functionality in response to RPN was not investigated here but may be worthy of exploration as provision of niacin has altered immune cell function (Wu Ben et al., 2010; Lipszyc et al., 2013) which may alter the cow's ability to clear a natural mastitis infection.

An additional question during this experiment was whether cows would exhibit LPS tolerance during the 2nd LPS challenge. Previous research in dairy cattle subject to repeated challenges with *E. coli* demonstrated a muted local and systemic inflammatory response after the 2nd challenge (Suojala et al., 2008). They also observed similar SCS during both challenges but a reduction in the *E. coli* CFU during the 2nd challenge, indicating a more robust immune response (Suojala et al., 2008). The robust immune response to the 2nd mastitis challenge with *E. coli* may be a result of the adaptive immune system contributing to the response but recent findings on innate training point to potential roles for this arm of the immune system to contribute as well (Byrne et al., 2020). We observed an increase in SCS during the 2nd challenge which indicates a heightened local immune response to the LPS. These results align with a greater increase in circulating immature neutrophils after the 2nd LPS challenge compared to the first. Increases in SCS after the 2nd challenge may have to do with the LPS load in the mammary gland. When cows were challenged for 2nd time with *E. coli* (Suojala et al., 2008), the intramammary CFU were much lower, reducing the LPS load. In contrast, we challenged cows with the same amount of LPS each time. The greater SCS and greater increase in immature nuetrophils after the 2nd challenge may be indicative of a trained innate immune response (Netea et al., 2020). Generally, trained innate responses are characterized by increased cytokine secretion from the immune

cells, which in this case may have driven greater recruitment of immune cells to the mammary gland. Suojala et al. (2008) also observed some signs of this innate training, as the cows had a greater and faster rebound in circulating leukocytes after the 2nd mastitis challenge. Functionality of these immune cells, both systemically and in the mammary gland, deserve investigation under this repeated challenge model to better undersand the dairy cow's response to repeated mastitis cases and determine whether the function of these cells is enhanced after repeated challenges as would be expected if innate training is occuring.

Plasma Hp was reduced after the 2nd LPS challenge compared to the 1st LPS challenge. Also, plasma LBP was reduced by 30% 24 h after the 2nd challenge compare to the 1st challenge in CON cows ($P < 0.01$); together these data suggest a degree of systemic tolerance occuring. These data are consistent with Suojala et al. (2008) who observed that Hp, LBP, and serum amyloid A (SAA) in both milk and serum of dairy cows were reduced after a 2nd *E. coli* mastitis challenge compared to the 1st challenge. They also observed that milk yield from the challenged quarter was greater after the 2nd challenge compared to the 1st. which is likely a result of more efficient clearance of *E. coli* from the udder. Since we observed an increased local response to LPS (as measured by SCS) we may have had similar or reduced milk yield from the challenge quarter during the 2nd challenge, but we did not measure quarter-level milk prodcution. Additionally, the overall milk yield loss was similar following each challenge in our experiment (~30%).

As hypothesized, RPN reduced markers of systemic inflammation during mastitis, indicating that it had anti-inflammatory benefits in cows. Peak Hp was reduced by 30% with RPN and the reduction was most pronounced during the 1st challenge. Likewise, RPN reduced

plasma LBP by 23% 24 h after the 1st challenge. We also observed reduced Hp for RPN compared to CON during the 2nd challenge indicating carryover effects of RPN as it was not being supplemented during the 2nd challenge. This reduced systemic inflammation resulting from RPN aligns with results from other species (Lipszyc et al., 2013; Wanders et al., 2013; Singh et al., 2014; Salem and Wadie, 2017) where niacin reduced inflammation and reduced pro-inflammatory cytokine secretion. Although RPN reduced peak Hp and LBP, indicating that it reduced inflammation, RPN did not change DMI, milk yield, or milk component yields which may limit its utility or economic viability as an anti-inflammatory feed additive. Stage of lactation should be considered here as well. Reducing inflammation during early lactation may be more impactful on milk prodution and feed intake than reducing inflammation during late lactation as tested here.

Previous research demonstrated that niacin modulates macrophage polarization toward a resolution-promoting phenotype (Wakade et al., 2018). Macrophage phenotypes have been investigated in cattle and seem to align with the classical ($CD14^+CD16^-$), non-classical ($CD14^-CD16^+$), and intermediate ($CD14^+CD16^+$) phenotypes discussed in humans and laboratory species (Talker et al., 2022). Generally, classical monocytes have anti-microbial activites while intermediate and non-classical monocytes have anti-inflammatory and regulatory functions (Talker et al., 2022). We did not observe alterations in the proportion of any of these monocyte populations in cattle as a result of RPN supplementation. It is worth noting that after LPS challenges, $CD14^+$ monocytes were dramatically reduced and almost absent in blood compared to $CD16^+$ monoctyes, which were more stable after each challenge. This is likely due to the function of the $CD14^+$ cells; they have greater antimicrobial and proinflammatory activity than

$CD16^+$ cells (Talker et al., 2022) and may have been more inclined to migrate out of circulation into the mammary system or undergo apoptosis.

We did not observe alterations of granulocyte populations in response to RPN supplementation. It is interesting, though, that RPN increased the population of immature neutrophils in blood post-challenge. Activity of neutrophils may be important to assess as activation of the HCA2 receptor has been shown to alter their function (Zandi-Nejad et al., 2013; Shi et al., 2017). Although populations of total granulocytes were similar, it may be that their function was reduced. Evaluating niacin's impacts on immune cell function *in vivo* or *ex vivo* may provide further insights about how RPN affects the dairy cow's immune response.

Interestingly, RPN affected $CD3^+$ populations, especially reducing $CD3^+CD4^+$ cells, which are considered T helper (Th) cells. These Th cells can differentiate into a variety of cell subsets with different functionalities (León, 2023), so deeper investigation into how RPN may alter these lineages would provide a more complete picture of its effects. Nonetheless, our data indicates that supplementing RPN may reduce T-cell populations and since niacin has reduced pro-inflammatory cytokines (Lipszyc et al., 2013), it may also increase the proportion of regulatory T-cell populations through modulating the cytokine mileu (León, 2023). Future experiments should focus on characterization of γδ T-cell responses to niacin, as this appears to be a major regulatory cell subset in dairy cattle (Guzman et al., 2014). It may also be useful to investigate whether RPN affects the response of macrophages to pathogens, because Th cells play a role in macrophage activation as well (Gordon, 2003).

These data demonstrate that nutritional interventions can alter the immune system of cattle and should be thoroughly investigated as strategies to improve livestock resilience. In future investigations, immune cell function, in additional to phenotype, should be assessed.

CONCLUSIONS

Feeding RPN to late-lactation dairy cows reduced SCS prior to LPS challenge, but did not affect SCS after the LPS challenge at either the quarter-level or in composite milk samples. Additionally, RPN blunted the systemic inflammatory response during induced mastitis, especially during the 1st LPS challenge, as demonstrated by reduced plasma Hp and LBP. Both CON and RPN had reduced systemic Hp after the 2nd LPS challenge, indicating a degree of systemic tolerance to LPS. Interestingly, the cows appeared to have a heightened local immune response in the mammary gland after the second challenge even though they had reduced Hp during the 2nd challenge. We conclude that RPN is a tool to improve milk quality and reduce systemic inflammation in late lactation dairy cows. It also modulates T-cell populations. Further investigation of how it may modulate immune cell function, especially of Th cells, may be warranted to better understand how RPN affects a dairy cow's response to mastitis.

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APPENDIX

Table 3.1. Effect of rumen-protected niacin (RPN) on feed intake, milk production, and milk composition prior to, during, and after intramammary challenges with lipopolysaccharide (LPS)[1].

Item	Treatment		SEM	*P*-values		
	CON	RPN		Trt	Day	Trt × Day
Pre-challenge						
DMI, kg/d	24.5	25.2	0.80	0.47	<0.01	0.75
Milk, kg/d	36.3	36.5	1.71	0.90	<0.01	0.32
ECM, kg/d	38.9	38.6	1.61	0.86	0.45	0.73
FE, ECM/DMI[2]	1.63	1.57	0.057	0.24	0.52	0.09
Fat, kg/d	1.43	1.44	0.062	0.94	0.03	0.54
Fat, %	3.98	4.10	0.198	0.59	<0.01	0.29
Protein, kg/d	1.20	1.18	0.065	0.78	0.87	0.64
Protein, %	3.34	3.34	0.085	0.99	0.04	0.66
SCS[3]	1.24	0.05	0.450	<0.01	<0.01	0.29
Milk conductivity, mOhm	91.6	94.4	2.38	0.26	<0.01	0.97
Post-challenge 1						
DMI, kg/d	25.3	24.8	0.82	0.59	<0.01	0.92
Milk, kg/d	33.2	33.4	2.18	0.93	<0.01	0.08
ECM, kg/d	38.4	38.9	2.64	0.76	<0.01	0.16
FE, ECM/DMI	1.47	1.48	0.034	0.89	0.54	0.75
Fat, kg/d	1.46	1.49	0.092	0.22	0.02	0.95
Fat, %	4.39	4.53	0.209	0.60	0.01	0.86
Protein, kg/d	1.18	1.20	0.094	0.63	0.09	0.27
Protein, %	3.51	3.56	0.099	0.59	0.08	0.87
SCS	2.78	2.51	0.337	0.46	<0.01	0.60
Milk conductivity, mOhm	93.2	94.5	2.88	0.68	<0.01	0.26
Post-challenge 2						
DMI, kg/d	23.9	22.6	0.85	0.19	<0.01	0.43
Milk, kg/d	29.8	30.0	2.29	0.91	<0.01	0.78
Milk conductivity, mOhm	95.0	95.0	3.56	0.98	<0.01	0.06

[1]n = 12/treatment before challenge and n = 10/treatment after challenges

[2]FE = Feed efficiency

[3]Somatic Cell Score (SCS) = log base 2 (SCC / 100,000) + 3

Table 3.2. Effect of rumen-protected niacin (RPN) on bodyweight (BW) and body condition score (BCS) change in late lactation cows challenged with intramammary lipopolysaccharide (LPS).

Item[1]	Treatment		SEM	*P*-values		
	CON	RPN		Trt	Week	Trt × Week
ΔBW, kg/d	0.62	0.64	0.151	0.84	0.12	0.99
ΔBCS, units/wk[2]	0.001	0.009	0.0058	0.21	0.15	0.57

[1]BW and BCS were measured weekly throughout the experiment. N = 10/treatment.
[2]Measured on a 1-5 scale (Wildman et al., 1982)

Table 3.3. Effect of rumen-protected niacin (RPN) on complete blood count of lactating dairy cows prior to repeated intramammary lipopolysaccharide challenges, change in complete blood count of lactating dairy cows 28 d after the 1st intramammary lipopolysaccharide (LPS) mastitis challenge, and changes in complete blood count of lactating dairy cows 8 h after intramammary lipopolysaccharide (LPS) mastitis challenges[1].

Item, cells/μL	Treatment		SEM	*P*-values	
	CON	RPN		Trt	Challenge
Total Leukocytes	8879	9722	947.4	0.48	-
Neutrophils	2934	3116	381.1	0.54	-
Immature Neutrophils	36	38	9.0	0.90	-
Eosinophils	90	71	34.2	0.63	-
Monocytes	531	576	63.2	0.53	-
Lymphocytes	5783	6290	1846.2	0.72	-
28 d after 1st LPS challenge[2]					
ΔTotal Leukocytes[3]	437	-1335	560.0	0.02	-
ΔNeutrophils[3]	512	59	276.6	0.21	-
ΔImmature Neutrophils	8	14	20.1	0.74	-
ΔEosinophils	-20	52	52.7	0.27	-
ΔMonocytes	62	-31	102.5	0.17	-
ΔLymphocytes[3]	-121	-1426	430.3	0.03	-
8 h after each LPS challenge[4]					
ΔTotal Leukocytes	-5622	-6031	882.7	0.71	0.33
ΔNeutrophils	-2211	-2666	524.3	0.35	0.14
ΔImmature Neutrophils	161	244	73.2	0.08	<0.01
ΔEosinophils	-54	-73	19.0	0.47	0.92
ΔMonocytes	-331	-339	13.5	0.92	0.19
ΔLymphocytes	-2863	-3080	659.9	0.70	0.31

[1]N = 12/treatment for prechallenge leukocytes and N = 10/treatment for change across challenges.

Table 3.3. (cont'd)

[2]Calculated as leukocytes prior to the 2nd challenge – leukocytes prior to the first challenge.

[3]Main effect of parity ($P \leq 0.05$)

[4]Calculated as the leukocytes after challenge – leukocytes before challenge.

Table 3.4. Effect of niacin (RPN) on circulating immune cell phenotype prior to and after intramammary lipopolysaccharide challenges[1].

Item[4], % live cells	Trt	Timepoint[2,3]				SEM	*P*-values		
		Challenge 1 0 h	Challenge 1 8 h	Challenge 2 0 h	Challenge 2 8 h		Trt	Time	Trt × Time
$CD21^+$	CON	33.3	46.1	40.0	41.4	10.68	0.59	0.07	0.35
	RPN	35.5	45.8	25.5	34.8				
$CD21^+CD11b^+$	CON	28.9	38.2	33.8	37.9	9.53	0.75	0.06	0.34
	RPN	32.0	38.9	23.9	34.9				
$CD3^+$	CON	7.8 ^{AB}	5.1^{B}	12.4^{A}	5.3 ^{B}	1.36	0.08	<0.01	0.08
	RPN	5.3 ^{B}	4.8 ^{B}	7.2 ^{B}	4.9 ^{B}				
$CD3^+CD4^+$	CON	2.8 $^{\alpha}$	2.3 $^{\alpha}$	2.1$^{\alpha}$	0.9 $^{\beta}$	0.48	0.06	<0.01	0.13
	RPN	1.2	1.6	1.0	0.6				
$CD3^+CD8^+$	CON	1.2^{B}	0.9 ^{B}	5.4^{A}	2.4 ^{B}	0.79	0.24	<0.01	0.05
	RPN	0.8 ^{B}	0.9 ^{B}	2.8^{AB}	2.0 ^{B}				
Granulocytes	CON	35.6	25.9	24.8	31.9	8.93	0.22	0.34	0.29
	RPN	35.6	26.3	46.8	42.3				
Eosinophils	CON	2.4 $^{\beta}$	1.5 $^{\gamma}$	3.0 $^{\alpha}$	0.9 $^{\gamma}$	0.55	0.95	<0.01	0.14
	RPN	2.2	0.6	3.9	1.0				
Neutrophils	CON	32.7	24.2	20.1	29.7	8.75	0.26	0.48	0.40
	RPN	33.7	25.9	39.9	38.4				
Monocytes	CON	17.7	17.8	18.2	17.9	3.95	0.70	0.87	0.83
	RPN	17.9	17.8	15.7	14.3				
$CD14^-CD16^-$	CON	7.6 $^{\beta\gamma}$	15.8$^{\alpha}$	7.9 $^{\gamma}$	17.4 $^{\alpha\beta}$	3.44	0.85	<0.01	0.43
	RPN	10.8	15.6	6.8	13.1				

Table. 3.4. cont'd

$CD14^+CD16^-$	CON	7.1 $^{\alpha}$	1.0 $^{\beta}$	7.2 $^{\alpha}$	0.0 $^{\beta}$	1.08	0.40	<0.01	0.15
	RPN	4.6	0.8	5.9	0.1				
$CD14^-CD16^+$	CON	1.1	0.9	1.5	0.8	0.25	0.60	0.09	0.19
	RPN	1.1	1.2	1.2	1.1				
$CD14^+CD16^+$	CON	2.0 $^{\alpha}$	0.1 $^{\beta}$	1.6 $^{\alpha}$	0.0 $^{\beta}$	0.25	0.70	<0.01	0.23
	RPN	1.4	0.2	1.8	0.0				
$CD11b^+$ Monocytes	CON	14.4	13.1	15.2	12.6	3.28	0.99	0.47	0.92
	RPN	14.1	14.2	14.5	12.7				

[1]N = 5/treatment

[2]Cells with different letters indicate significant differences within a cell type ($P \leq 0.05$)

[3]Columns with different symbols ($^{\alpha, \beta, \gamma}$) indicates a difference by the main effect of time ($P \leq 0.05$) within cell type.

[4]$CD21^+$ = B cells, $CD3^+CD4^+$ = T helper cells, $CD3^+CD8^+$ = Cytotoxic T cells

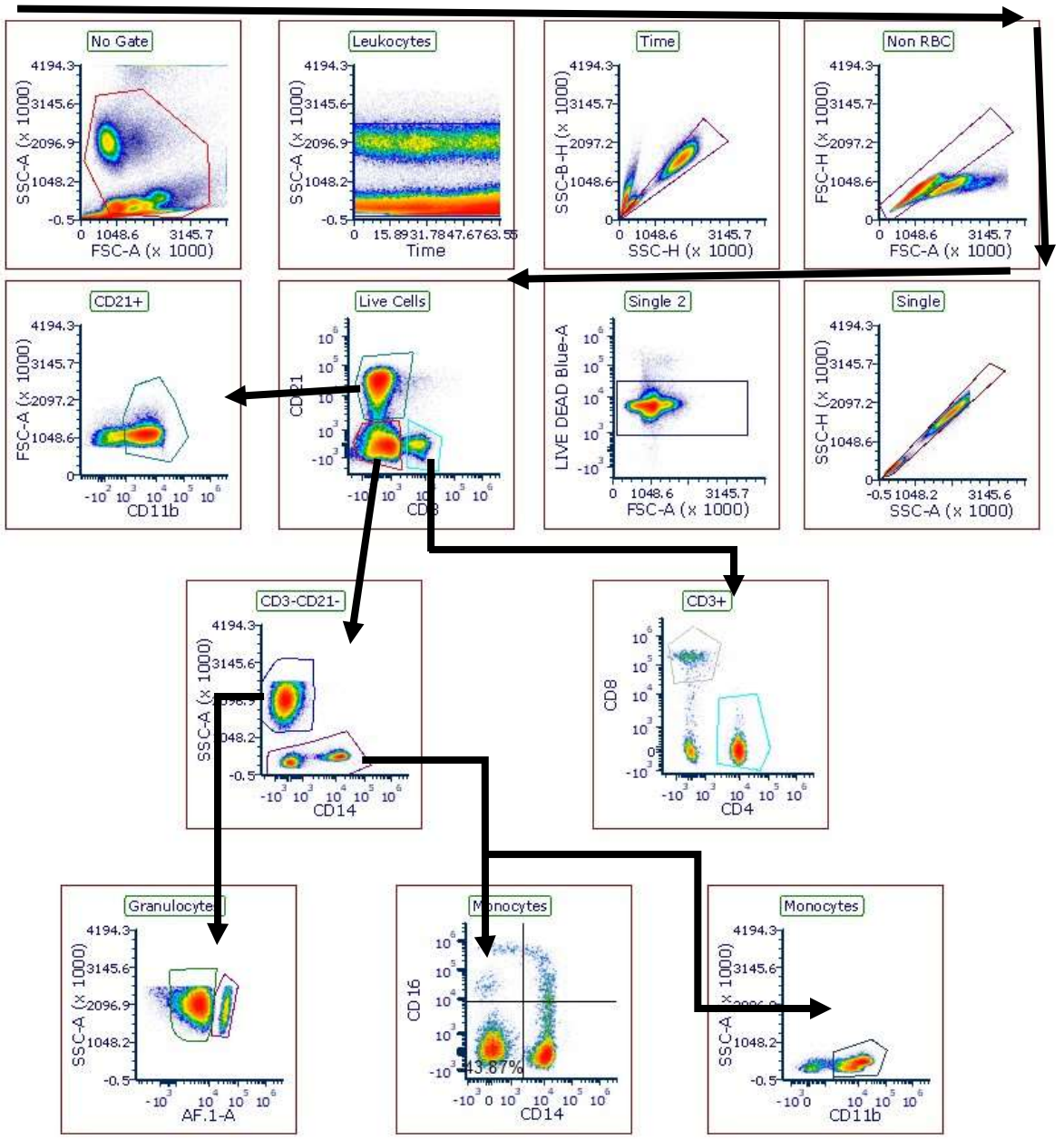

Figure 3.1. Gating strategy for whole blood immunophenotyping protocol used for lactating dairy cattle supplemented with rumen-protected niacin (RPN) and subject to repeated intramammary lipopolysaccharide challenges. SSC-A = side scatter – area; SSC-H = side scatter – hieght; SSC-B-H = side scatter blue-height; FSC-A = forward scatter-area; FSC-H = forward scatter-height; AF.1-A = autoflouresence 1 signiture indicating eosinophils.

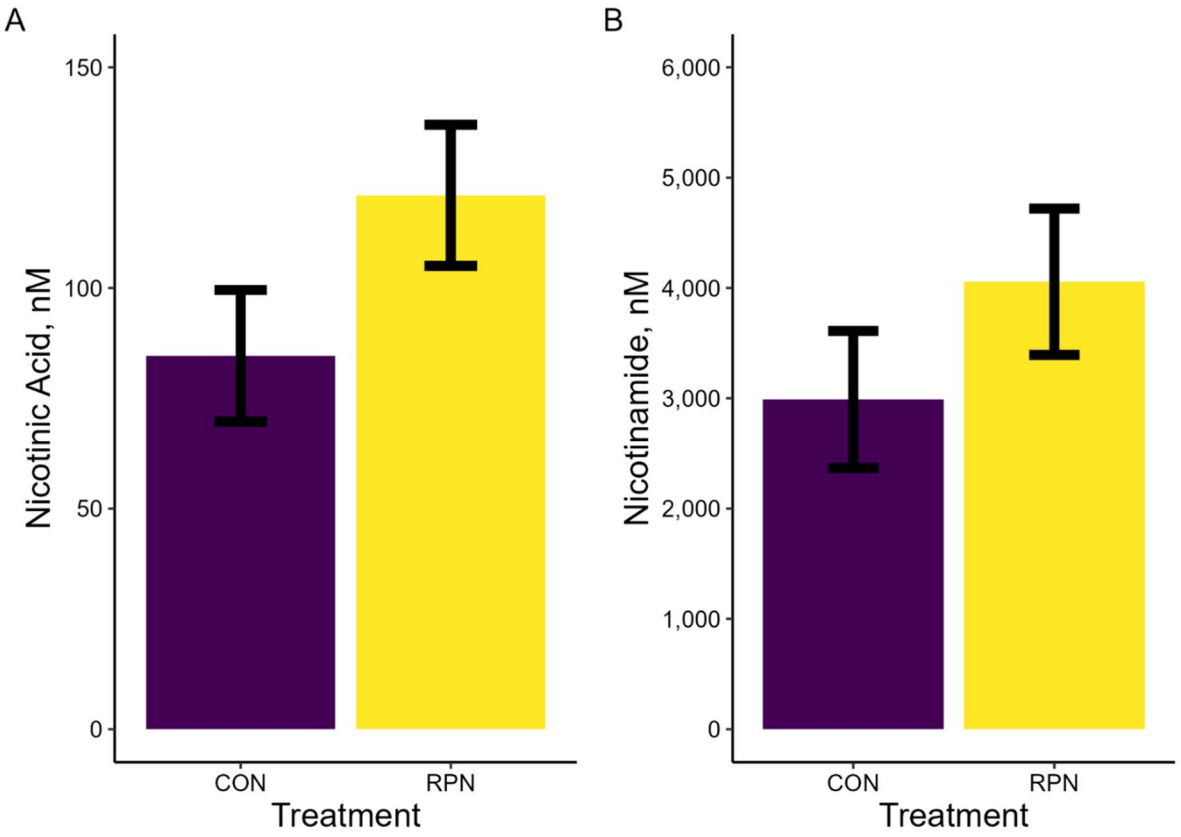

Figure 3.2. Effect of feeding rumen-protected niacin (RPN; n = 12/treatment group) on plasma nicotinic acid and nicotinamide in lactating dairy cows. A) RPN tended to increase plasma nicotinic acid (P = 0.06; 84.6 ± 14.91 nM vs. 121.0 ± 16.00 nM). B) RPN increased plasma nicotinamide ($P < 0.01$; 2989.6 ± 621.19 nM vs. 4056.5 ± 663.1 nM).

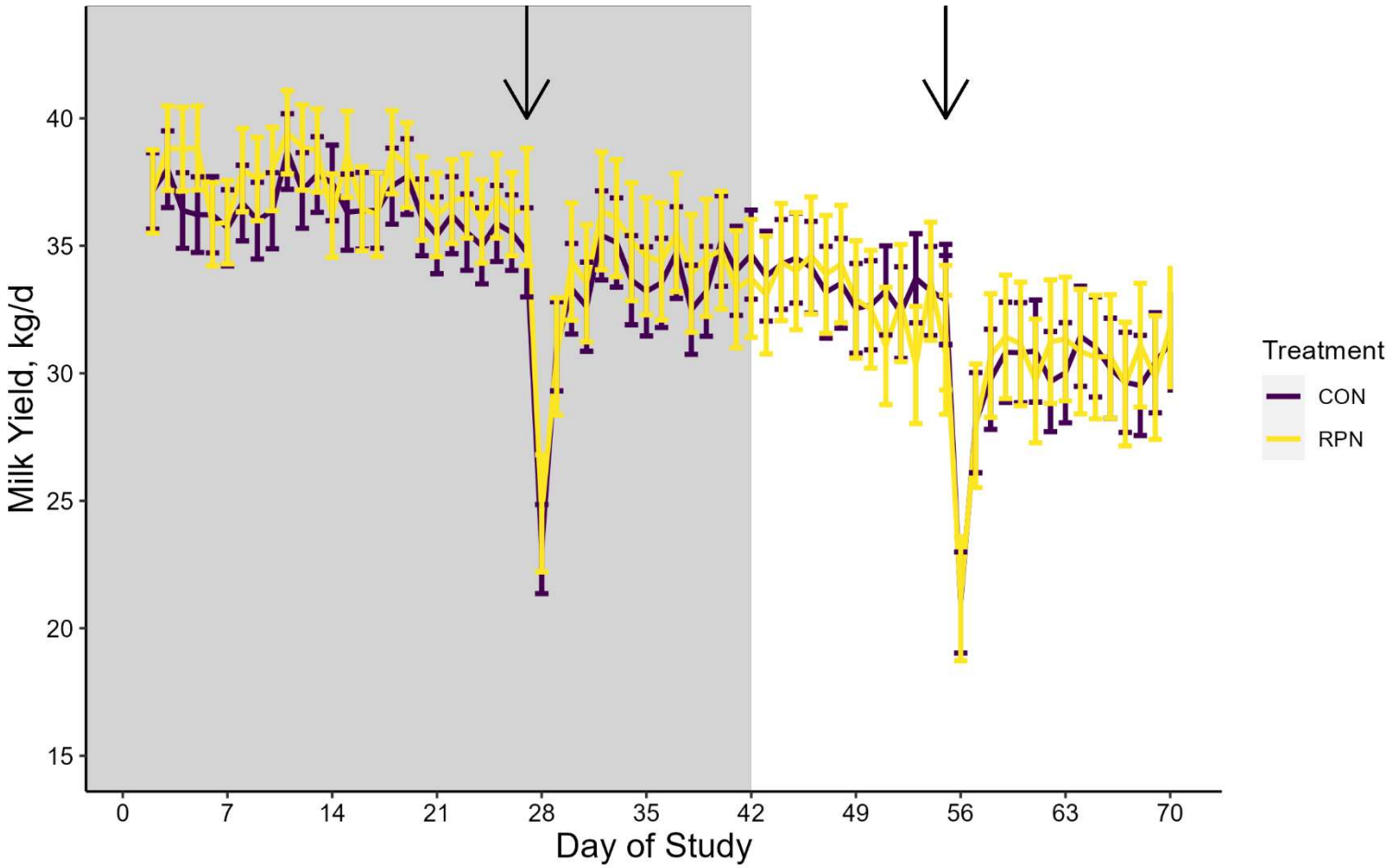

Figure 3.3. Effect of rumen-protected niacin (RPN; n = 12/treatment prior to 1st challenge and n = 10 after challenge) on milk yield prior to, during, and after repeated intramammary lipopolysaccharide challenges. Arrows indicate intramammary lipopolysaccharide challenges. The shaded area indicates when RPN was supplemented to the cows. Supplementing RPN did not affect milk yield prior to, after the 1st, or after the 2nd lipopolysaccharide challenge ($P \geq 0.47$).

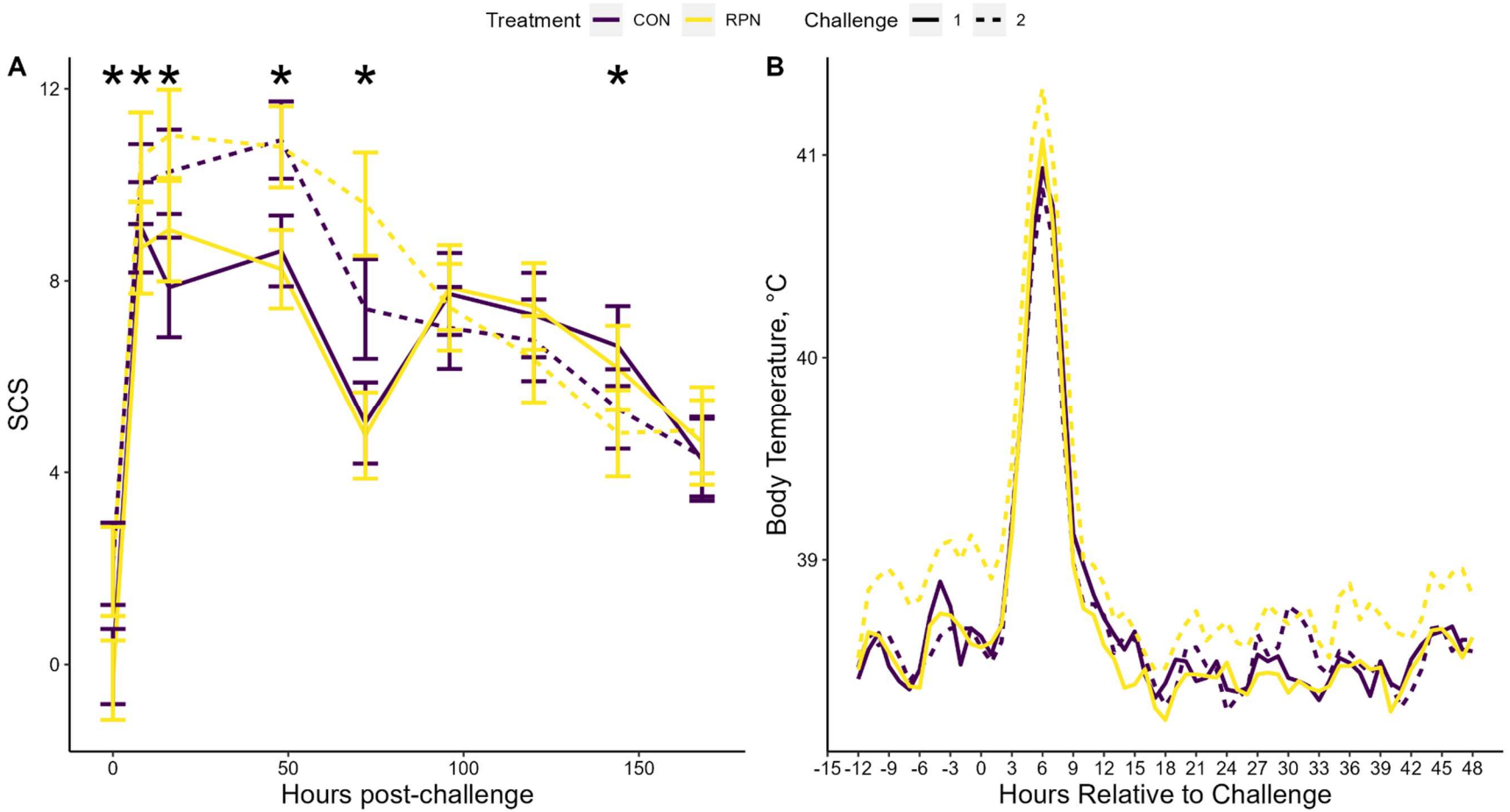

Figure 3.4. A) Quarter level somatic cell score [somatic cell score (SCS) = log base 2 (SCC / 100,000) + 3] of dairy cows fed supplemental rumen protected niacin (RPN; n = 10/treatment) or a no-treatment control (CON). Cows were challenged with lipopolysaccharide two times in their rear right quarter. Treatment did not affect SCS ($P = 0.71$). There was a significant effect of challenge × time ($P < 0.01$). During the 2nd challenge, SCS was increased at 0, 8, 16, 48, and 72 h post challenge ($P \leq 0.05$). At 144 h post-challenge, the 1st LPS challenge had greater SCS than the 2nd LPS challenge ($P = 0.03$). B) Intravaginal temperature during each challenge. AUC for each challenge was not affected by treatment ($P = 0.69$). RPN was supplemented during challenge 1 but not during challenge 2.

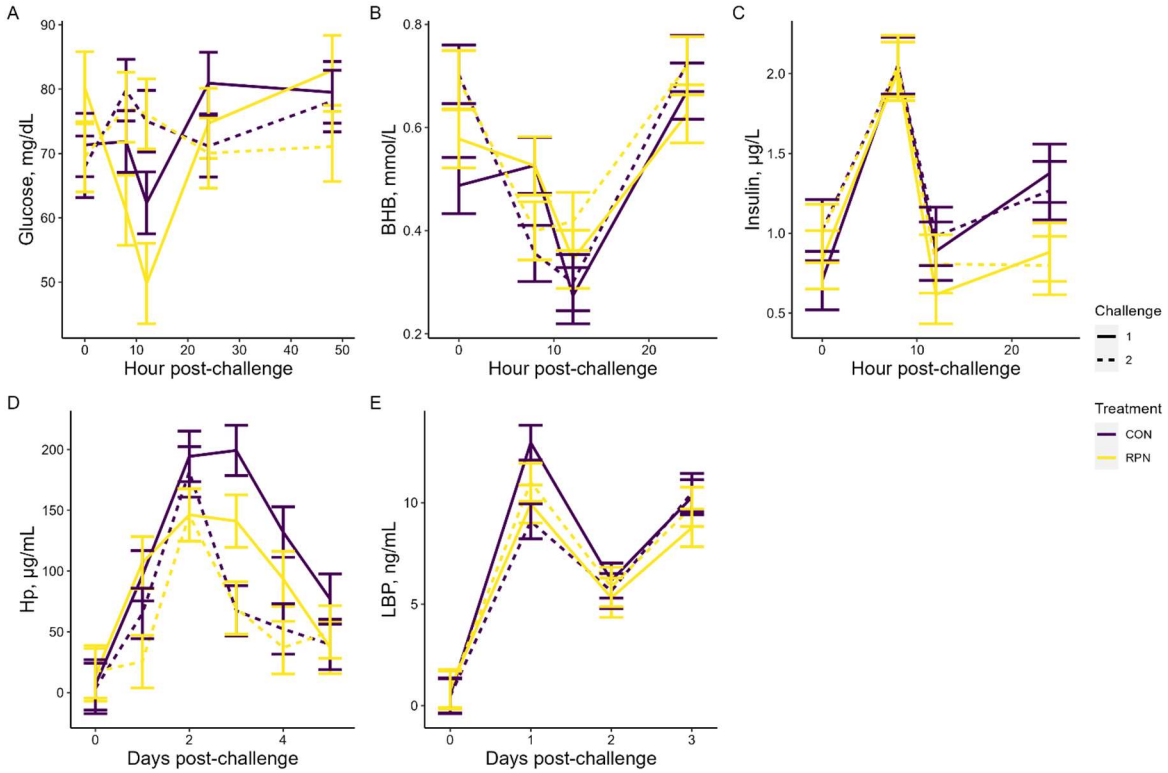

Figure 3.5. A) Effect of rumen-protected niacin (RPN; n = 10/treatment) on plasma glucose concentrations following intramammary lipopolysaccharide (LPS) challenges. Rumen-protected niacin did not affect plasma glucose ($P = 0.19$). B) Effect of RPN and challenge on plasma β-hydroxybutyrate (BHB). RPN did not affect BHB ($P = 0.32$). There was an LPS × time interaction ($P > 0.01$) such that BHB was greater during the 2nd LPS challenge at 0 h ($P = 0.01$) and less at 8 h ($P = 0.03$) after challenge. C) Effect of RPN on plasma insulin. Treatment did not affect plasma insulin ($P = 0.14$) and both challenges resulted in similar insulin dynamics ($P = 0.37$). D) Effect of RPN on plasma haptoglobin (Hp) concentrations. RPN tended to reduce Hp ($P = 0.08$). Hp during challenge 2 was reduced 1, 3, and 4 d compared to challenge 1 ($P \leq 0.01$). E) The LPS × time × treatment interaction tended to affect plasma lipopolysaccharide binding protein ($P = 0.08$) such that RPN tended to have reduced LBP 24 h after challenge 1 ($P = 0.08$). RPN was supplemented during challenge 1 but not during challenge 2.

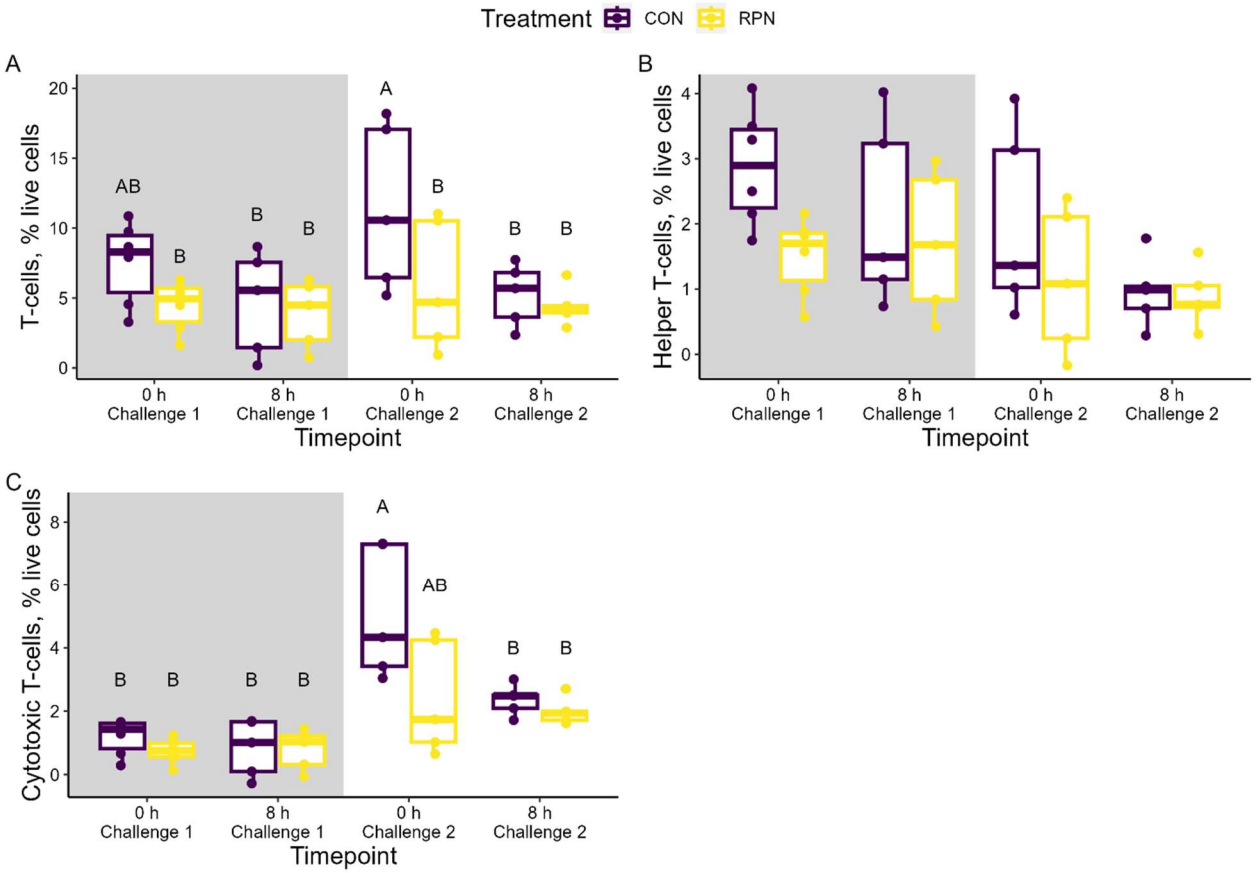

Figure 3.6. The effect of rumen-protected niacin (RPN; n = 5/treatment) on $CD3^+$ cell populations prior to and after intramammary lipopolysaccharide (LPS) challenges. The RPN was supplemented during challenge 1 only, indicated by the shaded region. A) T-cells ($CD3^+$) tended to be affected by a Trt × Time interaction ($P = 0.08$). Groups that do not share superscripts indicate significant differences between groups ($P \leq 0.05$). B) T-helper cells ($CD3^+CD4^+$) tended to be reduced by RPN ($P = 0.06$). C) Cytotoxic T-cells ($CD3^+CD8^+$) were affected by a Trt × Time interaction ($P = 0.05$). Groups that do not share superscripts indicate significant differences between groups ($P \leq 0.05$).

Table S3.1. Antibodies and fluorochromes used in whole blood immune phenotyping flow cytometry panel investigating the effects of rumen protected niacin on circulating immune cell populations.

Marker	Antibody Source[1]	Host	Isotype	Clone	Fluor	Final antibody concentration, μg/mL
CD3	WSU	Mouse	IgG1	MM1A	CF405L	25
CD4	BR	Mouse	IgG2a	CC8	AF 700	5
CD8	BR	Mouse	IgG2a	CC63	AF 647	1.25
CD21	BR	Mouse	IgG1	CC21	FITC	0.5
TCR-N24	KF	Mouse	IgG2b	GB21A	Dylight 594	5.2
CD11b	WSU	Mouse	IgG2b	MM10A	CF750	2.5
CD14	BL	Mouse	IgG2a	M5E2	BV 650	2.5
CD16	BR	Mouse	IgG2a	KD1	PE	1.75

[1]WSU = Washington State Antibody Center, Pullman, WA; BR = Bio-Rad, Hercules, California; KF = Kingfischer Biotech, Saint Paul, MN; BL = Biolegend, San Diego, CA

Table S3.2. Nutrient composition of the total mixed ration fed to lactating dairy cows throughout the experiment[1].

Item, % DM	Mean	SD
DM	50.70	2.195
CP	15.76	0.717
Starch	25.50	1.161
ESC[2]	4.64	1.224
NDF	32.92	1.843
Lignin	3.63	0.311
Crude Fat	3.79	0.229
Ash	5.85	1.374
Ca	0.80	0.197
P	0.38	0.097
Mg	0.34	0.085
K	1.23	0.246
S	0.19	0.010
Na	0.35	0.074
Cl	0.28	0.022

[1]n = 5

[2]ESC = ethanol soluble carbohydrates

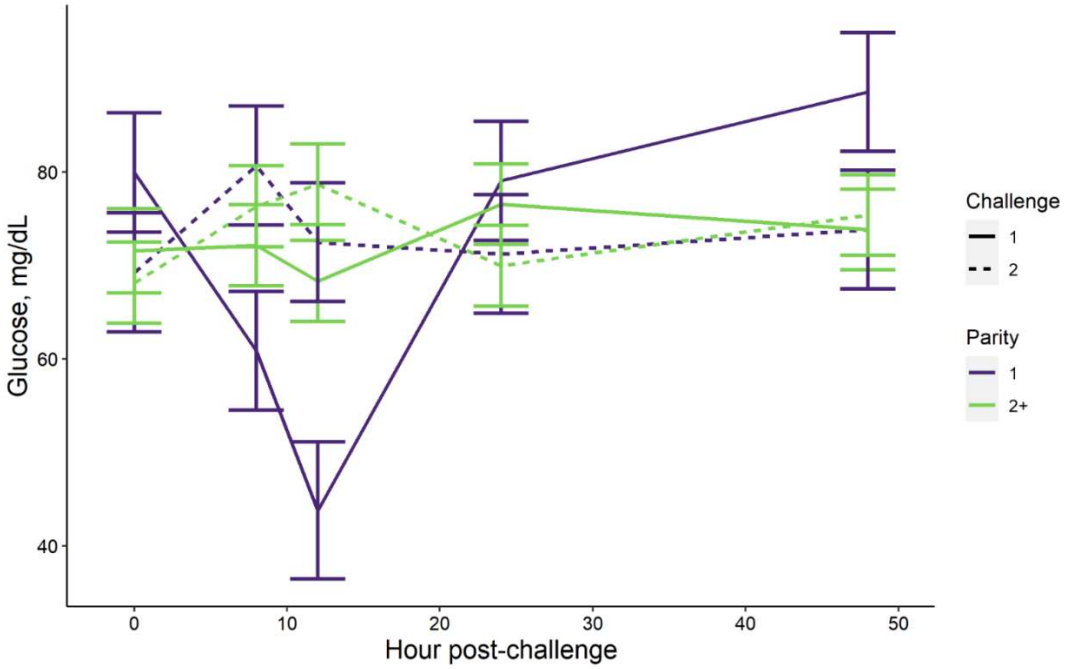

Figure S3.1. Plasma glucose concentration was affected by a LPS × time × parity interaction (P = 0.04). At 8 and 12 hr post-challenge, primiparous cows had reduced plasma glucose during the first LPS challenge compared to the 2nd challenge ($P \leq 0.05$). During the first challenge, primiparous cows also had reduced plasma glucose compared to multiparous cows at 12 hr post-challenge ($P < 0.01$). n = 4 primiparous and n = 16 multiparous.

CHAPTER 4: ON-FARM SUPPLEMENTATION OF RUMEN-PROTECTED NIACIN: A RANDOMIZED CLINICAL TRIAL

ABSTRACT

B-vitamins, including niacin (vitamin B_3), are synthesized by rumen microbes but supplementation may provide additional benefits. Supplementing rumen-protected niacin (RPN) during the transition period reduces lipolysis after calving and, consequently, may improve health and fertility of dairy cattle later in lactation. Our objective was to determine if supplementing RPN during the first 21 DIM reduced risk of leaving the herd, improved fertility, or reduced mastitis incidence of dairy cows on a commercial dairy farm. Holstein cows were blocked by parity and calving date then at parturition and were randomly assigned to receive RPN (n=481) in addition to their robot pellet through the automated milk systems (AMS) or the robot pellet only (CON; n = 593). Cows were followed for the rest of their lactation. Milk yield, milk components, pre- and postpartum body condition score, health records, and reproductive records were collected. Blood was collected from a random subset of 99 cows at 3 DIM and 97 cows at 10 DIM to assess plasma niacin concentrations, metabolic biomarkers, and biomarkers of inflammatory status. Culling, pregnancy, and mastitis risk were analyzed using Cox proportional hazard models. Mastitis incidence and conception risk were also analyzed using a χ^2 test where treatment, parity, and their interaction were included in the model. Continuous variables were analyzed with linear mixed models that included fixed effects of treatment, parity, and their interaction. Supplementing RPN increased plasma nicotinamide concentration by 1740 nM ± 410.0 nM but it did not plasma nicotinic acid concentrations. Supplementing RPN reduced plasma insulin concentrations at 3 and 10 DIM across all parities. Circulating β-hydroxybutyrate and free fatty acid concentrations were greater for cows receiving RPN, especially in 3+ parity

cows. Plasma haptoglobin was not affected by treatment. RPN increased milk yield for 1st and 2nd parity cows by week 9 and week 13 of lactation, respectively. Increased milk yield in those groups was sustained for the rest of lactation resulting in 639 kg ± 319.8 kg and 712 kg ± 364.4 kg more milk for RPN supplemented 1st and 2nd parity cows. The risk of leaving the herd, mastitis incidence, and risk of pregnancy were not affected by RPN. Inquiries into the effects of RPN supplementation on metabolism and timing of RPN supplementation relative to calving are necessary to understand optimal supplementation strategies for RPN in dairy cattle.

Key words: disease, fertility, health, management

INTRODUCTION

Research on nutritional strategies to improve dairy cow health and reproduction is often limited by the size (experimental units) of the experiments. In place of directly measuring binary outcomes like disease, culling, or pregnancy, investigators commonly resort to measuring biomarkers which are associated with health or fertility, like free fatty acids, β-hydroxybutyrate (BHB), or acute phase proteins such as haptoglobin (Ospina et al., 2010; Kerwin et al., 2022). Fortunately, the increased adoption of automated milking systems (AMS) on dairy farms provides new opportunities for controlled research into the effects of nutritional strategies, nutrients, or feed additives on these binary, cow-level outcomes. There is evidence that some supplements supplied to individual cows through an AMS may reduce disease or improve reproductive efficiency (Barkema et al., 2015; Olagaray et al., 2019). These examples also demonstrate the utility of AMS for controlled dairy cow nutrition and health research.

It is well understood that lactating dairy cows experience a plurality of their disease in the early postpartum period (Ingvartsen, 2006) and the risk of dairy cows experiencing disease was observed to be greater when plasma free fatty acids (≥0.6–1.0 mEq/L), BHB (≥1.2 mmol/L;

McArt et al., 2013), or haptoglobin increase (Hp; ≥0.45 g/L; Kerwin et al., 2022). Kerwin et al. (2022) observed that cows with elevated Hp made 492 kg less milk over their lactation and as post-partum BHB increased, milk yield linearly declined. These associations indicate that increased circulating ketones and inflammation are associated with poorer lactation outcomes. Which comes first, or how they are related, are topics of intense thought and study in the dairy science community (Contreras et al., 2017; Horst et al., 2021). Either way, the evidence suggests that reducing excessive lipolysis or inflammation or both may improve whole lactation animal performance and health.

Another factor influencing health during the transition period of dairy cattle is the weight loss and gain cycle which occurs through lactation. Excessive weight-cycling is implicated in metabolic and immune dysregulation. Anderson et al. (2013) observed that weight-cycled mice had the greatest plasma glucose after a glucose tolerance test and the greatest concentration of fasting insulin, indicative of greater dysregulation. Poorer glucose regulation and increased pro-inflammatory biomarkers in weight-cycled mouse immune cells were also observed by Caslin et al. (2023). Excessive weight-cycling that a minority of dairy cows experience through the course of lactation may promote inflammation and metabolic disruption, although that has not been directly investigated. Reducing body weight (BW) loss, or lipolysis, after calving and minimizing the required BW gain to replenish body nutrient stores may reduce inflammation and metabolic dysregulation during and after the transition period.

One nutritional strategy to reduce lipolysis and inflammation in dairy cattle during the postpartum period is through supplementation of rumen-protected niacin (RPN). Supplementing dairy cattle with RPN from 21 days prepartum to 21 days postpartum reduces peak free fatty acids by 40-50% (Morey et al., 2011; Yuan et al., 2012). The reduction in lipid mobilization

when supplementing RPN during the early lactation period may improve reproduction, health, and fertility. Fertility may be especially affected as increased exposure to free fatty acids during in vitro maturation of oocytes led to fewer cells and altered metabolism in the resulting blastocysts, both markers of reduced embryonic viability (Van Hoeck et al., 2011). Furthermore, increasing free fatty acids appeared to reduce follicle diameter and reduce oocyte development in mice (Valckx et al., 2014) which suggests that reducing circulating free fatty acids during oocyte maturation is an opportunity to improve oocyte quality in dairy cows later in lactation (Leroy et al., 2015).

Even though RPN's anti-lipolytic properties have been observed in multiple experiments with dairy cows, there is no evidence as to whether this alteration in lipolysis may improve health or reproduction of dairy cattle over the course of their lactation. Presented with this gap, our objectives were to determine if supplementing RPN to dairy cows in the immediate postpartum period would affect disease incidence, reproduction, or risk of leaving the herd. We hypothesized that supplementing RPN would reduce lipolysis and result in improved reproductive performance and reduced disease, therefore reducing risk of leaving the herd for lactating dairy cattle.

MATERIALS AND METHODS

Cows, treatments, design, and management

Cows were housed in sand-bedded free-stall barns at a dairy farm in western Michigan. Prepartum cows were fed once daily and postpartum cows were fed twice daily (Table 4.1). Cows were enrolled in the experiment at calving. The farm followed a just-in-time calving management system. Prepartum pens, one housing only nulliparous cows and one housed a mix of nulliparous, primiparous, and multiparous cows, were walked hourly. Cows expressing signs

of the birthing process were then moved to a straw-bedded calving pen. The cows were first milked in a conventional parlor to collect 1st milking colostrum prior to entering the regular lactating pens. First lactation cows and multiparous cows were placed in separate lactation pens for the first 3 wk of lactation. After that, cows were placed in a lactation pen based on body size and milk production. Once in the lactating pens, all fresh cows within 14 DIM were fetched twice per day. Also, the farm staff fetched cows and brought them to the AMS three times per day; cows were fetched if they had not been to the AMS within 12 h.

The voluntary waiting period prior to first breeding was 70 d with all cows being bred on a presynchonization and synchronization protocol at 1st service. The presynchronization began with PGF-2$_\alpha$ 24 d prior to 1st service and gonadotropin releasing hormone (GnRH) administered at 17 d prior to first service. Then the synchronization protocol included a GnRH injection 9 d prior to 1st service, a PGF-2$_\alpha$ injection 3 d prior to 1st service, GnRH 1 d prior to 1st service, and on the day of 1st service. Then, at 7 d prior to pregnancy diagnosis, cows were administered GnRH and if open at the pregnancy check between 28-33 d post-breeding, PGF-2$_\alpha$ was administered followed by GnRH 2 d after and breeding 24 h after that. Cows were rebred if they exhibited estrous prior to the pregnancy checks at 28-33 d or 68-72 d postbreeding. As mentioned previously, pregnancy was assessed between 28-33 d post-breeding through ultrasound by the farm veterinarian. Pregnancy status was confirmed by blood tests at 68-72 d post-breeding.

The experiment was a randomized complete block design. We blocked cows by parity and calving month. On a monthly basis, a list of cows due to calve in the next 30 days was extracted from PCDART (Dairy Record Management Systems, Raleigh, NC). The cows were stratified by parity and treatment was randomly assigned to cows within parity using a random

number generator (Microsoft Excel). Once enrolled, cows received one of two treatments; the control (CON) treatment was the regular robot pellet while treated cows received 26 g/d rumen-protected niacin (RPN; targeting 6 g of bioavailable niacin/d; Vetagro Inc, Chicago IL), in addition to the robot pellet through the AMS. Delivery of RPN was achieved with a hopper and calibrated delivery mechanism that was independent of that used to deliver the pellets.

The power analysis indicated that 484 cows per treatment group would be sufficient to detect a 9-unit change in conception at first service with 80% power ($\alpha = 0.05$). Thus, our desired sample size of the experiment was 968 cows. Cows were enrolled from March 2022 until January of 2023. We successfully enrolled 1,074 cows; 593 were on CON and 481 were on RPN. 363 cows were primiparous, 252 cows were second parity, and 459 cows were $\geq 3^{rd}$ parity. Treatments were intended to be perfectly balanced but not all cows were placed in the correct pre-assigned group at calving within the computer system. No systematic errors in treatment assignments were readily observable so misassigned animals were maintained on the study protocol and included in the data analysis.

Data collection, sample collection, and sample analysis

Body condition scores (BCS; Wildman et al., 1982) were observed once per week by the same trained investigator throughout the experiment. Prepartum BCS were taken within 14 days of the cow's projected calving date. Postpartum BCS was observed between 14 and 21 DIM. Change in BCS (ΔBCS) was calculated by subtracting the prepartum BCS from postpartum BCS.

Dairy farm staff collected blood samples via coccygeal venipuncture twice per week from multiparous cows between 3 and 7 DIM for analysis of whole blood ketone concentrations using a handheld ketone tester (Part No. 45824, Nova Max Plus, Waltham, MA). Research staff

collected additional blood samples once per week from cows at 3 and 10 DIM via coccygeal venipuncture. The blood was collected into K_2EDTA vacutainer tubes and centrifuged for 20 min at 1,350 × *g* to collect plasma within 30 minutes of blood sample collection. Plasma samples were stored at -20ºC for later analysis. Plasma samples were analyzed for glucose (kit #997-03001; Fujifilm, Osaka, Japan), free fatty acids (NEFA-HR, Fujifilm Wako Chemicals), insulin (no. 10–1201–01; Mercodia AB, Uppsala, Sweden), BHB (kit #H7587–58, Pointe Scientific Inc.), and Hp (kit # HAPT-11; Life Diagnostics, West Chester, PA). The inter-assay coefficients of variation (CV) were 6.3, 6.9, 6.8, 5.2, 4.1, and 6.9 for glucose, free fatty acids, insulin, BHB, and Hp, respectively. Intra-assay CV were 5.6, 6.3, 6.3, 3.2, and 6.3 for glucose, free fatty acids, insulin, BHB, and Hp, respectively.

A random subset of blood samples from 10 DIM (CON, n = 25; RPN, n = 26) were analyzed for plasma nicotinic acid and nicotinamide to assess niacin status of the dairy cows. Samples were analyzed by a method adapted from Lahély et al. (1999). Samples were analyzed by on a Waters Xevo TQ-S UPLC/MS/MS (Waters Corporation, Milford, MA) at the MSU Mass Spectrometry and Metabolomics Core (East Lansing, MI).

Milk samples were collected from cows between 5 and 21 DIM. Samples were collected using a milk sampling device that attaches to the AMS (Figure 4.1; Lely; Pella, IA). Individual milk samples were analyzed by Central Star Cooperative (Grand Ledge, MI) for milk fat, protein, and milk urea nitrogen (MUN) using near infrared spectroscopy. Somatic cell count (SCC) was measured by flow cytometry, also by Central Star Cooperative.

Daily milk yield was collected from management software which collects data from the AMS (T4C or Horizon; Lely, Pella, IA). Herd management records which included culling,

mastitis incidence, and reproduction were downloaded from PCDART upon completion of the experiment. Data from all cows enrolled were gathered until the cows reached at least 150 DIM.

Pre-fresh and lactating cow TMR samples were collected once per month. Samples of the pellet fed through the AMS were also sampled once per month. The samples were analyzed at Cumberland Valley Analytical Services (CVAS; Waynesboro, PA). All samples were analyzed for DM (AOAC International, 2000), CP (Leco FP-528 N Combustion Analyzer, Leco Corp., St. Joseph, MO), NDF (Van Soest et al., 1991), aNDFom (Mertens, 2002), ADF (method 973.18; AOAC International, 2000), starch (Hall, 2009), crude fat (2003.05; AOAC International, 2006), ash (AOAC International, 2000), and minerals (2003.05; AOAC International, 2006).

Statistical analysis

Milk and milk component yields were analyzed with a linear mixed model approach using the GLIMMIX procedures of SAS (9.4; SAS Institute, Cary NC). All models included the fixed effects of treatment, parity, week of lactation and their interactions, as well as the main effect of pen. These models were analyzed as repeated measures model with the random effect of cow. For all repeated measures, we evaluated compound symmetry, autoregressive, autoregressive heterogenous, and Toeplitz covariance structures for each variable and the structure with the lowest AIC was selected. All statistical models were visually evaluated to ensure normality of residuals. If the normality assumption was violated, the variable was log-transformed, and the model was re-fit. Furthermore, observations with a Studentized residual $\geq |4|$ they were removed, and the model was re-fit. For all statistical analysis, significance was declared at $P \leq 0.05$ and tendencies were declared at $P \leq 0.10$. Data from cows who were died or left the herd were included in each analysis.

Plasma metabolites and biomarkers, except for nicotinic acid and nicotinamide, were analyzed as repeated measures with cow as the random effect using the GLIMMIX procedures of SAS. Each model included the fixed effects of treatment, parity, DIM, and their interactions. Plasma nicotinic acid and nicotinamide were analyzed with a fixed effects model that included the effects of treatment, parity, and their interaction.

Body condition score and cow-side ketones were analyzed using the GLIMMIX procedures of SAS and included fixed effects of treatment, parity, and their interaction. The models for postpartum BCS and cow-side ketones also included DIM, the quadratic term for DIM, and their interaction with treatment and parity. The DIM and DIM^2 remained in the model if $P \leq 0.10$.

Mastitis incidence rates were calculated according to Kelton et al. (1998). Mastitis cases were defined as cows who were included in the AMS health report and upon physical examination presented with abnormal milk. A case was not included if it occurred within 2 weeks of a previous case of mastitis. The true incidence rate and first-case incidence rates were analyzed with a χ^2 test in the GLIMMIX procedure of SAS. The model included effects of treatment, parity, and their interaction.

Conception rates and pregnancy loss were analyzed using a χ^2 test with the GLIMMIX procedure of SAS. The model included effects of treatment, parity, and their interaction.

Risk of leaving the herd, risk of mastitis, and risk of pregnancy were analyzed using Cox Proportional Hazards analysis (Survival package; R, v. 4.0.3). The model included the effects of treatment and parity. Parity remained in the model if $P \leq 0.10$. The proportional

hazards assumption was checked using a Schoenfeld residuals test. If the proportional hazards assumption was violated, the model was stratified on parity and the model was re-fit.

Associations of BCS, blood, milk yield, and disease were analyzed in R using linear and generalized logistic regression models in a univariate fashion. For BCS variables, we also tested the quadratic association of BCS with each dependent variable. A Pearson correlation matrix of blood biomarkers and BCS was created using the corr.test and corplot functions of R (Psych package) and the asterisks indicate $P \leq 0.05$ for each respective correlation.

RESULTS

Body condition score and plasma analytes

Prepartum and postpartum BCS were similar for each treatment group within parity (Table 4.2). Additionally, BCS was lesser for primiparous cows compared to multiparous cows ($P < 0.01$) and 2nd parity BCS was less than BCS parity 3+ cows ($P < 0.01$) during the prepartum period. During the postpartum period, BCS for 1st parity cows was less than 2nd parity cows ($P = 0.03$) and 3rd+ parity cows ($P < 0.01$). 2nd parity cows had reduced postpartum BCS compared to 3rd+ parity cows ($P = 0.04$) during this period. The ΔBCS tended to be less negative in 1st parity cows than in 2nd parity cows ($P = 0.08$) and was significantly less negative for 1st parity than 3rd+ parity cows ($P < 0.01$). 2nd parity ΔBCS was less negative than 3rd parity ΔBCS ($P = 0.04$).

Cow-side blood ketones were not affected by treatment ($P = 0.20$) but were greater for 3rd+ parity cows than for 2nd parity cows ($P < 0.01$). Blood biomarker results are displayed in Figure 4.2A-E. Supplementing RPN did not affect plasma glucose at 3 or 10 DIM ($P = 0.20$), but glucose was reduced in 3rd+ parity cows compared to 1st or 2nd parity cows ($P <0.01$; Figure 4.2A). Plasma insulin was reduced in cows fed RPN ($P = 0.01$). Insulin was also lesser 3rd+ parity cows than 1st lactation cows ($P < 0.01$). Plasma free fatty acids were greater for cows fed

RPN ($P = 0.05$). Additionally, free fatty acid concentration was reduced at 10 DIM compared to 3 DIM ($P < 0.01$) and was greater for 3rd+ parity cows than for 1st and 2nd parity cows ($P < 0.01$). Plasma BHB was affected by an interaction of parity × treatment ($P < 0.01$); RPN increased BHB in 3rd+ parity cows ($P < 0.01$). The BHB at 10 DIM was lesser than BHB at 3 DIM ($P < 0.01$). Plasma Hp was not affected by treatment ($P = 0.93$). It was affected by parity × DIM interaction ($P = 0.03$) such that Hp was similar for all parities at 3 DIM, but by 10 DIM the 1st lactation cows had reduced Hp compared to multiparous cows ($P \leq 0.02$). Plasma nicotinic acid was not affected by RPN ($P = 0.72$; Figure 4.3A) but RPN significantly increased plasma nicotinamide concentrations ($P < 0.01$; Figure 4.3B; 3481 ± 339 nM vs. 5221 ± 305 nM).

Milk yield and milk composition.

During the immediate postpartum period, RPN tended to increase milk yield ($P = 0.10$; Table 4.3). It did not alter milk composition, milk component yields, or SCS during the immediate postpartum period ($P \geq 0.23$).

Milk yield throughout lactation was affected by the three-way interaction of treatment × wk × parity ($P < 0.01$; Figure 4.4). In first parity cows, milk yield tended to be greater during wk 9, 11, and 13 of lactation for RPN compared to CON ($P \leq 0.08$). Milk yield continued to be greater for RPN supplemented primiparous cows compared to CON primiparous cows from wk 15 until wk 42 of lactation ($P \leq 0.10$). In 2nd parity cows, RPN had greater milk yield in wk 13 of lactation ($P = 0.05$) and tended to have greater milk yield during wk 15 and 16 of lactation ($P \leq 0.08$). In 2nd parity cows, RPN increased milk yield from wk 18 through wk 40 and during wk 42 of lactation ($P \leq 0.05$). In 3+ parity cows, RPN tended to increase milk yield compared to CON during wk 11 ($P = 0.06$), otherwise milk yield was similar across treatment groups. In total, RPN

increased milk yield by 639 kg ± 319.8 kg and 712 kg ± 364.4 over the course of the lactation in 1st and 2nd parity cows, respectively.

Mastitis incidence, fertility, and risk of leaving the herd.

Supplementing RPN during the postpartum period did not affect the risk of pregnancy (Figure 4.5; $P = 0.84$), the risk of mastitis (Figure 4.6; $P = 0.56$), or the risk of leaving the herd (Figure 4.7; $P = 0.44$). The true incidence rate and first case incidence rate for mastitis were similar across treatments groups ($P \geq 0.25$; Table 4.4). First-case mastitis incidence rates increased with increasing parity ($P < 0.01$). The true incidence rate was greater for 3rd+ parity cows compared to both 1st and 2nd parity cows ($P \leq 0.03$). Additionally, RPN did not affect 1st or 2nd service conception rate across parity ($P \geq 0.58$; Table 4.5). Parity affected or tended to affect 1st and 2nd service conception, respectively ($P \leq 0.08$) such that 2nd parity cows had greater pregnancy risk at both 1st and 2nd service than 3rd+ parity cows ($P \leq 0.04$). The pregnancy loss at 68-72 d post breeding was unaffected by treatment, parity, or their interaction ($P \geq 0.38$).

BCS and blood biomarker associations with mastitis, risk of leaving the herd, fertility, and peak milk yield.

Prepartum BCS tended to be associated with the risk of experiencing mastitis ($P = 0.07$; Figure 4.8A) such that increasing prepartum BCS by 1 unit increased the odds of mastitis by 51% (Table 4.5). Conversely, increasing prepartum BCS tended to be associated with reduced risk of leaving the herd ($P = 0.08$; Figure 4.8B). Increasing prepartum BCS was also associated with increasing the risk of pregnancy ($P < 0.01$).

Greater postpartum BCS was associated with increased pregnancy risk ($P < 0.01$). Postpartum BCS had a quadratic association with risk of leaving the herd ($P = 0.05$) such that thinner cows were at much greater risk of leaving the herd compared to cows with a BCS of

3.25-3.75 and as BCS increased above 4, risk of leaving the herd began to increase again (Figure 4.8C).

The ΔBCS was associated with the risk of culling ($P = 0.05$) such that a 1 unit increase in ΔBCS (e.g. less BCS loss) was associated with 39% less risk of leaving the herd (Table 4.5; Figure 4.9B).

The only blood biomarker associated with mastitis, culling, or pregnancy was BHB. Cow-side ketones measured between 3 and 7 DIM were associated with culling and pregnancy ($P < 0.01$; Table 4.5). A 1 mM increase in the cow-side ketone test was associated with a 56% increase in risk of leaving the herd and a 44% reduced risk of pregnancy (Figure 4.10A, B). Additionally, plasma BHB at 3 DIM was associated with increased risk of mastitis ($P = 0.02$) such that a 1 mM increase in BHB was associated with a 4.63-fold increase in mastitis risk (Figure 4.10C).

Peak milk yield was associated with prepartum BCS, postpartum BCS, and ΔBCS ($P < 0.01$; Table 4.6). Peak milk yield was also associated with cow-side ketones ($P < 0.01$), plasma glucose at 10 DIM ($P < 0.01$), and free fatty acids at 10 DIM ($P = 0.04$).

The ΔBCS was correlated with pre (r = -0.54) and post-partum BCS (r = 0.37). Prepartum BCS and postpartum BCS were correlated with each other (r = 0.59). Also, insulin at 10 DIM was correlated with glucose (r = 0.30) and free fatty acids (r = -0.27) at 10 DIM. Glucose at 3 DIM was correlated with BHB (r = -0.48) and free fatty acids (r = -0.31) at 3 DIM. Insulin at 3 DIM was correlated with BHB (r = -0.34) and free fatty acids (r = -0.29) at 3 DIM as well as the cow-side ketone test (r = -0.39). Glucose at 10 DIM was correlated with BHB at both 3 (-0.50) and 10 DIM (-0.46). The Hp at 10 DIM was correlated with postpartum BCS (r = 0.27). The

cow-side ketone test was correlated with free fatty acids at 3 and 10 DIM (r = 0.56; r = 0.27). The BHB at 3 DIM was correlated with BHB at 10 DIM (r = 0.76), cow-side ketones (r = 0.45), and free fatty acids at 3 DIM (r = 0.59). The BHB at 10 DIM was correlated with cow-side ketones (r = 0.34) and free fatty acids at 3 DIM (r = 0.50).

DISCUSSION

Previous RPN investigations have included supplementation during both the pre- and post-partum periods (Morey et al., 2011; Yuan et al., 2012; Zeitz et al., 2018). These experiments were also much smaller and didn't allow for the investigation of RPN-mediated effects on health, disease, or fertility. Additionally, we followed cows well past the postpartum period which has not been done with supplementing RPN. Supplementing such a large cohort of cows only postpartum and following them through their whole lactation makes our experiment unique. We designed our experiment with the main objectives of investigating whether RPN reduced culling, increased pregnancy risk, and reduced mastitis risk.

One of the challenges of our experiment was ensuring there was minimal contamination of treatments through the AMS. Cows were assigned to receive RPN in two 13 g doses for the first 21 DIM through an automatic feed dispenser independent of the regular robot pellet within the AMS. Contamination of CON with RPN was a risk. We examined the niacin status of a subset of cows at 10 DIM to evaluate if the RPN cows had an altered niacin status compared to CON. Cows assigned to RPN had 50% greater nicotinamide than the CON which provides evidence that cows assigned to RPN had an altered niacin status compared to CON cows. It was not unexpected for nicotinic acid to remain unchanged as it is quickly metabolized (Henderson, 1983; Morey et al., 2011). Based on this data, we're confident that using the AMS to distribute

the treatments to cows on dairy farms is an adequate model for controlled research with dairy cows.

Cows who received RPN had reduced plasm insulin concentrations across all parities. This result aligns with RNA-seq analysis from Alfaro et al. (2023); they observed reduced enrichment of insulin signaling pathways in the liver as a result of 30 days of RPN supplementation in weaned beef cattle. This may be due to reduced insulin secretion. It should be noted that long-term niacin supplementation has reduced insulin sensitivity (Heemskerk et al., 2014; Montastier et al., 2019) in humans and mice but in certain scenarios niacin improved glucose metabolism and insulin sensitivity through modulation of the intestinal microbiome (Fangmann et al., 2018). A more thorough understanding of the effects that RPN may have on insulin signaling, gluconeogenesis, or glucose metabolism of dairy cattle would be useful. Understanding these alterations during the postpartum period are particularly important as cows undergo a period hypoglycemia after calving.

We suspect that the increased free fatty acids and BHB, which went against our hypothesis and previous data, may have been a consequence of the reduction in insulin that resulted from RPN supplementation. Also, we observed increased nicotinamide which does not activate the HCA2 receptor (Gille et al., 2008) and no change in nicotinic acid, which does activate the HCA2 receptor. This receptor mediates niacin's antilipolytic effects (Tunaru et al., 2003). Also, BHB, another HCA2 ligand (Gille et al., 2008), may have risen prior to RPN supplementation enough to have saturated the HCA2 receptor-mediated pathway, rendering the niacin's antilipolytic actions ineffective. For those reasons, the effects we observed within this experiment may be non HCA2-mediated effects of niacin.

We postulate that we may have missed the time period where supplementing RPN would be most effective at blunting lipolysis. In experiments where RPN reduced free fatty acids and BHB, it was supplemented prior to calving as well as after calving (Morey et al., 2011; Yuan et al., 2012). Since lipolysis accelerates rapidly with the onset of colostrum production and milk production, reducing lipolysis through RPN supplementation likely requires supplementation to begin prepartum. Another point of consideration with prepartum RPN supplementation is that supplementing RPN prior to calving reduced milkfat production in the immediate postpartum period (Yuan et al., 2012) another observation which we did not replicate. These changes in milk and component yields must be considered when investigating the role niacin plays in dairy cow metabolism.

Although there is little evidence to indicate supplemental RPN improves milk yield, no RPN studies have followed cows beyond the transition period. For example, Yuan et al. (2012) supplemented RPN both pre- and postpartum and observed a reduction of 9 kg/d of ECM in the first wk of lactation but by wk 3, milk production was similar. Unfortunately, cows in that study were not followed past the fresh period to determine if milk yield increased later in lactation when RPN was supplemented. All data investigating milk yield throughout lactation came from experiments using rumen-available niacin (Jaster et al., 1983; Skaar et al., 1989) which is almost completely degraded in the rumen (Santschi et al., 2005; Niehoff et al., 2013). Our data indicates that postpartum supplementation with RPN tended to increase milk yield in the immediate postpartum period but also resulted in greater peak milk yield and a more persistent milk yield after peak lactation in 1st and 2nd parity cows. In our study, RPN resulted in 639 kg ± 319.8 kg and 712 kg ± 364.4 more milk from 1st and 2nd parity cows, respectively. One limitation of our study is that we do not have milk composition data throughout lactation. As an alternative, the

AMS milk composition indicators, which estimate milk composition with near-infrared sensors, suggested milk fat % was reduced in primiparous cows and similar in multiparous cows (Supplementary Table 4.1). The data demonstrated that RPN increased fat and energy corrected milk yield in 1st and 2nd parity cows (Supplementary Figure 4.1 & 4.2). We are unsure why this increased milk yield occurred later in lactation. We suspect liver health or metabolic efficiency may have improved. Alfaro et al. (2023) observed metabolic and inflammatory alterations in the liver in response to RPN which may explain some of the increased milk yield we observed herein. Specifically, inflammatory immune cell signaling pathways were downregulated, which may have increased resources available for milk synthesis or resulted in improved liver function. Also, it may be that additional RPN increased NAD+ and NADP+ which may have improved metabolic efficiency on the cellular level. Supplemental niacin does increase NAD+ in the liver, but this form of stored NAD+ may not be active in redox metabolism (Henderson, 1983).

An additional mechanism that may be related to the increased milk yield is a family of enzymes called sirtuins. These proteins are metabolic sensors, largely under the influence of NAD+, of which niacin is a precursor (Romani et al., 2019; Rasti et al., 2020). Promotion of sirtuin-1 activity, specifically, may increase mitochondrial biogenesis and regulate glucose and lipid metabolism (Imai and Guarente, 2016; Romani et al., 2019). Our observation of reduced insulin along with increased nicotinamide, which is a precursor of NAD+, led us to hypothesize that RPN supplementation may have increased sirtuin-1 activity, resulting in increased mitochondrial biogenesis and improved glucose and lipid metabolism throughout lactation, which may have resulted in increased milk yield and a more persistent lactation. There is not currently research investigating the role of sirtuins in dairy cow milk production or metabolism, but it may be a rich area of future inquiry.

Deeper investigation into the metabolic alterations triggered by postpartum RPN supplementation would clarify why milk yield was increased in this experiment. Regardless of the mechanism, our study demonstrates the value of following cows throughout their whole lactation. The effect of RPN on milk yield was not readily apparent until at least the 9th week of lactation, an effect that would have remained unrealized had we not continued to follow the cows through their lactation.

Our main objectives were to investigate whether RPN altered culling, pregnancy, or mastitis. We postulated that a positive effect of RPN would be downstream of reductions in lipolysis as measured by free fatty acids and BHB, effects we did not observe. Given the apparent lack of anti-lipolytic effects, it was not unexpected that supplementing RPN did not alter mastitis, pregnancy, or culling. Repeating a similar experiment which includes RPN supplementation to prepartum cows may yield different results. We suspect that supplementing RPN during only the postpartum period missed the window of supplementation that would be necessary to harness niacin's potential antilipolytic-mediated health effects.

CONCLUSIONS

Supplementation of RPN during the postpartum period did not reduce biomarkers of lipolysis or inflammation by 3 or 10 DIM. Both BHB and free fatty acids were increased as a result of RPN. Supplementing during the postpartum period only is likely too late to stem lipolysis, which begins during the prepartum period. The RPN treatment did reduce plasma insulin across all parities which indicates that some metabolic effects are present and should be investigated further. RPN increased milk yield, especially later in lactation in 1st and 2nd parity cows. Supplemental RPN did not alter mastitis incidence, fertility, or risk of leaving the herd as we hypothesized. The lack of effect may be related to our supplementation timing. Since RPN

did not reduce biomarkers of lipolysis, we did not expect any follow-on effects on health and fertility. Additional research should investigate how RPN supplementation, and its timing, may influence metabolism, especially glucose metabolism, to better understand the cause for increased milk yield later in lactation.

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APPENDIX

Table 4.1. Nutrient composition of total mixed ration, partial mixed rations, and pellets fed to dairy cows supplemented with rumen-protected niacin for the first 21 days of lactation (n = 10).

Item, % DM unless noted otherwise	Pre-partum total mixed ration		Robot pellet[1]		1st parity partial mixed ration		Mature cow partial mixed ration	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
DM	46.3	4.03	86.2	0.65	44.8	4.14	46.1	3.41
CP	14.7	1.45	20.9	0.53	15.3	0.63	15.9	0.98
NDF	37.4	2.30	27.6	0.98	30.6	1.56	31.3	2.15
Starch	21.2	2.36	32.9	2.54	27.5	1.81	27.1	2.30
Ash	8.41	1.318	7.8	0.68	7.85	0.996	8.63	0.748
Ca	0.55	0.174	1.04	0.058	0.83	0.046	0.88	0.100
P	0.32	0.030	0.76	0.020	0.31	0.020	0.35	0.032
Mg	0.41	0.147	0.34	0.011	0.34	0.015	0.37	0.033
K	1.52	0.140	1.25	0.028	1.51	0.085	1.53	0.113
Na	0.36	0.174	0.06	0.043	0.64	0.064	0.68	0.085
Fe, mg/kg	252.5	50.74	133.3	12.70	220.2	36.95	220.6	45.76
Mn, mg/kg	85.7	43.38	84.3	5.23	82.1	9.11	84.8	10.65
Zn, mg/kg	105.2	61.48	66.9	3.21	95.3	6.19	108.3	17.33
Cu, mg/kg	23.0	11.04	15.6	2.46	17.3	1.23	18.7	2.00

[1]Partial mixed rations were formulated with 3.8 kg/d of pellets for primiparous cows and 4.2 kg/d of pellets for multiparous cows

Table 4.2. Effects of rumen-protected niacin (RPN) supplementation vs. no-treated control (CON) during the first 21 days of lactation on body condition score (BCS) and body condition score change (ΔBCS) of dairy cows.

Item	N[4]	1st Lactation		2nd Lactation		3+ Lactation		SEM	*P*-value		
		CON	RPN	CON	RPN	CON	RPN		Trt	Parity	Trt × Parity
Pre-partum BCS[1]	964	3.28	3.28	3.43	3.37	3.55	3.51	0.040	0.32	<0.01	0.62
Post-partum BCS	961	2.79	2.86	2.88	2.92	2.97	2.95	0.031	0.35	<0.01	0.24
ΔBCS[2]	961	-0.49	-0.45	-0.54	-0.50	-0.57	-0.59	0.033	0.27	<0.01	0.48
Ketones, m*M*[3]	566			0.73	0.68	0.78	0.80	0.035	0.20	<0.01	0.25

[1] BCS was measured on a 1-5 scale (Wildman et al., 1982). Prepartum BCS was observed within 14 days of the projected calving date while postpartum BCS was measured between 14 and 21 days in milk.

[2] ΔBCS = Postpartum BCS – Prepartum BCS

[3]Cow-side whole blood ketone test (Part No. 45824, Nova Max Plus, Waltham, MA) conducted by on farm personnel on multiparous cows between 3 and 7 DIM.

[4]N = total number of individual cows observed for each variable.

Table 4.3. Effects of rumen-protected niacin (RPN) supplementation vs. no-treated control (CON) during the first 21 days of lactation on milk yield, milk composition, and milk component yields of dairy cows during the 1st 21 days of lactation.

Item[1]	N[2]	1st Lactation		2nd Lactation		3+ Lactation		SEM	*P*-value						
		CON	RPN	CON	RPN	CON	RPN		Trt	Parity	Wk	Trt × Parity	Trt × Wk	Parity× Wk	Trt × Wk × Parity
Milk yield, kg/d	1056	24.8	26.1	35.0	35.6	35.5	36.0	1.63	0.10	<0.01	<0.01	0.74	0.56	<0.01	0.58
Fat, %	482	4.87	4.62	4.42	4.52	4.49	4.68	0.189	0.92	0.21	<0.01	0.21	0.84	0.22	0.28
Fat, kg/d	483	1.33	1.32	1.23	1.26	1.25	1.30	0.125	0.63	0.77	0.64	0.84	0.95	0.54	0.51
Protein, %	483	3.17	3.24	3.26	3.35	3.30	3.27	0.110	0.23	0.64	<0.01	0.21	0.58	0.89	0.49
Protein, kg/d	481	0.94	0.99	0.98	0.97	0.93	0.97	0.081	0.42	0.67	<0.01	0.64	0.13	0.47	0.33
Lactose, %	480	4.82	4.87	4.78	4.78	4.74	4.72	0.058	0.64	0.03	<0.01	0.23	0.82	<0.01	0.24
Lactose, kg/d	481	1.48	1.54	1.41	1.32	1.30	1.34	0.131	0.91	0.41	<0.01	0.38	0.13	0.45	0.22
MUN, mg/dL	482	14.71	13.99	12.28	12.2	12.54	12.36	1.133	0.41	0.18	<0.01	0.71	0.93	0.15	0.40
SCS	482	2.56	2.32	2.59	2.86	3.19	3.29	0.688	0.82	0.09	<0.01	0.64	0.31	0.43	0.22

[1] MUN = milk urea nitrogen. Somatic Cell Score (SCS) = log base 2 (SCC / 100,000) + 3

[2]N = total number of individual cows observed for each variable.

Table 4.4. Effects of rumen-protected niacin (RPN) supplementation vs. non-treated control (CON) during the first 21 days of lactation on clinical mastitis incidence rate during lactation.

Item[1]	1st Lactation		2nd Lactation		3+ Lactation		SEM	*P*-value		
	CON	RPN	CON	RPN	CON	RPN		Trt	Parity	Trt × Parity
True mastitis incidence rate[2,3]	1.02	0.61	1.79	0.67	2.31	2.39	0.632	0.25	<0.01	0.52
First-case incidence rate (%)	24/201 (12%)	15/163 (9%)	25/142 (18%)	17/110 (16%)	63/250 (25%)	55/208 (26%)	-	0.48	<0.01	0.65

[1]Incidence rates calculated according to Felton et al. (1998)

[2]Clinical mastitis cases/100 cow-days

[3]1st parity CON n = 201, 1st parity RPN n = 163, 2nd parity CON n = 142, 2nd parity RPN n = 110, 3rd+ CON n = 250, 3rd+ RPN n = 208

Table 4.5. Effects of rumen-protected niacin (RPN) supplementation vs. non-treated control (CON) during the first 21 days of lactation on first and second service conception rate of lactating dairy cows.

Item	1st Lactation		2nd Lactation		≥ 3rd Lactation		*P*-value		
	CON	RPN	CON	RPN	CON	RPN	Trt	Parity	Trt × Parity
1st service P/AI[1]									
d 28-32	95/165 (58%)	74/136 (54%)	66/121 (55%)	62/100 (62%)	102/197 (52%)	77/172 (45%)	0.81	0.03	0.23
d 68-72	88/164 (54%)	70/135 (52%)	64/121 (53%)	60/100 (60%)	91/194 (47%)	70/170 (41%)	0.97	<0.01	0.32
PG loss[2]	6/88 (7%)	3/70 (4%)	3/66 (4%)	3/60 (5%)	7/100 (7%)	5/75 (9%)	0.74	0.38	0.88
2nd service P/AI									
d 28-32	34/75 (45%)	27/62 (44%)	24/56 (43%)	22/40 (55%)	36/98 (36%)	33/94 (35%)	0.58	0.08	0.49
d 68-72	31/75 (41%)	23/62 (37%)	23/56 (41%)	19/39 (49%)	36/98 (36%)	31/94 (33%)	0.95	0.26	0.60
PG loss	3/34 (9%)	4/27 (15%)	1/24 (4%)	2/21 (9%)	0/36 (0%)	2/33 (6%)	0.98	0.68	0.98

[1]P/AI = Pregnancy/Artificial insemination at first (28-32 d postbreeding) and second (68-72 d) pregnancy check following 1st or 2nd service.

[2]PG loss defined as cows that were rebred after their 28-32 d pregnancy confirmation or declared open at the 68-72 d pregnancy check after an initial pregnancy confirmation at 28-32 d.

Table 4.6. Univariate associations of plasma metabolites, cow-side ketones measured on farm, body condition score (BCS) and change in BCS (ΔBCS) with culling, reproduction, and disease.

Item[1]	OR[2]	95% CI	*P*-value
Pre-partum BCS			
Culling	0.67	0.43-1.04	0.08
Pregnancy	1.78	1.16-2.75	<0.01
Mastitis	1.51	0.96-2.38	0.07
Post-partum BCS			
Culling[3]	2.02	0.84-4.63	0.10
Pregnancy	2.47	1.57-3.94	<0.01
Mastitis	1.20	0.75-1.90	0.45
ΔBCS			
Culling	0.61	0.37-1.00	0.05
Pregnancy	1.39	0.86-2.26	0.18
Mastitis	0.65	0.38-1.11	0.11
Cow-side ketones, m*M*[4]			
Culling	1.56	1.17-2.09	<0.01
Pregnancy	0.56	0.41-0.75	<0.01
Mastitis	1.17	0.86-1.58	0.30
Plasma analytes at 3 DIM			
Glucose, mg/dL			
Culling	0.98	0.95-1.01	0.15
Pregnancy	0.99	0.97-1.02	0.49
Mastitis	0.98	0.95-1.01	0.17
BHB, m*M*			
Culling	2.29	0.69-7.50	0.15
Pregnancy	1.32	0.40-6.19	0.68
Mastitis	4.63	1.45-17.95	0.02
Free fatty acids, *M*			
Culling	2.51	0.47-12.85	0.26
Pregnancy	1.20	0.26-6.90	0.82
Mastitis	2.03	0.37-10.22	0.39
ln[Hp, ng/mL]			
Culling	1.04	0.87-1.23	0.70
Pregnancy	1.02	0.86-1.19	0.78
Mastitis	0.91	0.78-1.07	0.22
Plasma analytes at 10 DIM			
Glucose, mg/dL			
Culling	0.98	0.96-1.01	0.23
Pregnancy	0.99	0.96-1.01	0.25
Mastitis	0.98	0.95-1.01	0.22
BHB, m*M*			

Table 4.6. (cont'd)

Culling	1.80	0.65-8.35	0.36
Pregnancy	1.32	0.40-6.19	0.68
Mastitis	1.05	0.33-2.45	0.93
Free fatty acids, *M*			
Culling	0.73	0.10-3.63	0.22
Pregnancy	1.28	0.26-9.53	0.78
Mastitis	0.20	0.01-1.68	0.20
ln[Hp, ng/mL]			
Culling	1.03	0.92-1.17	0.62
Pregnancy	1.02	0.90-1.14	0.79
Mastitis	1.10	0.96-1.27	0.18

[1]BCS determined on a 1-5 scale according to Wildman et al. (1982). Prepartum BCS was measured within 14 days of projected calving date, postpartum BCS was measured between 14-21 d after calving, ΔBCS = postpartum BCS – prepartum BCS. BCS prepartum n = 964, BCS postpartum n = 961, ΔBCS n = 961 cow-side ketones n = 566, plasma 3 DIM n = 99, plasma 10 DIM n = 97.

[2]Odds ratio (OR) indicates odds an event occurs with a 1-unit change in each independent variable

[3]Indicates quadratic association estimates between dependent and independent variable displayed.

[4]Blood for cow-side ketones is from multiparous cows only.

Table 4.7. Univariate associations of plasma metabolites, cow-side ketones, body condition score (BCS) and change in BCS (ΔBCS) with peak weekly milk yield (kg/d).

Item[1]	Coefficient	SEM	Adjusted R^2	*P*-value
Pre-partum BCS	9.59	1.151	0.07	<0.01
Post-partum BCS	6.59	1.200	0.03	<0.01
ΔBCS	-5.68	1.352	0.02	<0.01
Cow-side BHB, m*M*	-4.23	0.866	0.04	<0.01
Plasma analytes at 3 DIM				
Glucose, mg/dL	-0.07	0.065	<0.01	0.31
BHB, m*M*	4.36	3.256	0.01	0.18
Free fatty acids, *M*	-0.01	4.328	-0.01	0.99
ln[Hp, ng/mL]	-0.005	0.4403	-0.01	0.99
Plasma analytes at 10 DIM				
Glucose, mg/dL	-0.21	0.069	0.06	<0.01
BHB, m*M*	2.85	2.567	<0.01	0.27
Free fatty acids, *M*	9.93	4.646	0.03	0.04
ln[Hp, ng/mL]	0.06	0.341	-0.01	0.85

[1]BCS determined on a 1-5 scale according to Wildman et al. (1982). Prepartum BCS was measured within 14 days of projected calving date, postpartum BCS was measured between 14-21 d after calving, ΔBCS = postpartum BCS – prepartum BCS. BCS prepartum n = 964, BCS postpartum n = 961, ΔBCS n = 961 cow-side ketones n = 566, plasma 3 DIM n = 99, plasma 10 DIM n = 97.

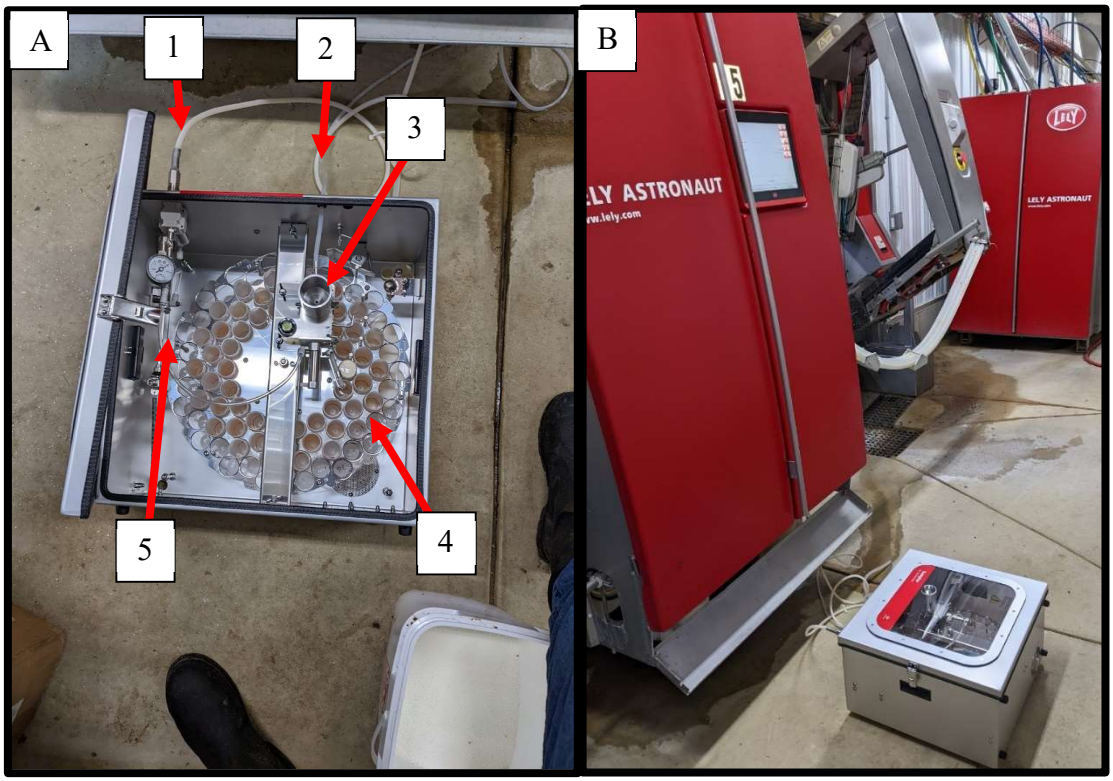

Figure 4.1. Photos displaying the milk sampler connected to the automated milking system (AMS). A) Diagram of parts of the milk samples. The air hose [1] connects the milk sampler to the AMS. The milk shuttle uses air pressure from the AMS to automatically rotate the turn table to collect an individual milk sample into an individual cup after each cow completes milking. Milk line [2] connected to the AMS which is gravity fed from the AMS to the milk sample collector. 3) Milk sample collection cup [3] which holds the milk before it is released into the sample container below. 4) The turntable [4] that holds individual milk sample tubes. Hydraulic system [5] that allows the milk sample cup to open and release the sample into the sample tube in the turntable. B) Photo of milk sampler attached to AMS.

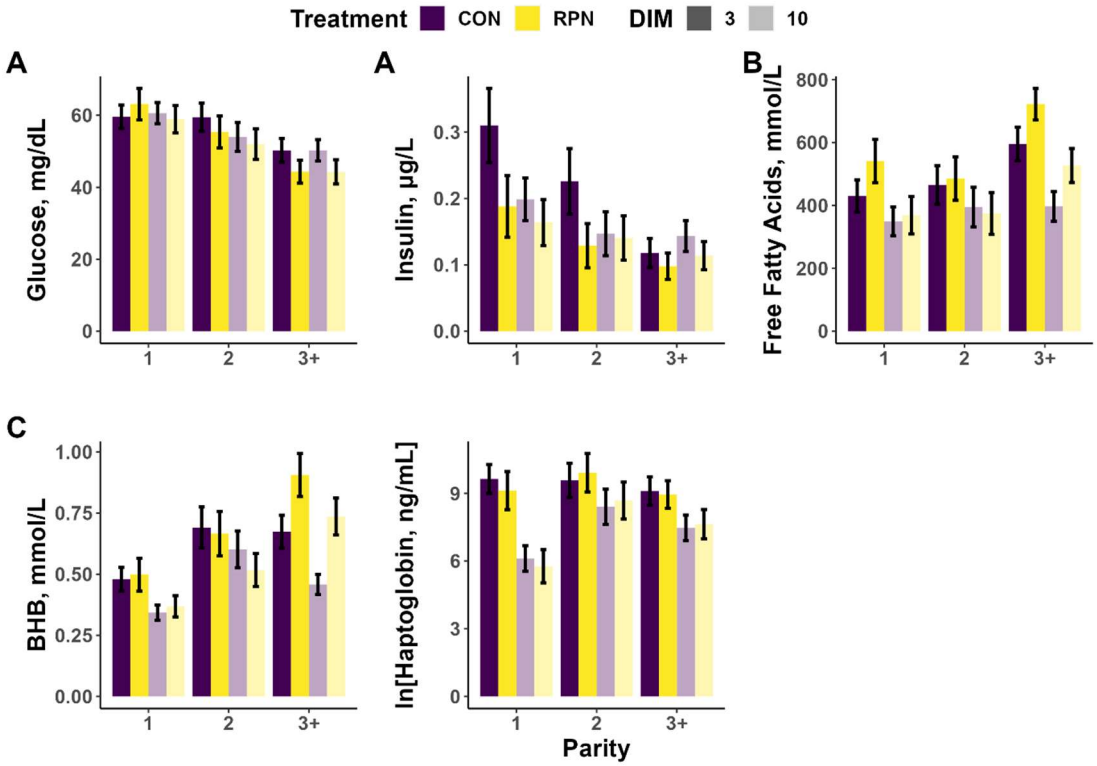

Figure 4.2. Effects of rumen-protected niacin (RPN) vs. no-treated control (CON) supplemented for 21 d post-partum on lactating dairy cow plasma biomarkers at 3 and 10 DIM. The n for each group at 3 DIM: CON 1st Parity = 21, RPN 1st parity = 11, CON 2nd Parity =14, RPN 2nd parity = 11, CON 3rd+ parity = 20, RPN 3rd+ parity = 22. The n for each group at 10 DIM: CON 1st Parity = 27, RPN 1st parity = 16, CON 2nd Parity =13, RPN 2nd parity = 12, CON 3rd+ parity = 29, RPN 3rd+ parity = 20. A) Glucose was not affected by treatment ($P = 0.20$). Glucose was reduced in parity 3+ cows compared to 1st and 2nd parity cows ($P < 0.01$). B) Insulin was reduced for cows fed RPN ($P = 0.01$) and reduced in 3+ parity compared to 1st parity cows ($P < 0.01$). C) Plasma free fatty acids were increased for RPN (P = 0.05). The free fatty acids were reduced by 10 DIM ($P < 0.01$). Parity 3+ had greater free fatty acids than 1st or 2nd parity ($P < 0.01$). D) β-hydroxybutyrate (BHB) was affected by an interaction of parity × treatment ($P < 0.01$); RPN increased BHB in 3rd+ parity cows ($P < 0.01$). BHB was reduced at 10 DIM compared to 3 DIM ($P < 0.01$). E) Plasma haptoglobin (Hp) was affected by the interaction of parity and DIM ($P = 0.03$) such that at 3 DIM all parity had similar Hp but by 10 DIM, 1st parity cows had reduced Hp compared to 2nd or 3+ cows ($P \leq 0.02$).

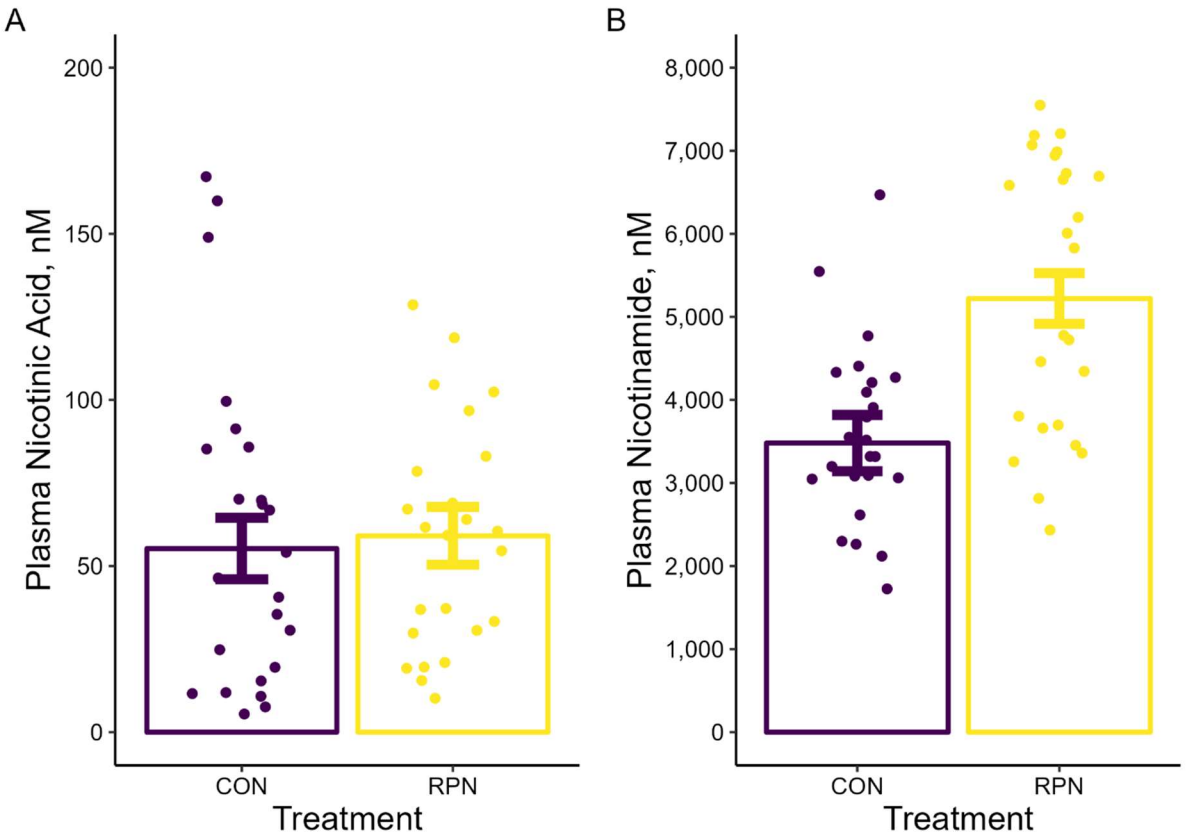

Figure 4.3. Effects of feeding rumen-protected niacin (RPN) vs. no-treated control (CON) on lactating dairy cow on plasma nicotinic acid and nicotinamide. The n for CON = 24 and n for RPN = 25. A) RPN did not affect plasma nicotinic acid concentration (P = 0.72; 55.3 ± 9.26 vs. 59.1 ± 8.68). B) RPN increased plasma nicotinamide concentration (P < 0.01; 3480.5 ± 338.99 vs. 5220.9 ± 305.44).

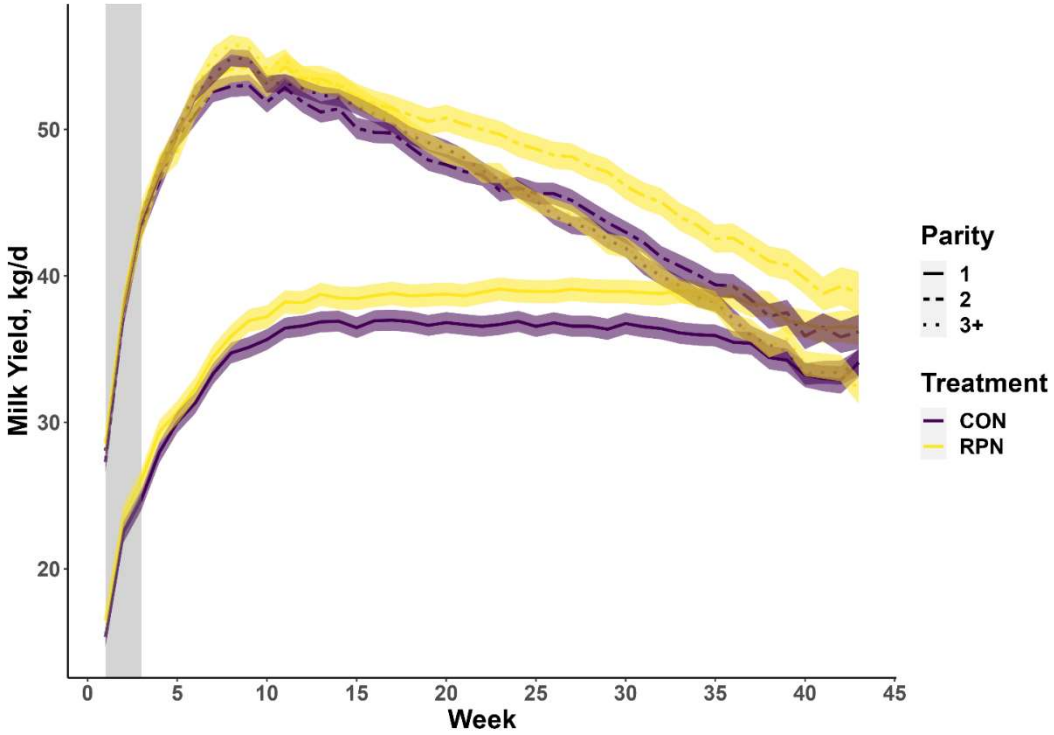

Figure 4.4. Weekly lactation milk yield from cows supplemented with rumen-protected niacin (RPN) vs. no-treated control (CON) for 21 d post-partum. The shaded area indicates the RPN supplementation period (0-21 DIM). The n for each group: CON 1st Parity = 197, RPN 1st parity = 162, CON 2nd Parity =140, RPN 2nd parity = 110, CON 3rd+ parity = 244, RPN 3rd+ parity = 205. Milk yield was affected by the Trt × Wk × Parity interaction ($P < 0.01$). 1st parity cows had similar milk yield for the first 8 wk of lactation ($P \geq 0.17$); during wk 9, 11 and 13 RPN tended to have greater milk yield than CON ($P \leq 0.08$). From week 15 through 42 RPN had significantly greater or tended to have greater milk yield ($P \leq 0.10$). 2nd parity cows had similar milk yield through 12 wk of lactation ($P \geq 0.16$). RPN had greater milk yield during wk 13 ($P = 0.05$), tended to have greater milk yield during wk 15 and 16 ($P \leq 0.08$), had greater milk yield from wk 18 through wk 40 ($P \leq 0.05$), and greater milk yield during wk 42 ($P = 0.04$). In 3+ parity cows, milk yield was similar except for wk 11, where RPN tended to have greater milk yield ($P = 0.06$).

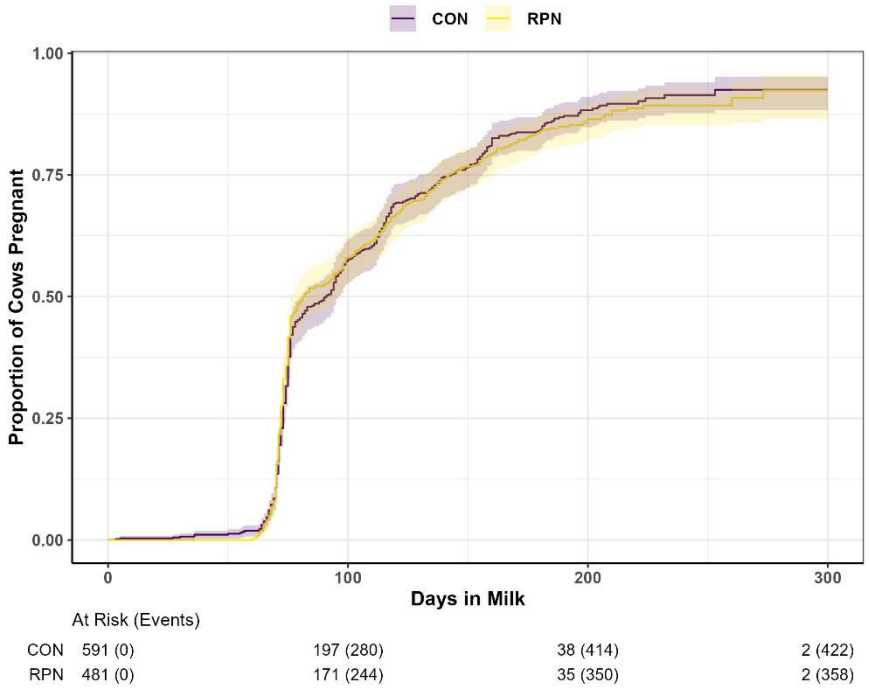

Figure 4.5. Effects of rumen-protected niacin (RPN) vs. no-treated control (CON) supplemented for 21 d post-partum on pregnancy risk. The RPN did not affect cumulative pregnancy risk ($P = 0.84$; 95% CI: 0.88 – 1.17). The table lists the cows at risk and the events that have occurred at 0 , 100, 200, and 300 DIM, respectively.

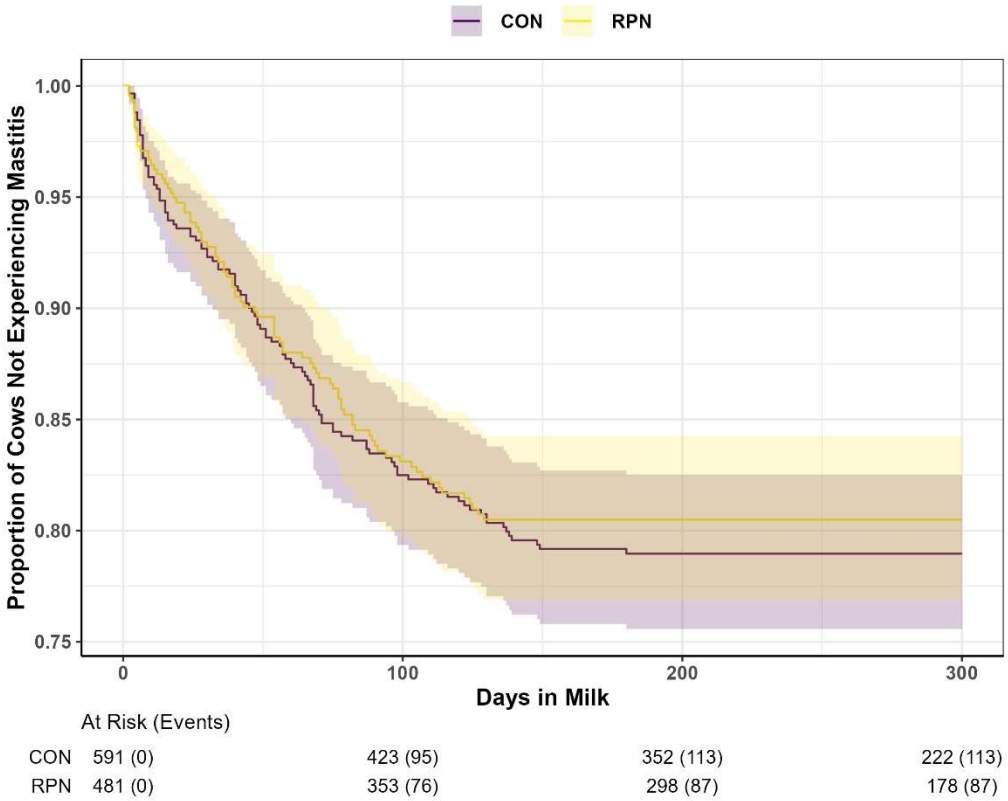

Figure 4.6. Effects of rumen-protected niacin (RPN) vs. no-treated control (CON) supplemented for 21 d post-partum on on first-case mastitis risk. The RPN did not affect first-case mastitis risk (P = 0.56; 95% CI: 0.69-1.22). The table lists the cows at risk and the events that have occurred at 0 , 100, 200, and 300 DIM, respectively.

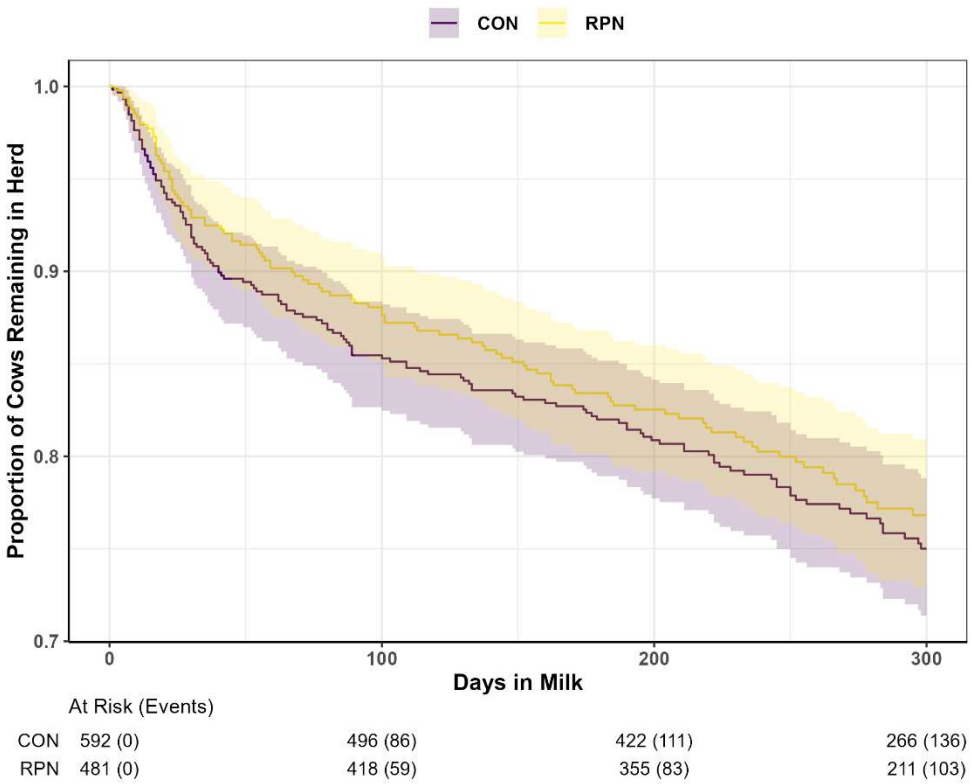

Figure 4.7. Effects of rumen-protected niacin (RPN) vs. no-treated control (CON) supplemented for 21 d post-partum on whole lactation survival. The RPN did not affect lactation survival ($P = 0.44$; 95% CI: 0.69 – 1.17). The table lists the cows at risk and the events that have occurred at 0 , 100, 200, and 300 DIM, respectively.

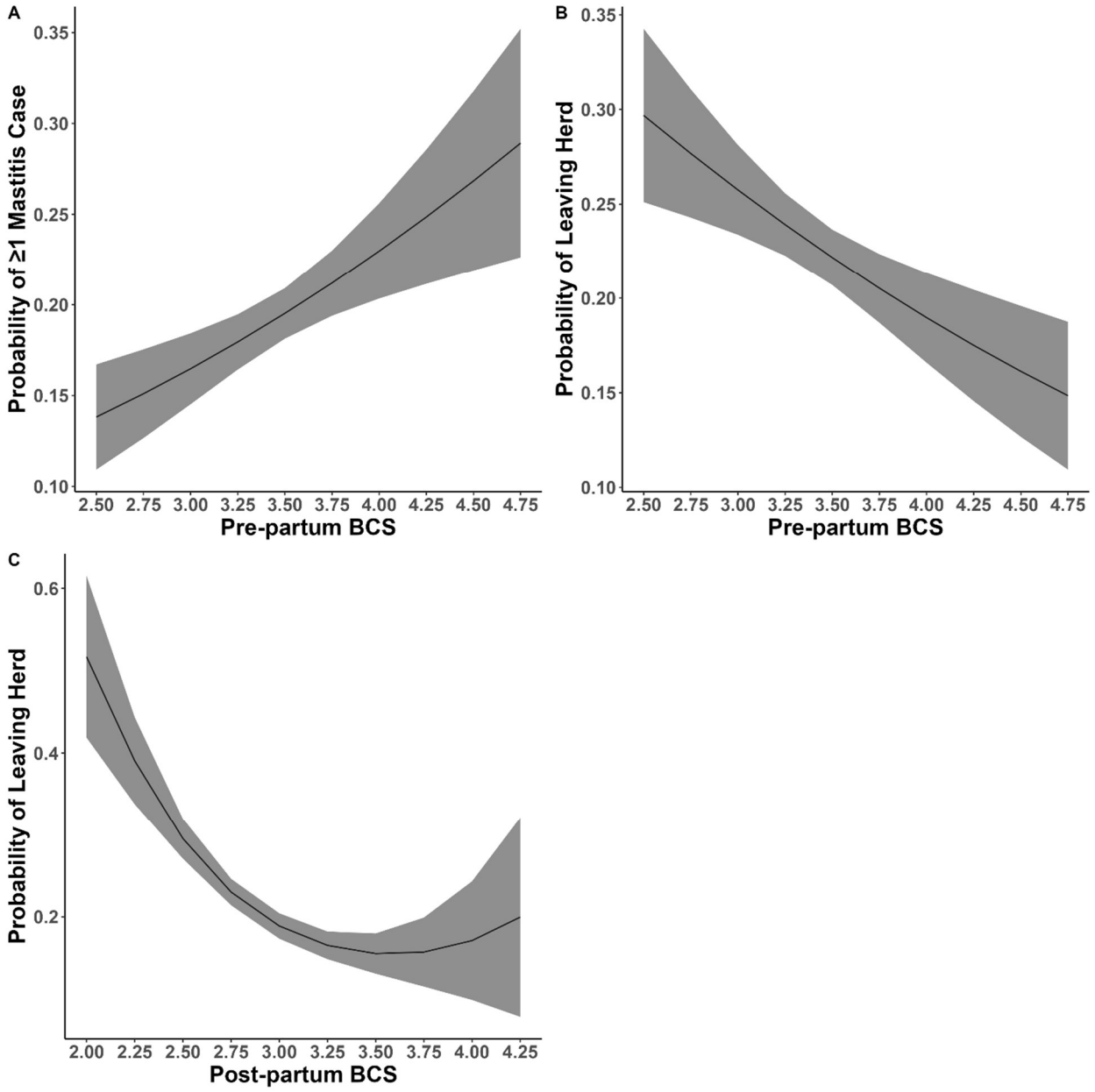

Figure 4.8. Associations of body condition score (BCS) with mastitis, risk of leaving the herd, and pregnancy. BCS prepartum n = 964 and BCS postpartum n = 961. Prepartum BCS was measured within 14 days of expected calving while postpartum BCS was measured 14-21 days after calving. A) Association of pre-partum BCS with mastitis ($P = 0.07$). B) Association of pre-partum BCS with risk of leaving the herd ($P = 0.08$). C) Association of post-partum BCS with probability of leaving the herd ($P = 0.05$).

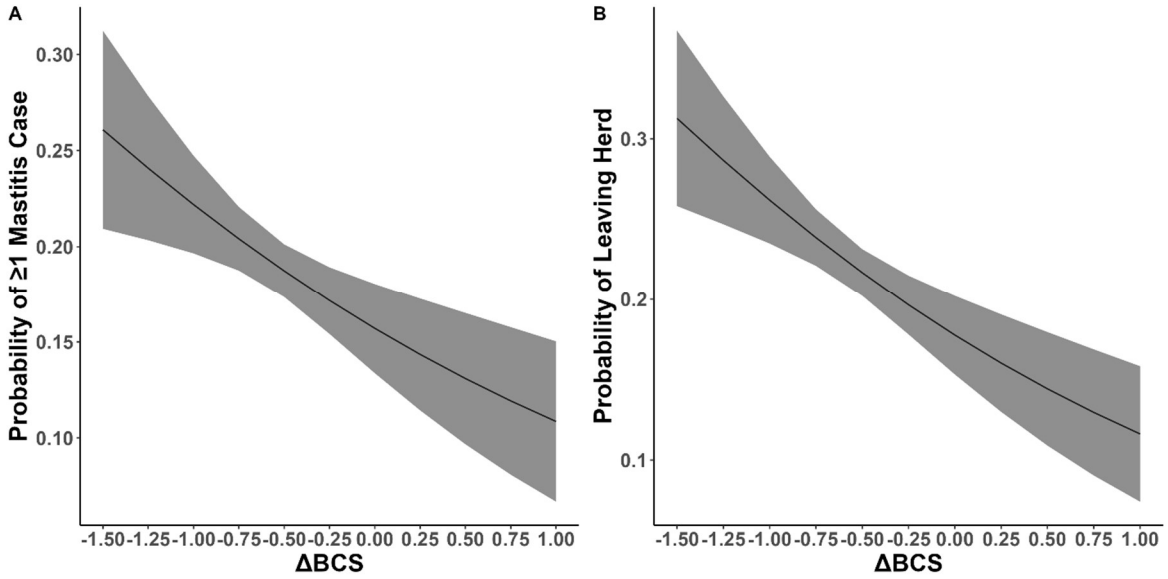

Figure 4.9. Associations of body condition score change (ΔBCS; n = 961) with risk of experiencing mastitis and leaving the herd. ΔBCS = Postpartum BCS – Prepartum BCS (prepartum BCS was measured within 14 days of expected calving and postpartum BCS was measured between 14 and 21 days after calving. A) ΔBCS is negatively associated with risk of mastitis (P = 0.11). B) ΔBCS is negatively associated with risk of leaving the herd (P = 0.05).

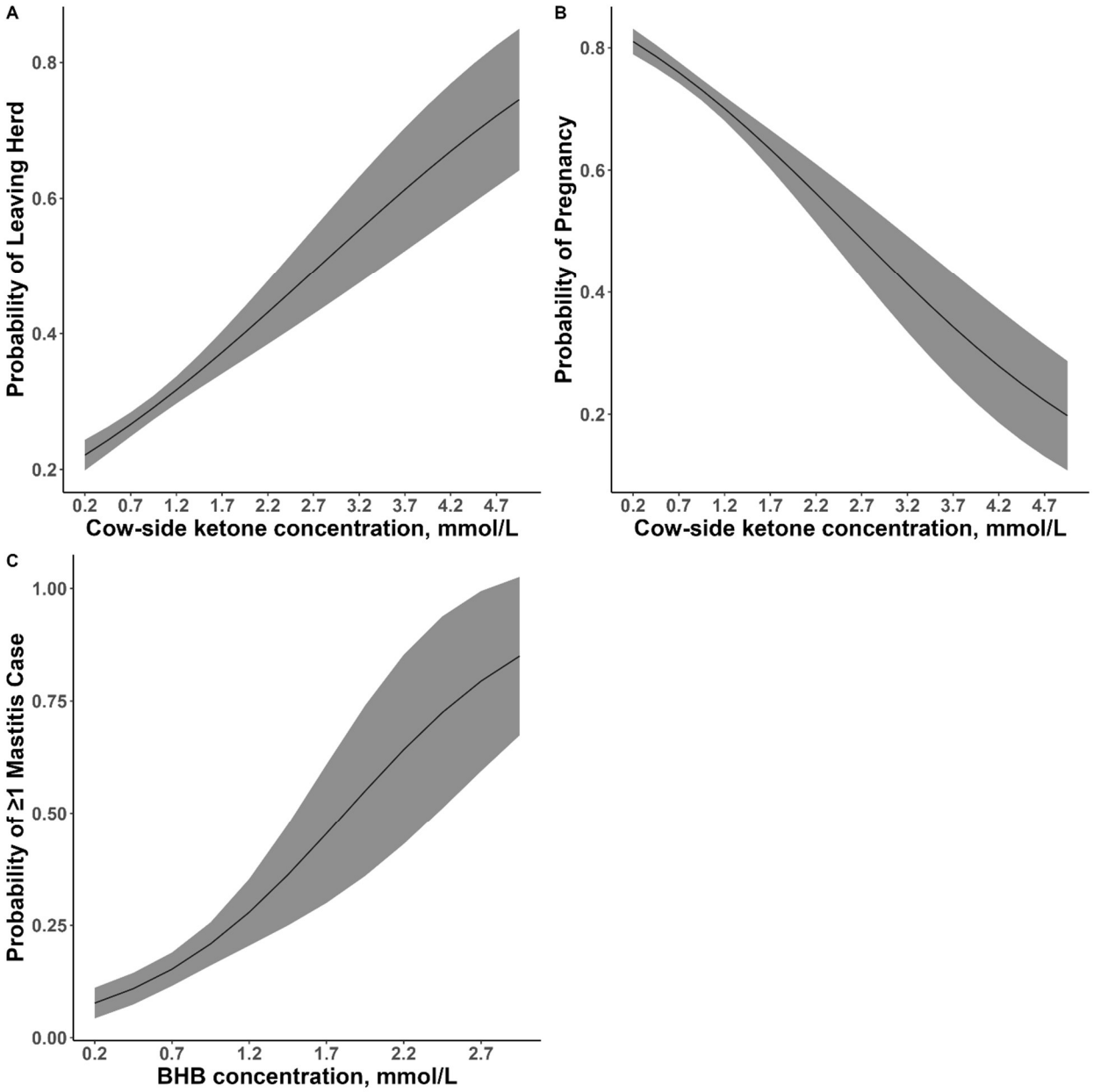

Figure 4.10. Associations of cow-side plasma ketones (n = 566) measured on farm or plasma β-hydroxybutyrate (BHB at 3 DIM; n = 99) with mastitis, risk of leaving the herd, and pregnancy. A) Association of cow-side ketones concentration with probability of leaving the herd ($P < 0.01$). B) Association of cow-side ketone probability of pregnancy ($P < 0.01$). C) Association of BHB at 3 days in milk with probability of mastitis ($P = 0.02$).

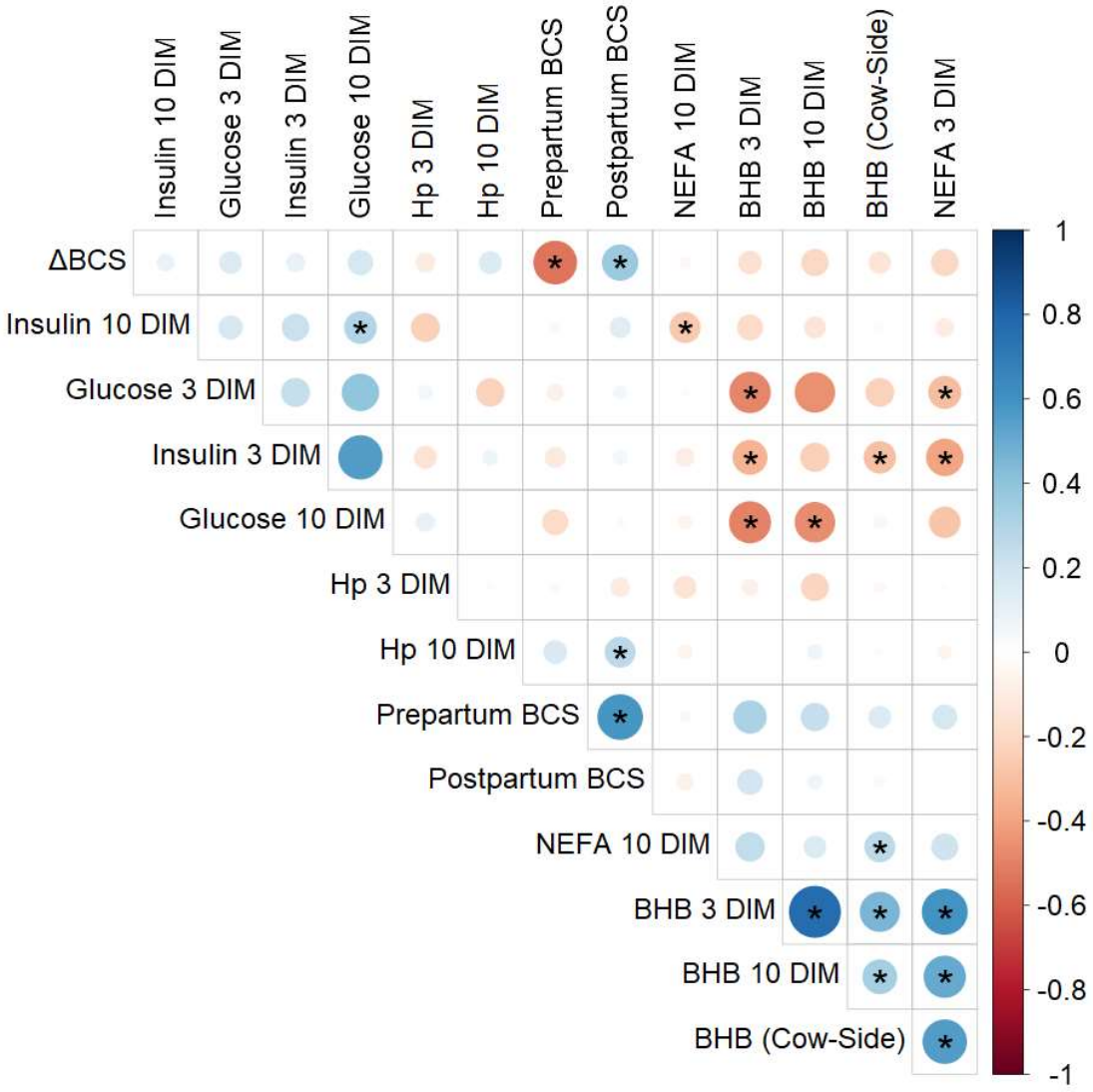

Figure 4.11. Correlation matrix of plasma biomarkers, prepartum body condition score, postpartum body condition score, and change in body condition score (ΔBCS; Postpartum BCS – Prepartum BCS). * indicates a significant correlation between variables ($P < 0.05$). BCS prepartum n = 964, BCS postpartum n = 961, ΔBCS n = 961 cow-side ketones n = 566, plasma at 3 DIM n = 99, plasma 10 DIM n = 97..

Table S4.1. Effect of rumen-protected niacin (RPN) supplementation vs. no-treated control (CON) during the first 3 wk of lactation on milk yield, milk composition, and milk component yields of dairy cows for their entire lactation.

Item[1]	1st Lactation		2nd Lactation		3+ Lactation		SEM	*P*-value						
	CON	RPN	CON	RPN	CON	RPN		Trt	Parity	Wk	Trt × Parity	Trt × Wk	Parity × Wk	Trt × Wk × Parity
Milk yield, kg/d	34.2	36.4	44.6	46.9	44.1	44.4	0.79	<0.01	<0.01	<0.01	0.18	<0.01	<0.01	<0.01
FCM, kg/d[3]	33.9	35.0	44.0	46.9	45.4	45.4	0.81	<0.01	<0.01	<0.01	0.09	<0.01	<0.01	<0.01
Fat, %	3.75	3.50	3.60	3.67	3.89	3.84	0.054	0.04	<0.01	<0.01	<0.01	0.84	<0.01	0.23
Fat, kg/d	1.17	1.19	1.52	1.63	1.62	1.61	0.032	0.05	<0.01	<0.01	0.07	0.70	<0.01	<0.01
Pellets, kg/d	5.3	5.4	5.6	5.7	5.7	5.7	0.076	0.03	<0.01	<0.01	0.80	<0.01	<0.01	0.16
PE, kg/kg[4]	6.4	6.7	8.3	8.5	8.0	8.0	0.081	0.03	<0.01	<0.01	0.19	0.28	<0.01	0.24

[1]All data were measured from Lely automated milking system.

[2]ECM (energy corrected milk yield) = 0.327 × milk yield + 12.95 × milk fat yield + 7.95 × milk protein yield

[3]3.5% FCM (fat corrected milk yield) = 0.4324 × milk yield + 16.216 × milk fat yield.

[4]PE (Pellet efficiency) = kg milk yield/kg pellet

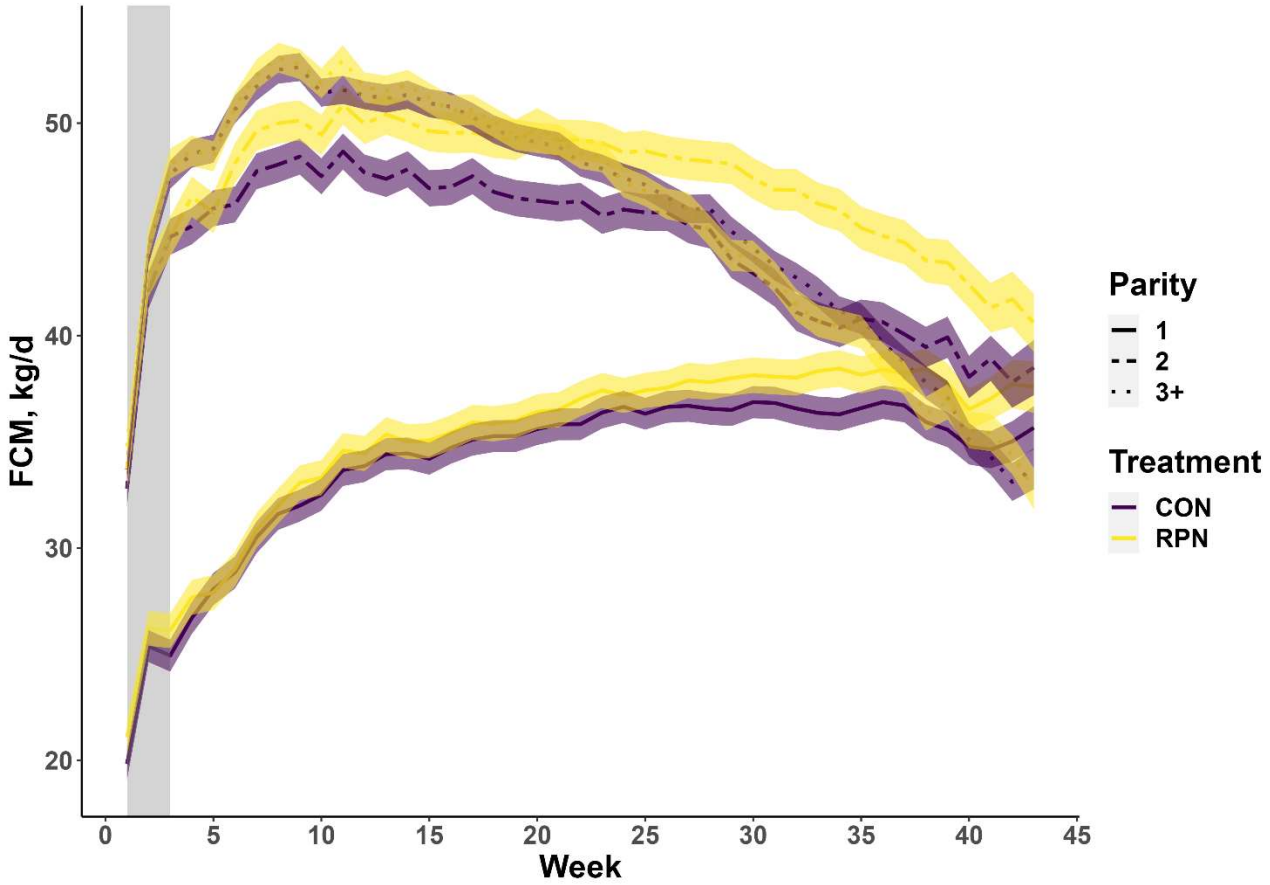

Figure S4.1. Weekly lactation fat corrected milk (FCM) from cows supplemented with rumen-protected niacin (RPN) during the first 21 days in milk. The shaded area indicates the RPN supplementation period (0-21 DIM). The n for each group: CON 1st Parity = 197, RPN 1st parity = 162, CON 2nd Parity =140, RPN 2nd parity = 110, CON 3rd+ parity = 244, RPN 3rd+ parity = 205. Fat-corrected milk yield was affected by the Trt × Wk × Parity interaction ($P < 0.01$). 1st parity cows had similar FCM for the first 32 wk of lactation ($P \geq 0.16$), during wk 33, 34 and 39 RPN tended to have greater FCM than CON ($P = 0.09$). During wk 38 RPN had greater ECM than CON ($P= 0.03$). From wk 11 until wk 42, RPN had or tended to have greater FCM than CON in 2nd parity cows ($P \leq 0.10$), except during wk 41 ($P = 0.13$). In 3+ parity cows, FCM was similar throughout lactation ($P \geq 0.16$).

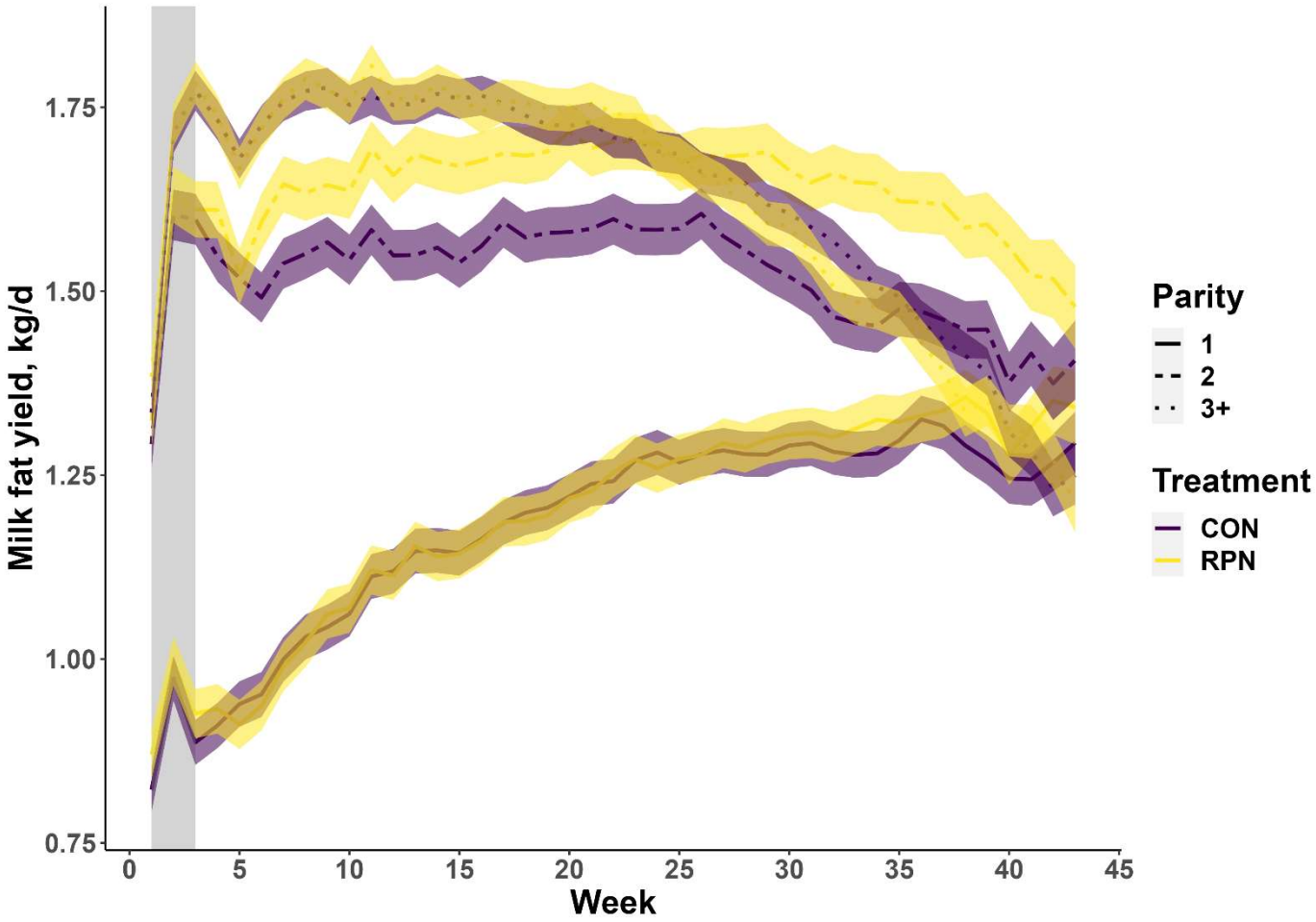

Figure S4.2. Weekly lactation milk fat yield from cows supplemented with rumen-protected niacin (RPN) during the first 21 days in milk. The shaded area indicates the RPN supplementation period (0-21 DIM). The n for each group: CON 1st Parity = 197, RPN 1st parity = 162, CON 2nd Parity =140, RPN 2nd parity = 110, CON 3rd+ parity = 244, RPN 3rd+ parity = 205. Milk fat yield was affected by the Trt × Wk × Parity interaction ($P < 0.01$). 1st parity cows had similar milk fat yield throughout lactation ($P \geq 0.15$). In 2nd parity cows, RPN had or tended to have greater milk fat yield from wk 6 until wk 42 ($P \leq 0.10$), except for wk 8, 9, and 26 ($P \geq 0.11$) In 3rd parity cows, CON tended to have greater milk fat yield than RPN during wk 38 of lactation ($P = 0.08$).

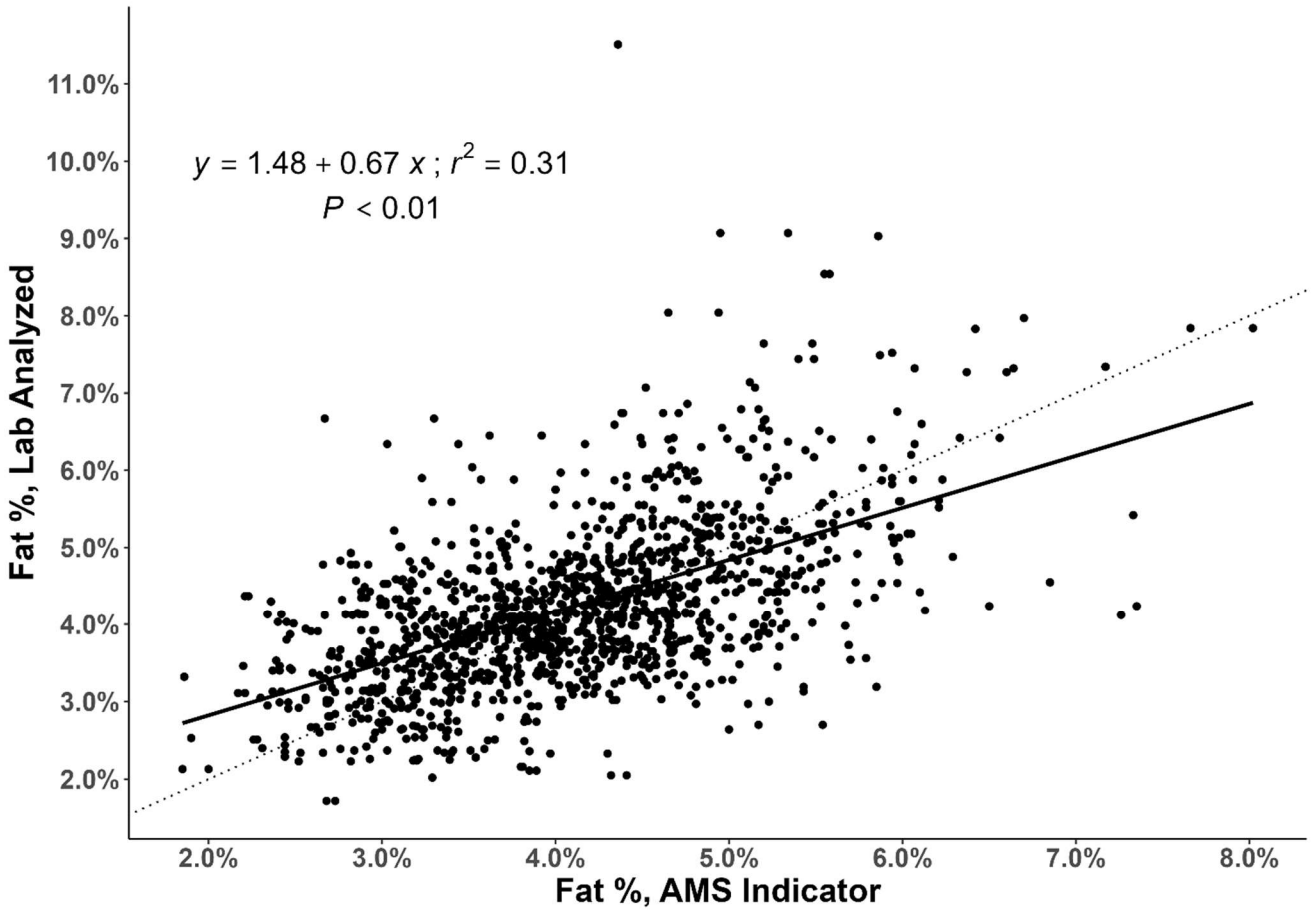

Figure S4.3. Regression analysis comparing the milk fat % measured from the automated milk system (AMS) indicator and from the lab analyzed milk fat % at Central Start Cooperative (Grand Ledge, MI).

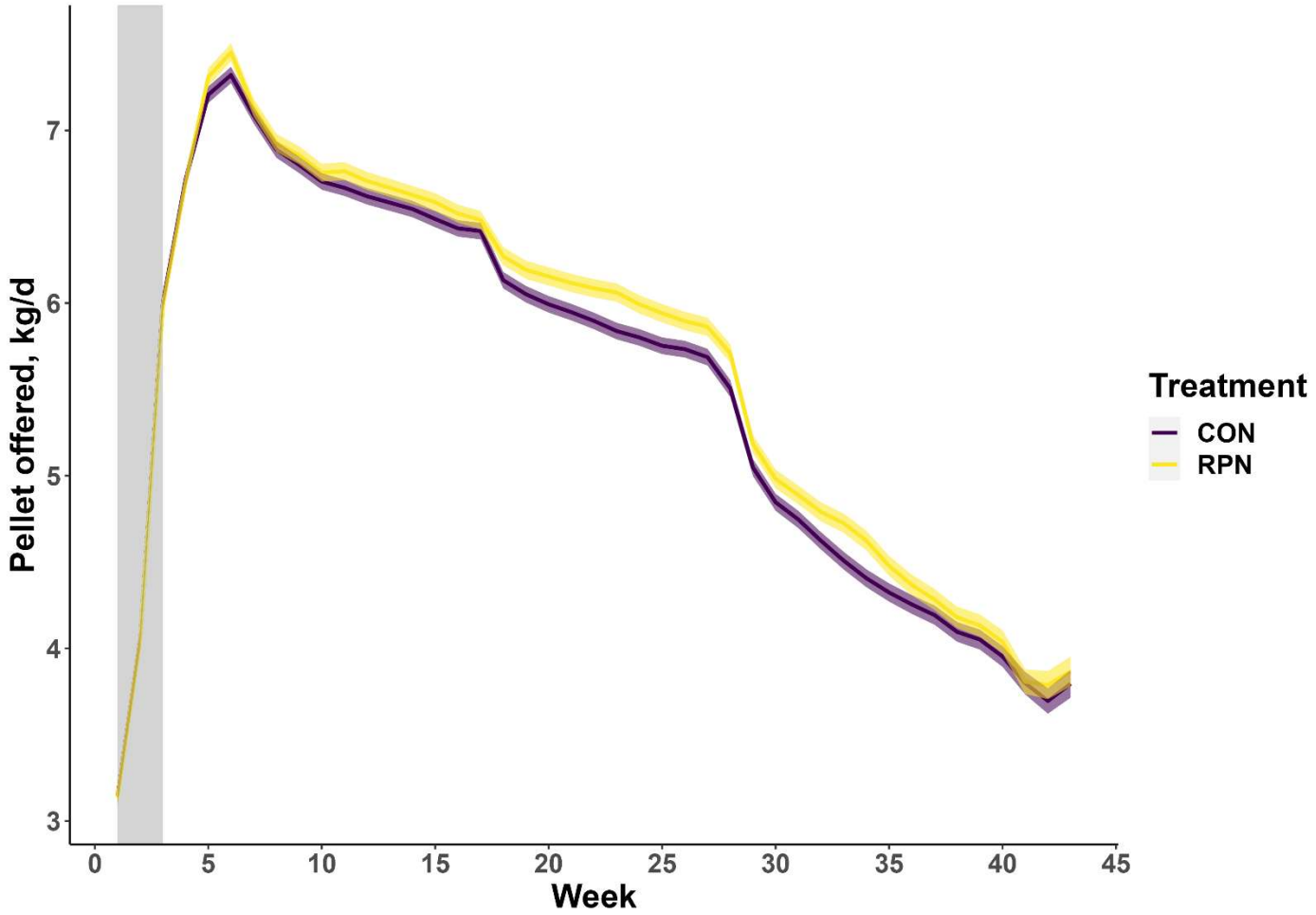

Figure S4.4. Weekly pellet offering for cows supplemented with rumen-protected niacin (RPN) during the first 21 days in milk. The shaded area indicates the RPN supplementation period (0-21 DIM). The n for each group: CON 1st Parity = 197, RPN 1st parity = 162, CON 2nd Parity =140, RPN 2nd parity = 110, CON 3rd+ parity = 244, RPN 3rd+ parity = 205. Pellet offering was affected by a Trt × Wk interaction ($P < 0.01$). The RPN tended to recieve more pellets during wk 6 ($P = 0.06$) and from wk 18 until 35 they were offered or tended to have been offered more pellet ($P \leq 0.06$).

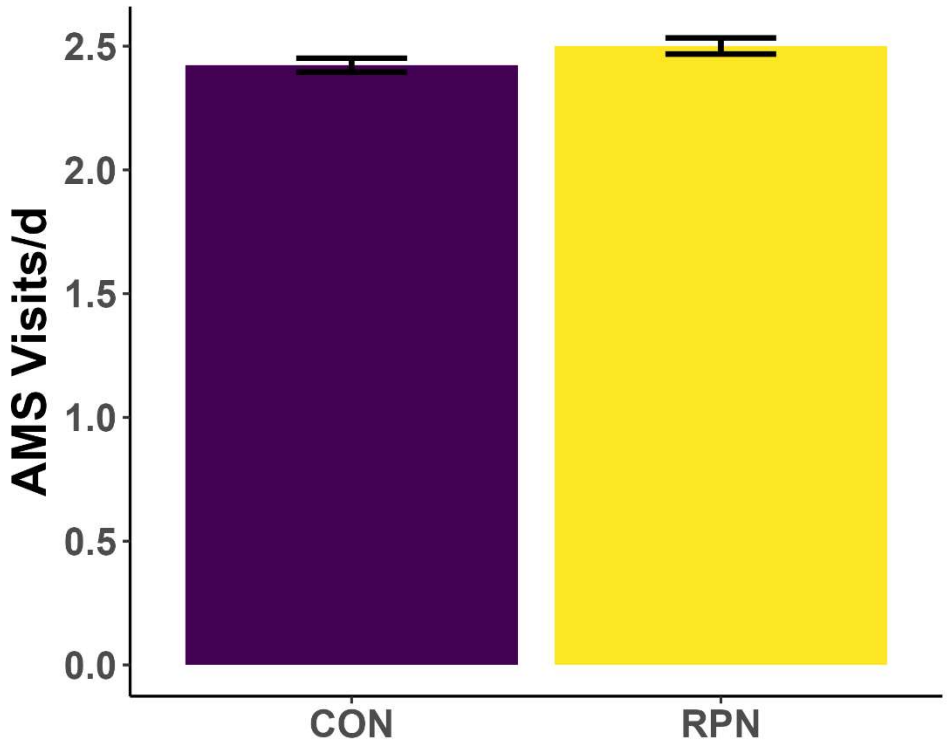

Figure S4.5. Daily automated robot visits for cows supplemented with rumen-protected niacin (RPN) during the first 21 days in milk. The n for each group: CON 1st Parity = 197, RPN 1st parity = 162, CON 2nd Parity =140, RPN 2nd parity = 110, CON 3rd+ parity = 244, RPN 3rd+ parity = 205. RPN tended to increase visits to the AMS (P = 0.07; 2.4 ± 0.03 vs 2.5 ± 0.03).

CHAPTER 5: EFFECTS OF β-CARYOPHYLLENE ON BOVINE MONOCYTE-DERIVED MACROPHAGE PROTEOME AND PHOSPHOPROTEOME IN RESPONSE TO REPEATED LIPOPOLYSACCHARIDE CHALLENGES *IN VITRO*

ABSTRACT

The endocannabinoid system (ECS) has an important role in modulating immune function. The ligands of the ECS exert both anti- and pro-inflammatory effects. However, the role of the ECS on immune modulation in cattle is currently unexplored. The cannabinoid-2 receptor (CB2) is expressed in immune cells, including monocytes, and its activation has mostly anti-inflammatory effects. Reducing inflammation in cattle, especially under stressful conditions, may improve the efficiency of milk production, animal welfare, and farm profitability. Our approach was to use a naturally occurring plant cannabinoid [β-caryophyllene (BCP)] that is a specific ligand of CB2 to modulate the inflammatory response in vitro. Our objective was to investigate the effects of BCP on the response of bovine monocyte-derived macrophages (BMDM) to two lipopolysaccharide (LPS) challenges, in order to investigate the effects of ECS modulation on immune response in dairy cows. We utilized high throughput proteomic and phosphoproteomics analyses and bioinformatics to gain molecular insight on the immune proteins that are affected by BCP. The BMDM from mid-lactation dairy cows were incubated with or without 1 μM of BCP for 7 d. Following two consecutive LPS-challenges, cell lysates were analyzed by proteomic and phospho-proteomic analyses to assess the effects of BCP vs. control (CTL). We quantified 650 proteins and 1,406 phosphosites, from which 11 proteins and 22 phosphopeptides were differential [$P \leq 0.05$ and fold change (FC) ± 1.5] or tended to be affected ($P \leq 0.10$) by treatment with BCP. Immune related proteins reduced by BCP were protein tyrosine phosphatase (FC = -2.7; $P = 0.01$) and latexin (FC = -3.9; $P = 0.05$). Calpain-1

catalytic subunit (FC = 3.2; $P = 0.03$) and fatty acid binding protein (FC = 2.2; $P = 0.07$) were increased by BCP. In phosphoproteomics, a tendency for lower phosphorylation of the phosphopeptides in secreted phosphoprotein 1 (FC = -6.3; $P = 0.04$) and Serine/Threonine Kinase 10 (FC = -1.8; $P = 0.08$) was found in BCP compared to control (CTL). Top canonical pathways enriched in BCP vs. CTL were related to immune function and migration, such as IL-8 signaling, leukocyte extravasation signaling, integrin signaling, and LXR/RXR signaling. Immunoblots demonstrated the presence and abundance of CB2 in BMDM cells. Our results indicate that targeting the ECS by providing BCP to BMDM may affect the immune response to LPS and reduce immune cell migration and inflammatory responses. These results suggest that modulating the ECS system may be a therapeutic target for dairy cows experiencing chronic inflammation.

Key words: proteomics, phosphoproteomics, cannabinoids, inflammation, β-caryophyllene

INTRODUCTION

The endocannabinoid system (ECS) is an important regulator of inflammation and metabolism through its associated receptors and enzymes that synthesize or degrade endogenous cannabinoids (Klein et al., 2003; Myers et al., 2021; Busquets-García et al., 2022). The ECS has two main receptors, cannabinoid receptor 1 and 2 (CB1; CB2). The TRPV1 (Mazier et al., 2015) and GPR55 (Lauckner et al., 2008) receptors also interact with endocannabinoids. The CB1 receptor is mainly expressed throughout the central nervous system (Tsou et al., 1998) but is also expressed in other tissues like liver and adipose tissue (Roche et al., 2006; Teixeira-Clerc et al., 2006). CB1 is expressed on mitochondrial membranes as well (Busquets-García et al., 2022). Activation of the CB1 receptors have increased feed intake, increased lipogenesis, increased glucose uptake, and promote energy storage [reviewed by Mazier et al. (2015)]. The CB2

receptor is mainly expressed in immune cells (Montecucco et al., 2008; Turcotte et al., 2016) but has also been detected in other tissues like adipose, liver, and the colon (Wright et al., 2005; Gong et al., 2006; Deveaux et al., 2009). Activation of the CB2 receptor has been shown to modulate the immune response; generally, it seems to encourage anti-inflammatory effects (Lindsey et al., 2019; Wei et al., 2021; Zhang et al., 2021). Specifically targeting this receptor in dairy cattle may have promising effects on animal health and welfare by reducing excess inflammation during the peripartum period or during times of stress.

Investigating selective activation of the CB2 receptor is valuable because it has anti-inflammatory effects without the CB1-associated metabolic effects. In THP-1 cells (human monocytic cell line), activation of CB2 with anandamide (endogenous cannabinoid which binds to both CB1 and CB2) or JWH-015 (synthetic CB2 agonist) reduced TNF-α and IL-1β secretion by up to 35% (Klegeris et al., 2003). In addition to reducing cytokine secretion, CB2 activation reduces human monocyte migration in response to CCL2 and CCL3 (Montecucco et al., 2008) and encouraged a pro-resolving macrophage phenotype in macrophages collected from patients with celiacs disease. Additionally, activation of CB2 increased IL-10 secretion while reducing IL-6 and TNF-α; chemokine receptor 7 was also reduced while CD206 was increased in response to CB2 activation (Tortora et al., 2022). Furthermore, co-culturing macrophages with Caco-2 cells and treating them with a CB2 ligand reduced IL-6 concentrations which was ablated with a CB2 antagonist (Tortora et al., 2022). Investigation of CB2 activation on bovine immune cells has not been conducted yet and is important as species differences in the ECS may exist. Investigation with bovine immune cells may demonstrate whether targeting the ECS in cattle is a promising approach to improving animal health and welfare.

Characterization of the ECS in cattle is at an early stage of investigation. Both CB1 and CB2 receptors have been observed in adipose tissue of dairy cattle (Daddam et al., 2021; Kra et al., 2022a; Kra et al., 2022b). The CB1 receptor was also present in liver tissue of peripartum dairy cattle (Kra et al., 2022b). Additionally, the CB1 receptor has been observed in the hypothalamus of dairy cattle (Kuhla et al., 2020) indicating its potential in controlling feed intake. The presence of CB2 on primary bovine immune cells has not yet been reported in the literature.

Current research investigating the effects of activating ECS in dairy cattle through supplementation of cannabinoids is limited. The ECS may play a role during the dairy cows' transition from gestation to lactation as AEA and 2-arachaodonylglycerol (2AG), increase in adipose tissue during the postpartum period; the increase was greatest in cows experiencing greater bodyweight loss and inflammation (Zachut et al., 2018). Thus far, there has been no investigation of whether supplementation of cannabinoids or different ligands for the ECS receptors modulate dairy cow inflammatory responses even though it has been demonstrated in other species (Rajesh et al., 2007b; Varga et al., 2018). Zachut et al. (2022) suggest that this approach may have great potential for dairy cattle. Reducing excessive inflammation in dairy cattle is important because cows with greater concentrations of inflammatory biomarkers produce less milk, have poorer fertility, and have increased incidence of disease (Bertoni et al., 2008; Kerwin et al., 2022). Targeting the ECS to reduce inflammation may be an effective means of improving dairy cow health, production, and sustainability.

One potential way to selectively activate the CB2 receptor is through supplementation of β-caryophyllene (BCP). This compound is a sesquiterpene found in spices and plants like oregano, cinnamon, and black pepper (Gertsch et al., 2008). Researchers demonstrated that BCP

activates the CB2 receptor with similar potency to the endogenous cannabinoid, 2AG, while not activating the CB1 receptor (Gertsch et al., 2008). They also demonstrated that the addition of BCP reduced IL-1β and TNF-α which was reversed by a CB2 antagonist (Gertsch et al., 2008). Providing BCP also reduced neutrophil accumulation after a challenge with *Mycobacterium bovis* in mice; the reduced accumulation of neutrophils seems to result from reduced cell adhesion and migratory capacity (Andrade-Silva et al., 2016). Also in mice, Varga et al. (2018) demonstrated that neutrophil infiltration into the liver was reduced in an alcohol-induced steatohepatitis model when BCP was supplemented. The response seems to be partially related to reduced integrin and selectin expression which reduces migration capability of immune cells. Formation of regulatory lymphocyte subsets may be dependent on CB2 activation (Ziring et al., 2006). In one example, activation of CB2 increased the proportion T-regulatory cells in an asthma model. In this same experiment, BCP reduced the infiltration of immune cells and reduced TNF-α, IL-6 and IL-8 in the lung tissue. BCP also increased IL-10 secretion (Wei et al., 2021). Supplementation of BCP to activate the ECS may be a novel strategy to reduce inflammation and improve health of dairy cattle.

The primary objective of this research was to investigate the effect of BCP on the proteome and phosphoproteome of bovine monocyte-derived macrophages (BMDM) when cultured *in vitro*. We hypothesized that the addition of BCP during lipopolysaccharide (LPS) challenges of BMDM would reduce proteins and phosphopeptides related to cell migration and cytokine secretion. We also hypothesized that pathways associated with cell migration, cytokine signaling, and immune cell functions would be reduced by BCP compared to control (CTL). To meet our objective and test our hypothesis, we utilized novel high throughput proteomic and phosphoproteomic analysis to obtain molecular insight on the processes that were affected by

BCP. These techniques were recently used to demonstrate the effects of other nutritional bioactive components, such as conjugated linoleic acid (Daddam et al., 2021) and omega-3 fatty acids (Kra et al., 2023) on the proteome and phosphoproteome of adipose tissues of dairy cows. The current investigation is the first to explore the effects of a bioactive compound on the proteome and phosphoproteome of BMDM.

MATERIALS AND METHODS

Cows, blood collection, and PBMC isolation

The experiment was approved by the Michigan State University Institutional Animal Care and Use Committee. Blood was sampled from the jugular vein of 5 mid-lactation cows averaging 176 ± 33.7 days in milk, 47 ± 8.2 kg/d of milk, and 12,400 ± 6,400 somatic cells/mL. Blood was collected into 50 mL tubes containing 1mL of 1000 IU/mL of heparin. Collected blood samples were immediately transferred, on ice, to the laboratory and processed within 30 min. Diluted blood (2 x with 2 mM EDTA in Ca++, Mg++ free Dulbecco's phosphate-buffered saline) was centrifuged at 700 × g for 30 min at 20°C using Ficoll-paque PLUS (GE Healthcare, Uppsala, Sweden). After centrifugation, plasma was removed and the opaque phase containing peripheral blood mononuclear cells (PBMC) was collected in a separate tube and washed with Ca++, Mg++ free DPBS two times to remove platelets and retain monocytes and lymphocytes. Cell viability by trypan blue exclusion was measured using Corning Cell Counter Cytosmart software (99.52 ± 0.86; Corning Inc., Corning, NY).

Monocyte cell culture and maturation

Briefly, PBMC were diluted and seeded at 1×10^6 cells/mL in RPMI 1640 media with 10% FBS, 2 mM glutamine and 2% Penicillin/Streptomycin (Thermo Fisher Scientific, Waltham, MA) and incubated at 37°C and 5% CO_2 for 2 h. After incubation, which allows

monocytes to adhere, the medium containing lymphocytes was removed, and the plate was washed twice with warm Ca++, Mg++ free DPBS to remove any remaining lymphocytes. Adhered PBMC were treated with 1 μM BCP dissolved in ethanol (final concentration of ethanol in media 1%) for 7 d (catalog #21572; Cayman Chemicals, Ann Arbor, MI). Media was refreshed on d 2, 4 and 6, continuing treatment with 1 μM BCP. On d 6 cells were challenged with 0.1 μg/mL of LPS (cat# L4516; Sigma-Aldrich Inc., St. Louis, MO) for 24 hours to induce differentiation into polarized macrophages. On d 7, cells were challenged again with LPS for 30 min to stimulate an immune response, and then cells were collected, and proteins were extracted for proteomic and phosphoproteomic analysis using 5% SDS Lysis buffer with a protease and phosphoprotease inhibitor Cocktail (PhosphoStop and cOmplete ULTRA Tablets, Roche, Germany) and 0.2 M PMSF (Sigma-Aldrich Inc., St. Louis, MO). Lysates were kept at -80°C pending analysis. All 5 samples were used for immunoblots but samples from 2 cows were compromised prior to proteomic and phosphoproteomic analysis so there is n = 3 for proteomic and phosphoproteomic analysis and n = 5 for immunoblots. Additionally, a separate 96-well cell culture plate was used to assess cell viability at the time of cell harvest (Figure 5.1).

Proteomics and phosphoproteomics

Proteomic and phosphoproteomic analyses were conducted at G-INCPM (Weizmann Institute of Science, Rehovot, Israel). The samples were lysed and digested with trypsin using the S-trap method (Elinger et al., 2019). For proteomics, the resulting peptides were analyzed using nanoflow liquid chromatography (nanoAcquity) coupled to high resolution, high mass accuracy mass spectrometry (TIMS-ToF Pro). Each sample was analyzed on the instrument separately in a random order in discovery mode. Raw data was processed with the Fragpipe package version 18. The data was searched with the MS Fragger search engine against the

bovine proteome database appended with common lab protein contaminants. Quantification was based on the LFQ method, based on unique peptides (Cox et al., 2014).

For phosphoproteomic analysis, the resulting peptides were enriched for phosphorylation using Fe-III IMAC columns on a Bravo liquid handling system. The resulting phosphopeptides were analyzed using nanoflow liquid chromatography (nanoAcquity) coupled to high resolution, high mass accuracy mass spectrometry (Q Exactive HFX). Each sample was analyzed on the instrument separately in a random order in discovery mode. Raw data was processed with MetaMorpheus version 0.0.320 (Wenger and Coon, 2013). The data was searched against the bovine proteome database appended with common lab protein contaminants. Quantification was performed using the embedded FlashLFQ (Millikin et al., 2018) and protein inference algorithms (Miller et al., 2019). The processing included the unique G-PTM-D method for identification of dozens of PTMs, including phosphorylation of S, T, Y (Li et al., 2017).

Immunoblots

For immunoblot analysis, 30 µl from each cell lysate sample was prepared with Laemmli loading buffer and resolved by SDS-PAGE under reducing conditions and transferred to a nitrocellulose membrane with antibodies to: TNF-α (1:1000, OACAO4183, Aviva System Biology, San Diego, CA), SPP1 (1:1000, 22952-1-AP, Proteintech, Rosemont, IL) and CB2 (1 µg/ml, ADI-905-820-100, Enzo, NY, USA). Goat anti-rabbit HRP-conjugated secondary antibody (Jackson Immunoresearch 111-035-003, West Grove, PA) at a concentration of 1:10,000 was used in an ECL reaction for protein detection. Data were processed and analyzed by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD). Chemiluminescence signals were measured after at least five consecutive exposure times to

determine each antibody's linear range of signal intensity. Specific band signals were normalized to total protein in each sample according to Ponceau staining.

Data Analysis and statistical analysis

The IDEP9.5 server tool was used to perform unsupervised hierarchical clustering of the individual samples. The clustering was based on the relative abundances of the differentially expressed proteins or phosphopeptides in each sample. Kendall's tau distance calculation process was used, and average linkage clustering was employed. The results were displayed using a heat map and PCA, which showed the protein and phosphopeptide expression. Proteins or phosphopeptides with a fold-change of ±1.5 were analyzed by Qiagen's Ingenuity® Pathway Analysis (IPA, Redwood City, CA, USA; www.qiagen.com/ingenuity) to determine the most relevant pathways that were altered by BCP as compared to the CTL. Also, the location and type of protein and phosphopeptides in BCP vs CTL were identified using IPA software. Networks were generated for protein and phosphopeptides using STRING database and visualized in Cytoscape software. The effect of BCP on proteins, phosphopeptides, and immunoblots were analyzed by student t-test. Significance was declared at $P \leq 0.05$ and trends at $P \leq 0.10$.

Prediction of Secreted Phosphoprotein 1 (SPP1) structure by modeling

Modeling the protein structure of SPP1 from Bos taurus involved several steps. Initially, the sequence was collected from the UNIPROT database using an identified peptide (Accession number: P31096). The SMART server was utilized to identify protein domains and BLAST was used to locate a related protein structure in the PDB database with the SPP1 domain. The ClustalX program was used with default parameters to align the SPP1 and template sequences. Homology modeling was carried out using the MODELLER9V7 software and the model with the lowest modeller objective function was chosen (Webb and Sali, 2016). The selected model

underwent further stabilization through molecular dynamics, utilizing NAMD 2.8 and CHARMM27 force field. To ensure the stereochemical quality of the protein structure, the Ramachandran plot server in PROCHECK was used. Finally, the ERRAT structure evaluation server was utilized to check the environment profile of the protein structure (Colovos and Yeates, 1993). The molecular dynamics studies helped to stabilize the Root Mean Square Deviation (RMSD) of SPP1.

RESULTS

Location and function of proteins and phosphopeptides

The proteins and phosphopeptides observed were mostly cytoplasmic proteins. The 2nd most abundant location of phosphopeptides was the nucleus while the 2nd most abundant location for proteins was the extracellular space (Figure 5.2A, B). Enzymes, transcription regulators, and kinases were the most abundant phosphopeptide functions. Enzymes, transporters, and peptidases were the most abundant functions of the proteins (Figure 5.2C, D).

Effects of BCP on Proteomics & Phosphoproteomics of BMDM

In total, 650 proteins and 1,406 phosphosites were quantified. Four proteins were affected ($P \leq 0.05$) and 7 proteins tended to be affected by BCP supplementation ($P \leq 0.10$). 7 phosphopeptides were affected ($P \leq 0.05$) and 16 phosphopeptides tended to be affected ($P \leq 0.10$) by BCP (Table 5.1; Figure 5.3). Immune-related proteins reduced by BCP were protein tyrosine phosphatase (PTPRC; FC = -2.7; $P = 0.01$) and latexin (LXN; FC = -3.9; $P = 0.05$). Calpain-1 catalytic subunit (CAPN1; FC = 3.2; $P = 0.03$) and fatty acid binding protein (FABP1; FC = 2.2; $P = 0.07$) were increased by BCP. There was reduced phosphorylation of the phosphopeptides in secreted phosphoprotein 1 (SPP1; FC = -6.3; phosphosite 293;297; $P =$

0.04) and tendency to reduce Serine/Threonine Kinase 10 (STK10; FC = -1.8; phosphosite 449; $P = 0.08$) was observed in BCP macrophages compared to control (CTL).

Enriched pathways in BCP vs. CTL in proteomics and phosphoproteomics

According to proteomic analysis, various immune and inflammation related pathways were affected by BCP. IL-8 signaling, leukocyte extravasation signaling, signaling by Rho family GTPases, TCA cycle II, ILK signaling, Fcγ receptor-mediated phagocytosis, and HIF1α signaling were reduced by BCP. LXR/RXR signaling, and production of ROS and NOS were increased by BCP (Table 5.2).

Analysis of phosphopeptides also demonstrated that various immune and inflammation pathways were differentially regulated. Leukocyte extravasation was increased while apoptosis signaling, integrin signaling, ERK/MAPK signaling, acute phase response signaling, and ILK signaling were all reduced because of BCP (Table 5.3).

Network Analysis of proteome and phosphoproteome in BCP vs. CTL samples

Figure 5.4 displays the network relationships between peptides affected by BCP vs. CTL. In the Ingenuity analysis, peptides CYBB, GNB2, GPLD1, ICAM1, IQGAP1, ITGAM, MMP9, MYL12B, RHOA and RHOG were commonly found in the IL-8 signaling, leukocyte extravasation signaling, signaling by Rho family GTPase pathways and connected to peptides IL1RN, LCAT, MMP9, NOS2, SAA1 and SAA4 of ILK signaling, LXR/RXR signaling, and production of ROS and NOS pathways. The interaction of these molecules involved in the pathways formed a hub of proteins that were connected.

Similarly, Figure 5.5 presents the phosphopeptides networks. Phosphopeptides of ITGB2, MAPK1, PXN, RAF1, and RRAS2 of leukocyte extravasation, integrin signaling, ERK/MAPK

signaling, and acute phase response signaling, were commonly found and linked to IRS2 and VCL of ILK signaling.

Immunoblots

We detected CB2 in BMDM but its relative abundance was not affected by treatment with BCP ($P = 0.21$). Neither TNF-a ($P = 0.14$) or SPP1 ($P = 0.17$) were affected by treatment with BCP (Figure 5.6).

Phosphorylation and structural analysis of SPP1

The MS/MS spectra of phosphorylated SPP1 is shown in Figure 5.7A. The peptide was matched to the SPP1 sequence of *Bos taurus* (Fig 5.7B), and a three-dimensional structure was developed using a sequence collected from the Uniprot database. The predicted SPP1 structure showed 14 helices and no sheets in quaternary structure (Figure 5.8C).

DISCUSSION

Use of nutritional supplements to reduce inflammation or improve immune function and health are critical for livestock and may lead to improved animal resilience. Modulating the ECS via CB2 maybe a novel strategy to improve immune function and resilience in livestock. Targeting this receptor may be especially useful in dairy cattle because dairy cows with a dysregulated immune system and excess inflammation have poorer health and performance throughout their lactation (Bertoni et al., 2008; Kerwin et al., 2022). The selective activator of CB2, BCP, is promising because it promotes anti-inflammatory effects in various disease challenge models (Bento et al., 2011; Lindsey et al., 2019; Zhang et al., 2021). Currently, there is no data investigating how BCP may affect primary bovine immune cells or other bovine cell types. Additionally, this is the first experiment were aware of that characterized the proteome

and phosphoproteome of primary bovine monocyte-derived macrophages cultured *in vitro* and stimulated with LPS.

First, our data demonstrate that the CB2 receptor is expressed in BMDM. This is critical because it demonstrates that BCP, or other cannabinoids like AEA and 2AG, may have immunomodulatory effects in bovines through altering cell migration, cell signlaing, and cell function which is supported with results from other species (Rajesh et al., 2007a; Rajesh et al., 2007b; Gertsch et al., 2008; Hashiesh et al., 2021). Thus, theraputic approaches targeting the CB2 receptor may imrpove the inflammatory status or immune function of dairy cattle.

The BCP affected proteins and phosphopeptides that were important for immune function when BMDM cells were stimulated with LPS *in vitro*. Proteins of note are protein tyrosine phasphatase (PTPRC), latexin (LXN), and CD5 molecule like (CD5L). PTPRC, which is also known as CD45, is compelling because it is one of the most abundant glycoproteins present in immune cells and has demonstrated positive and negative impacts on immune responses (Alexander, 2000). In macrophages and T-cells, CD45 knockout resulted in increased adherence of immune cells but in macrophages the ability to maintain adhesion was reduced over time (Roach et al., 1997). The receptor also has a role in signal transduction and T-cell proliferation (Pingel and Thomas, 1989; Koretzky et al., 1990). Reducing expression of this protein may dysregulate cell adhesion while also reducing lympocyte signaling and proliferation. LXN plays a role in inflammation by suppressing hematopoetic stem cell population and renewal (Zhang and Liang, 2018). Deficiency of latexin increases severity of colitis (Li et al., 2020) but also may promote a pro-resolving macrophage phenoteype and reduce T-cell functions (Li et al., 2022).

The affects on these proteins supports previous evidence that BCP may promote immune cells with resolving functions and reduce excess immune cell responses.

Previous studies predicting protein structures in cattle suggest that modelling is a reliable method to understand the structural variations in proteins (Daddam et al., 2020; Kra et al., 2021; Ben Meir et al., 2022; Kra et al., 2022a). We have predicted the phosphorylated SPP1 structure using mass spectra data and confirmed the osetopontin domain in SPP1 from *Bos taurus*. The reduction in secreted phosphoprotein 1 (SPP1) phosphopeptides by BCP in BMDM is compelling because of its ability to regulate a variety of immune functions, cytokines, and cell populatioions (Ashkar et al., 2000; Kahles et al., 2014). SPP1, also known as osteopontin, is a glycoprotein that is secreted by various cells, including osteoblasts, macrophages, and T-cells (Kahles et al., 2014). SPP1 contains several domains, including an RGD motif that allows it to bind to integrins, which are involved in cell adhesion and signaling. SPP1 promotes immune cell migration and adhesion (Giachelli et al., 1998) through interactions with various integrins and CD44 (Weber et al., 2002; Lund et al., 2013). Knock-out of SPP1 reduced macrophage infiltration into adipose tissue of mice fed high fat diets (Schuch et al., 2016) which demonstrates the role SPP1 plays in cell migration. It also may be necessary for both IL-12 and INF-γ production (Ashkar et al., 2000; O'Regan et al., 2000; Weber et al., 2002). SPP1 augments CD3+ interferon-γ production (O'Regan et al., 2000). In addition to lymphocyte functions, it modulates macrophage functions and polarization (O'Regan et al., 2000; Kahles et al., 2014; Zhang et al., 2017). Alterations of LXN, SPP1, and CD45 indicate that cell adhesion, migration, and lymphocyte function may be modulated. Effects of CB2 activation on lymphocyte function was also observed by Jackson et al. (2014); CB2 activation reduced IL-17 producing T-cells along with systemic reductions of INF-γ and IL-17A. Investigating effects of CB2 actinvation on

cell functions or effects on populations like γδ-T cells, which have regulatory roles in cattle (Hoek et al., 2009; Talker et al., 2022), may be fruitful areas of investigation. Whether these immune modulatory effects would be beneficial for cattle is likely dependent on context; preventing excess or pathological inflammation is beneficial but reducing the cow's ability to mount a sufficient immune response would be detrimental.

CD5L is a secreted glycoprotein that when exposed to macrophages results in a similar mRNA expression and cytokine profile to macrophages induced by IL-10 (M2 polarization) indicatng a more pro-resolving and wound healing phenotype (Sanjurjo et al., 2018). In an ischemic stroke model, CD5L reduced sterile inflammation by increased phagocytosis of cell debris. CD5L also increased survival in this stroke model (Maehara et al., 2021). In the present study, the tendency for an increase in CD5L from BCP may inidcate enhanced funciton or phagocytosis of BMDM while also promoting a pro-resolving macrophage phenotype. The effect CD5L has on macrophage function and polarization, along with reduced LXN and reduced phosphopeptides associated with SPP1, strongly suggest that BCP may increase abundance of pro-resolving macrophages which may improve resolution of inflammation or aid in regulating inflammaton in dairry cattle experiencing stress (Hoek et al., 2009). Investigation as to whether supplementing BCP may encourage a pro-resolving non-classical macrophage phenotype *in vivo* may be a fruitful area of investigation as these macrophage classifications and their functions seem to be consistent across species (Talker et al., 2022).

Interestingly, our investigation of enriched pathways based on proteomic and phosphoproteomic analyses also demonstrated alterations of immune signaling, cell migration, and cell function as result of BCP. Leukocyte extravasation appeared to be reduced according to

proteomic results in BCP treated lysates. Previous research in other species suggests that CB2 activation reduces migration of leukocytes (Andrade-Silva et al., 2016; Varga et al., 2018; Zhang et al., 2021). Reduced integrin signaling as a result of BCP supplementation according to our phosphoproteomic results also suggests reduced cell migration capacity. Reducing leukocyte adhesion and migration may leave cows more susceptible or tolerant to a bacterial infection or it may reduce excessive inflammation during times of stress; investigation of BCP's effects on disease responses or during times of stress like the transition from gestation to lactation in cattle would demonstrate whether this alteration in cell migration may be beneficial or problematic. Prevoious research indicates that the ECS does play a role during times of stress like the transition from gestation to lactation as endogenous cannabinoids increase during the postpartum period (Zachut et al., 2018; Kuhla et al., 2020). Research investigating supplementation of cannabinoids during this period has not been done.

Reduced IL-8 signaling and reduced acute phase response signaling demonstrate potential anti-inflammatory effects of BCP. IL-8 signaling increases immune cell activity through increasing nuetrophil activation, migration, and CD11b/CD18 expression (Lotz et al., 1992; Harada et al., 1994; Yang et al., 1999). Blocking this signaling pathway improves outcomes by reducing these functions of immune cells, specifically by reducing neutrophil function and migration (Yang et al., 1999). These results also align with other data as BCP often reduces inflammatory signaling and migration of immune cells (Bento et al., 2011; Varga et al., 2018; Lindsey et al., 2019). The ILK signaling, according to both proteomic and phosphoproteomic analyses, was reduced by BCP supplementation. This may downregulate inflammatory signals by reducing TNF-α, MCP-1, CCL2, IL-6, and IL-1β (Assi et al., 2011; Ahmed et al., 2017). Reductions of these signaling pathways indicate that BCP may reduce excess inflammation and

deserves closer investigation in cattle. Specifically, it seems that cell migration and adhesion may be downregulated in response to BCP which merits more rigorous investigation, especially whether it is beneficial or detrimental during different disease scenarios.

BCP also altered LXR/RXR signaling, ROS/NOS production, and phagocytosis pathways which suggests that macrophage antimicrobial capactiy, in addition to the chemotactic and cell migration capacity, was affected. LXR/RXR are nuclear receptors that control transcription of different lipid metabolism and inflammation related genes (Joseph et al., 2003; Núñez et al., 2010; Rőszer et al., 2013). For example, LXR knockout reduced expression of SPα in mice, which inhibited macrophage apoptosis. They demonstrated that signaling and activation of LXR was important in the innate immune response by inhibiting apoptosis and improving bacterial clearance during a listeria infection (Joseph et al., 2004). Knockout of RXR impairs leukocyte migration and recruitment likely through reduced CCL6 and CCL9 expression (Núñez et al., 2010). These phosphoproteomic pathway analyses also indicated reduced apoptosis signaling which suggests increased cell survival as a result of BCP. Coupled with increased LXR/RXR signaling, our data suggest reduced apoptosis and increased cell survival as a results of BCP supplementation. LXR activation also reduces myeloperoxidase expression (Reynolds et al., 2006) which may reduce inflammation. In addition to these LXR/RXR mediated effects, enriched ROS/NOS signaling and phagocytosis signaling are indicative of increase bovine immune cell function and increased ability to clear pathogens. Based on these results, investigations as to whether BCP alters dairy cattle's response to disease or stress is warranted; it may be particulary impactful to investigate whether BCP atlers a dairy cows response to mastitis or other bacterial infections that require immune cells to phagocytise and clear pathogens.

CONCLUSIONS

Our results demonstrate that the CB2 receptor is present in BMDM that have been challenged with LPS in vitro; therefore, CB2 activation may be a target for improving immune function and reducing pathological inflammation in cattle. We demonstrated that an exogenous CB2 ligand, BCP, altered the proteome and phosphoproteome of BMDM in response to LPS challenges in vitro. Generally, BCP reduced pathways and proteins associated with cell migration, cell signaling, and cell function. Our results align with previous data sugggesting that BCP may promote pro-resolving cell types, like M2-polarized macrophages, through differential effects on CD5L, LXN, and SPP1. These results demonstrate that targeting the ECS through exogenous supplementation with BCP may be a promising approach to modulating the bovine immune system, it may be particulary impactful to investigate whether BCP atlers a dairy cows response to mastitis or other bacterial infections that require immune cells to phagocytise and clear pathogens. Future research investigating how BCP and other cannabinoids may alter bovine's immune cell phenotype, function, or systemic response to stress may be beneficial and lead to strategies and feed supplements that improve dairy cow health and welfare.

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APPENDIX

Table 5.1. Effect of β-caryophyllene on protein and phosphoprotein abundance according to proteomics and phosphoproteomics analyses from bovine monocyte-derived macrophages after repeated lipopolysaccharide challenges (n = 3/treatment).

Genename[1]	Protein	Fold change	*P*-value
Proteomics			
ATP6V1E1	V-type proton ATPase subunit E 1	-2.5	<0.001
PTPRC	Protein-tyrosine-phosphatase	-2.7	0.011
CAPN1	Calpain-1 catalytic subunit	3.2	0.032
LXN	Latexin	-3.9	0.050
ALDH2	Aldehyde dehydrogenase, mitochondrial	-6.3	0.058
CD5L	CD5 molecule like	1.2	0.064
FABP1	Fatty acid-binding protein, liver	2.2	0.068
PSMD3	26S proteasome non-ATPase regulatory subunit 3	-2.0	0.069
DCXR	L-xylulose reductase	1.8	0.081
ARSB	ARSB protein	2.6	0.102
SPP2	Secreted phosphoprotein 24	-2.0	0.104
Phosphoproteomics			
SRRM2	Serine/Arginine Repetitive Matrix 2	7.9	0.008
UBR4	Ubiquitin Protein Ligase E3 Component N-Recognin 4	2.6	0.013
CBX2	Chromobox 2	-6.8	0.019
CHGB	Chromogranin B	-3.9	0.037
SPP1	Secreted Phosphoprotein 1	-6.3	0.038
RBM17	RNA Binding Motif Protein 17	-2.1	0.050
IQCA1L	IQ Motif Containing with AAA Domain 1 Like	-3.6	0.054
RIC1	RIC1 Homolog, RAB6A GEF Complex Partner 1	1.6	0.059
MYO18A	Myosin XVIIIA	1.8	0.067
VAV3	Vav Guanine Nucleotide Exchange Factor 3	-5.9	0.067
DDX27	DEAD-Box Helicase 27	-2.1	0.070
STK10	Serine/Threonine Kinase 10	-1.8	0.075
ASL	Arginosuccinate Lyase	2.4	0.076
MARCKS	Myristoylated Alanine Rich Protein Kinase C Substrate	-7.8	0.081
ANKRD12	Ankyrin Repeat Domain 12	-2.2	0.085
BCLAF1	BCL2 Associated Transcription Factor 1	2.0	0.086
C11H9orf78	Chromosome 9 Open Reading Frame 78	-4.9	0.087
CWC22	CWC22 Spliceosome Associated Protein Homolog	3.5	0.088

Table 5.1. (cont'd)

ACSS2	Acyl-CoA Synthetase Short Chain Family Member 2	1.4	0.089
LIMK1	LIM Domain Kinase 1	1.5	0.091
RPS6KA1	Ribosomal Protein S6 Kinase A1	1.9	0.091
BIN2	Bridging Integrator 2	-3.0	0.099
CHD4	Chromodomain Helicase DNA Binding Protein 4	1.8	0.100

[1]Gene name for the associated protein and phosphopeptides measured by proteomics and phosphoproteomic analysis.

Table 5.2. List of enriched canonical pathways according to proteomics analysis in bovine monocyte-derived macrophage (BMDM) were treated with β-caryophyllene during repeated LPS challenges (n = 3/treatment).

Pathway	z-score	Molecules involved	-log(*P*-value)
IL-8 Signaling	-0.302	CSTB, CYBB, GNB2, GPLD1, ICAM1, IQGAP1, ITGAM, LASP1, MMP9, MYL12B, RHOA, RHOG	7.80
Leukocyte Extravasation Signaling	-1.414	ACTG1, ACTN1, CYBB, EZR, ICAM1, ITGAM, MMP12, MMP9, RHOA	5.21
Signaling by Rho Family GTPases	-0.707	ACTG1, ARPC5, CYBB, EZR, GNB2, IQGAP1, ITGAM, MYL12B, RHOA, RHOG	4.98
Integrin Signaling	0	ACTG1, ACTN1, ARPC5, CAPN1, ITGAM, MYL12B, RHOA, RHOG	4.12
TCA Cycle II	-2.00	ACO1, CS, DLST, MDH2	3.85
LXR/RXR Signaling	1.633	IL1RN, LCAT, MMP9, NOS2, SAA1, SAA4	3.69
Production of ROS and NOS	1.134	IL1RN, LCAT, MMP9, NOS2, SAA1, SAA4	3.52
ILK Signaling	-0.47	IL1RN, LCAT, MMP9, NOS2, SAA1, SAA4	3.44
Fcγ Receptor-mediated Phagocytosis	-1.342	ACTG1, ARPC5, EZR, GPLD1, RAB11B	3.32
HIF1α Signaling	-0.378	CYBB, HK1, HSPA1A/HSPA1B, HSPA9, MMP12, MMP9, NOS2	3.31

Table 5.3. List of enriched canonical pathways according to phosphoproteomics analysis in bovine monocyte-derived macrophage (BMDM) were treated with β-caryophyllene during repeated LPS challenges (n = 3/treatment).

Pathway	z-score	Molecules involved	-log(*P*-value)
Apoptosis signaling	-0.277	ACIN1, CASP8, CDK1, LMNA, MAP4K4, MAPK1, RAF1, RPS6KA1, RRAS2	5.32
Integrin signaling	-1.508	ASAP1, CRKL, FNBP1, ITGB2, MAPK1, MYLK, PAK4, PPP1R12A, PXN, RAF1, RRAS2, VCL	4.94
ERK/MAPK signaling	-0.302	CRKL, ELF1, HSPB1, ITGB2, MAPK1, MKNK1, PAK4, PPP1R12A, PXN, RAF1, RPS6KA1, RRAS2	4.82
Leukocyte extravasation signaling	1.265	AFDN, CD44, CRKL, CTNND1, ITGB2, MAPK1, MSN, NCF4, PXN, VAV3, VCL	4.50
Acute phase response signaling	-0.816	AHSG, CEBPB, HRG, MAPK1, RAF1, RRAS2, SERPINA1, SERPIND1, SERPINF2, TTR	4.02
ILK signaling	-2.12	ARHGEF6, FNBP1, IRS2, ITGB2, MAPK1, MYH9, MYO18A, PPP1R12A, PXN, VCL	3.73

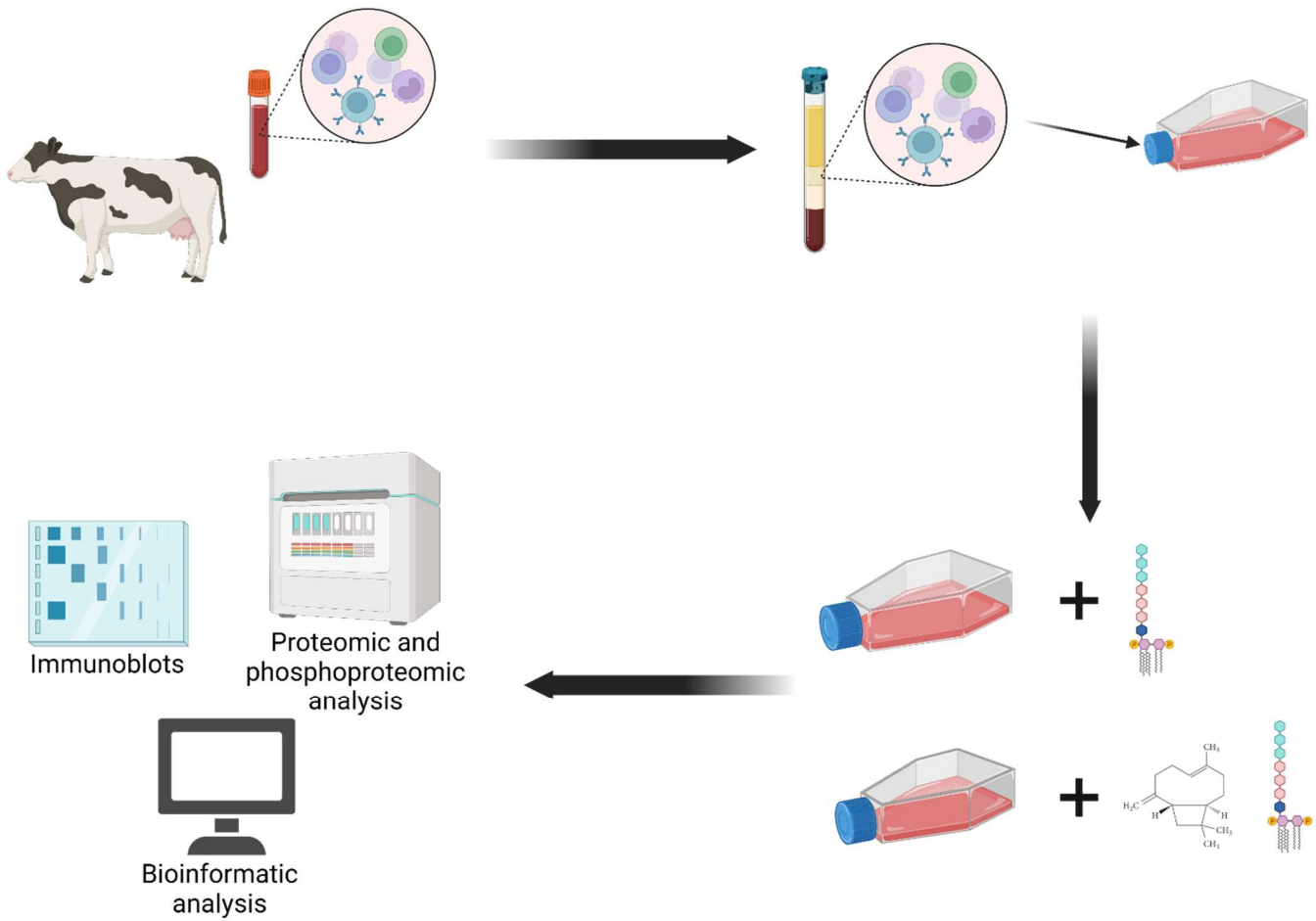


Figure 5.1. Diagram of experiment evaluating the effect of β-caryophyllene (BCP) on the proteome and phosphoproteome of bovine monocyte-derived macrophages stimulated with LPS in vitro. Blood was collected from 5 mid-lactation Holstein cows for isolation of PBMC for culturing. PBMC were harvested from buffy coat and plated for 2 hr to allow monocytes to adhere. Monocytes were cultured for 6 days in the presence or absence of BCP then challenged with LPS on d 7 and again 24 hours later. Cells were collected for proteomic and phosphoproteomic analysis, immunoblots, and bioinformatic analysis. Image created with Biorender.com.

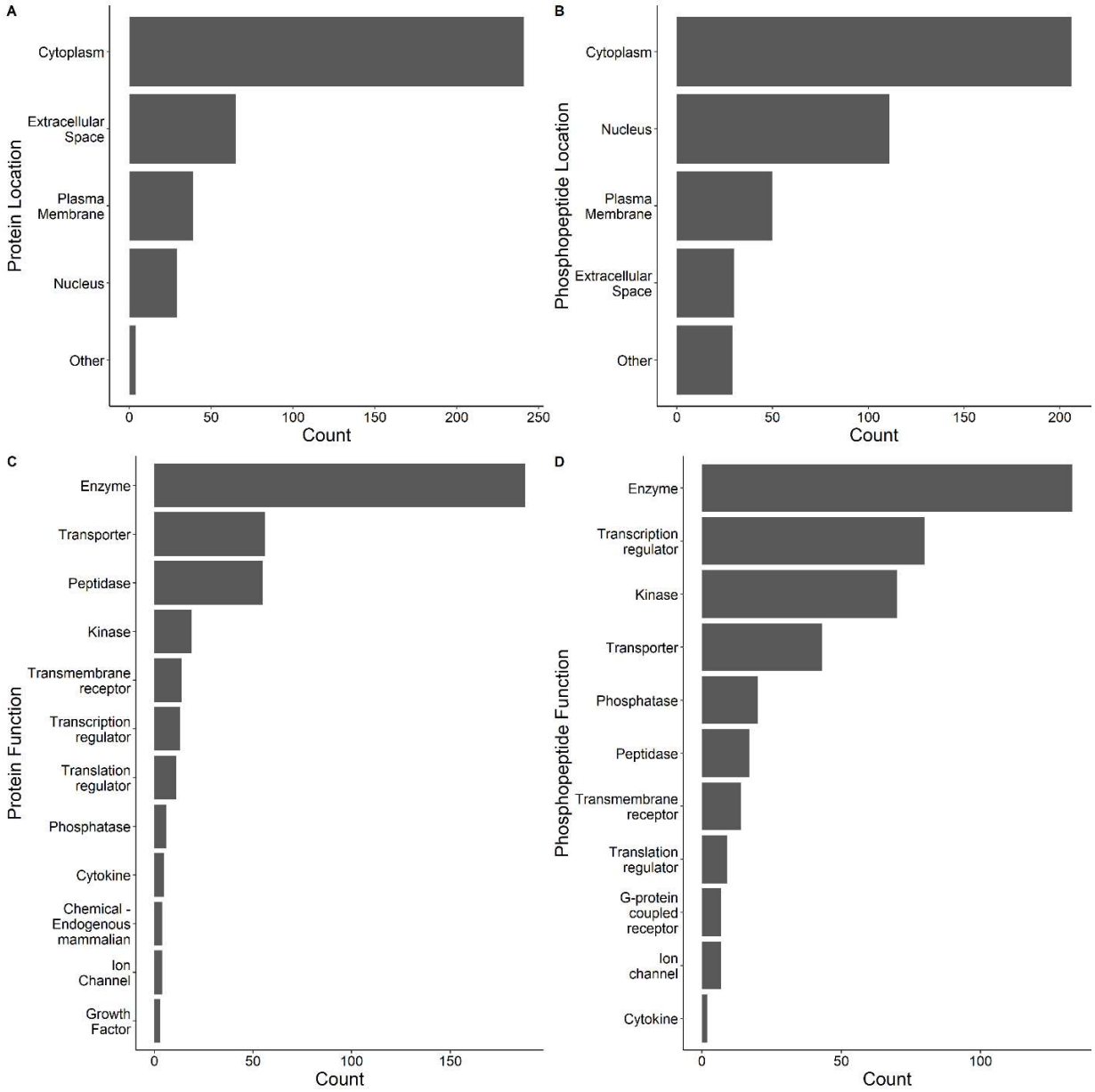


Figure 5.2. Location of A) proteins and B) phosphopeptides observed in bovine monocyte-derived macrophages that were challenged with lipopolysaccharide. Functions of C) proteins and D) phosphopeptides observed in bovine monocyte-derived macrophages that were challenged with lipopolysaccharide. N = 3/treatment.

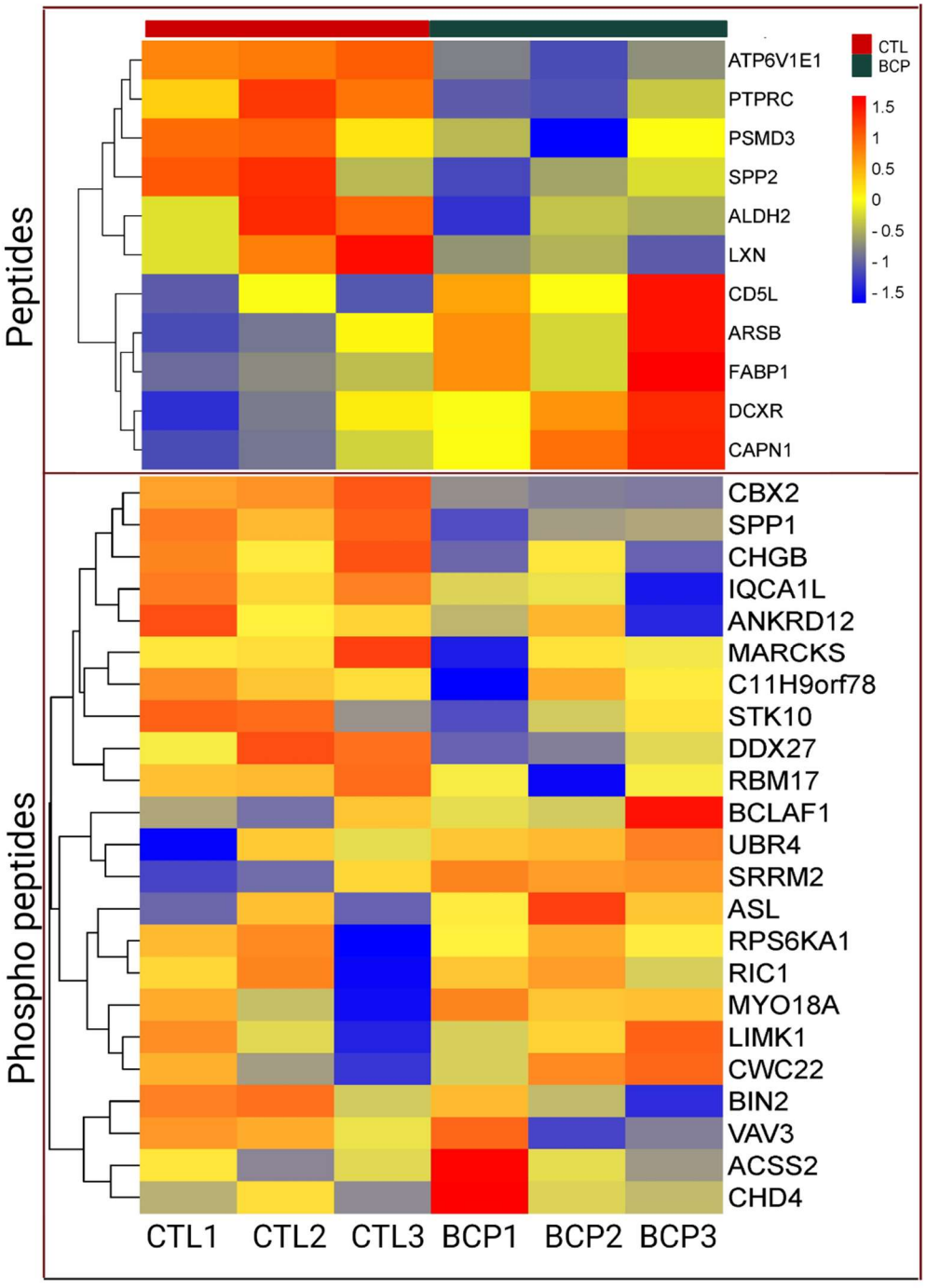


Figure 5.3. Heat map for proteins and phosphopeptides that were differentially affected by β-caryophyllene (BCP; n = 3/treatment) in bovine monocyte-derived macrophages (BMDM) that were challenged with lipopolysaccharide. Color legend: red - increased abundance or phosphorylation; blue – reduced abundance or phosphorylation.

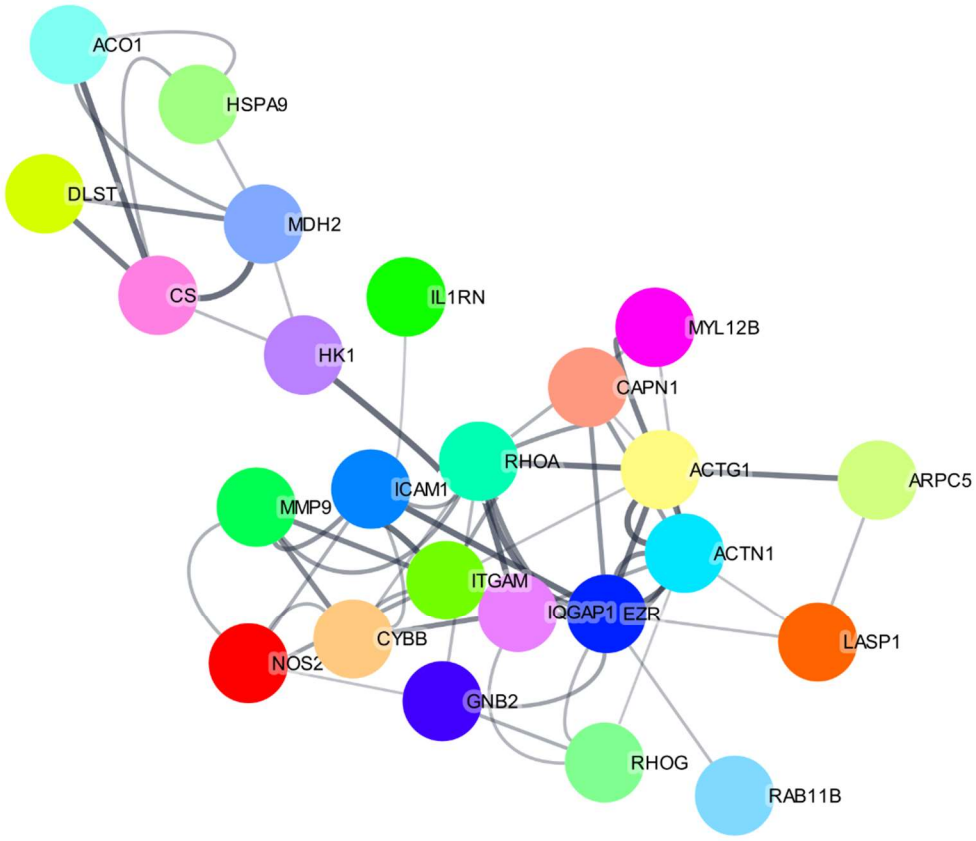


Figure 5.4. Network analysis of proteins involved pathways that were affected by β-caryophyllene (BCP; n = 3/treatment) in bovine monocyte-derived macrophages (BMDM) that were challenged with lipopolysaccharide according to proteomic analysis using Cytoscape software.

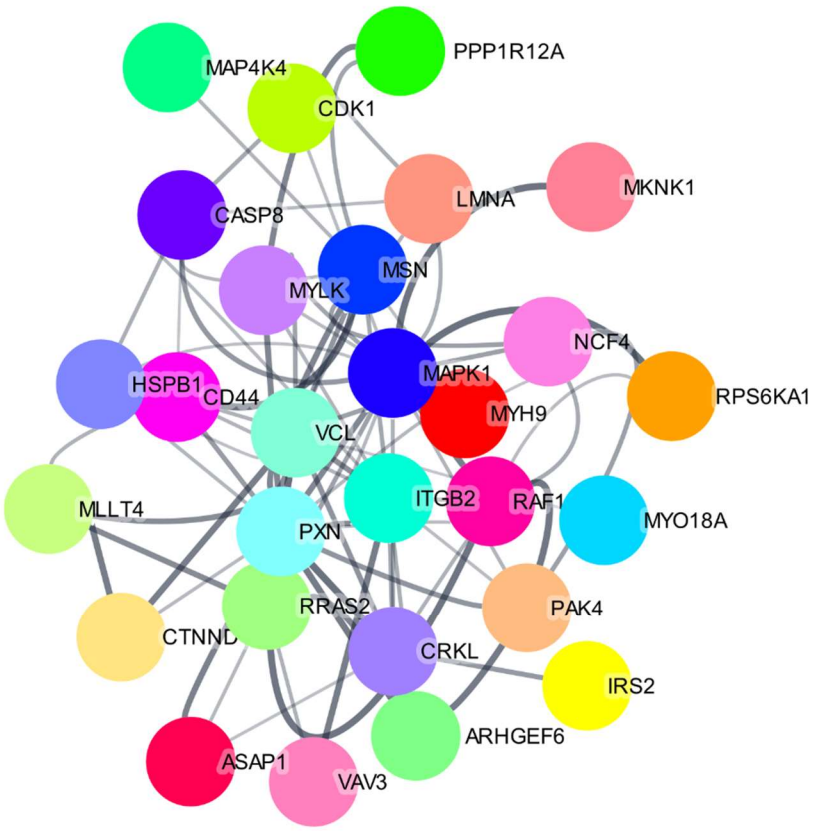


Figure 5.5. Network analysis of phosphopeptides involved pathways that were affected by β-caryophyllene (BCP; n = 3/treatment) in bovine monocyte-derived macrophages (BMDM) that were challenged with lipopolysaccharide according to phosphoproteomics using Cytoscape software.

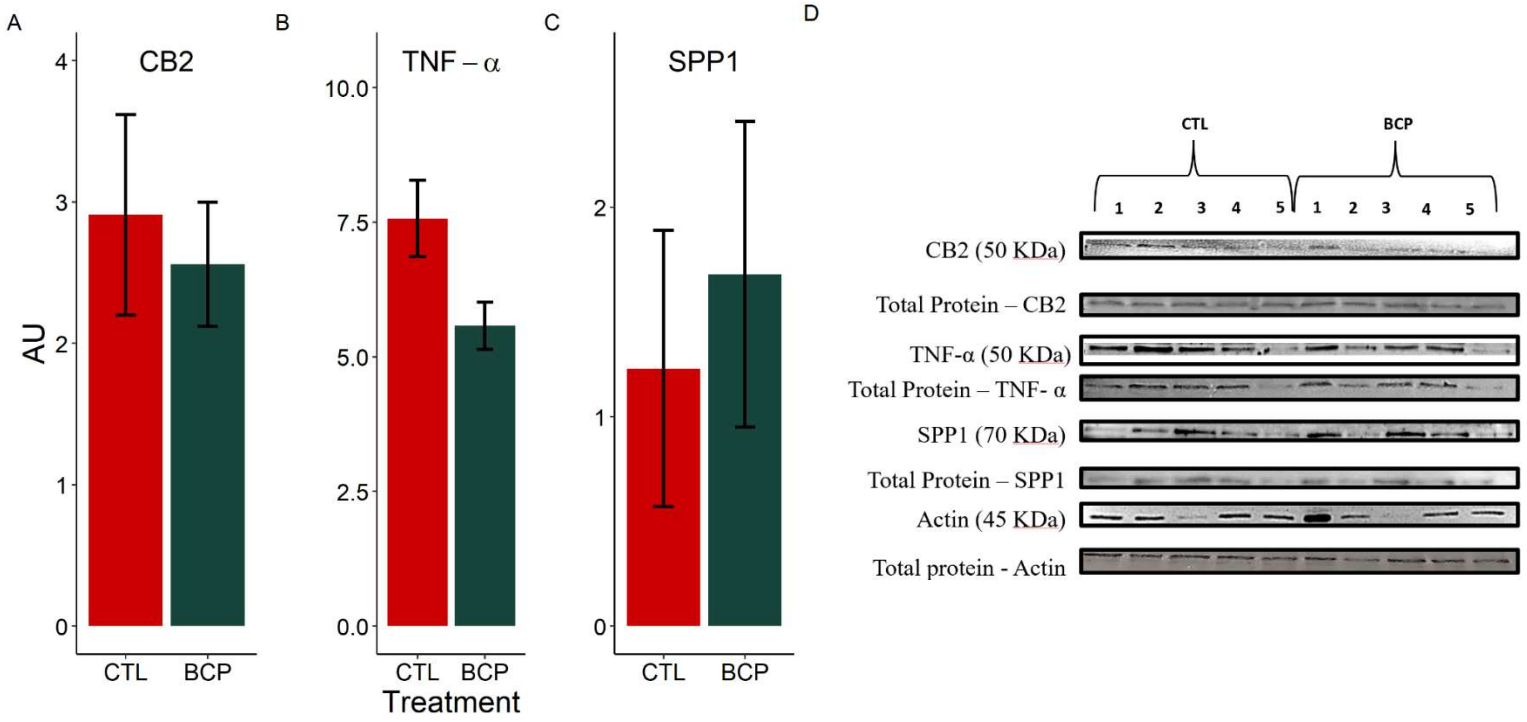
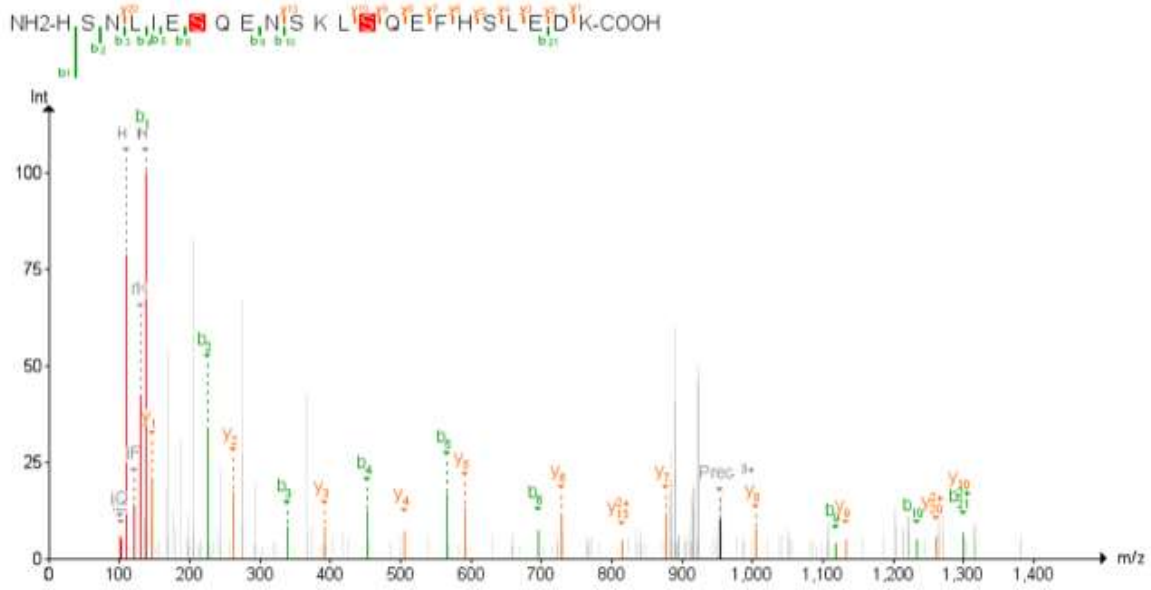


Figure 5.6. Effect of β-caryophyllene (BCP; n = 5/treatment) on protein expression in bovine monocyte-derived macrophages (BMDM) after challenges with Lipopolysaccharide (LPS). A) BCP did not affect CB2 expression ($P = 0.21$). B) BCP did not affect TNF-α expression ($P = 0.14$). C) BCP did not affect CB2 expression ($P = 0.21$). D) Protein bands corresponding to each panel.

A



B

MRIAVICFCLLGIASALPVKPTSSGSSEEKQLNNKYPDAVATWLKPDPSQKQTFLAPQNS

VSSEETDDNKQNTLPSKSNESPEQTDDLDDDDDNSQDVNSNDSDDAETTDDPDHSDESHH

SDESDEVDFPTDIPTIAVFTPFIPTESANDGRGDSVAYGLKSRSKKFRRSNVQSPDATEE

DFTSHIESEEMHDAPKKTSQLTDHSKETNSSELSKELTPKAKDKNKHSNLIESQENSKLS

QEFHSLEDKLDLDHKSEEDKHLKIRISHELDSASSEVN

P P

C

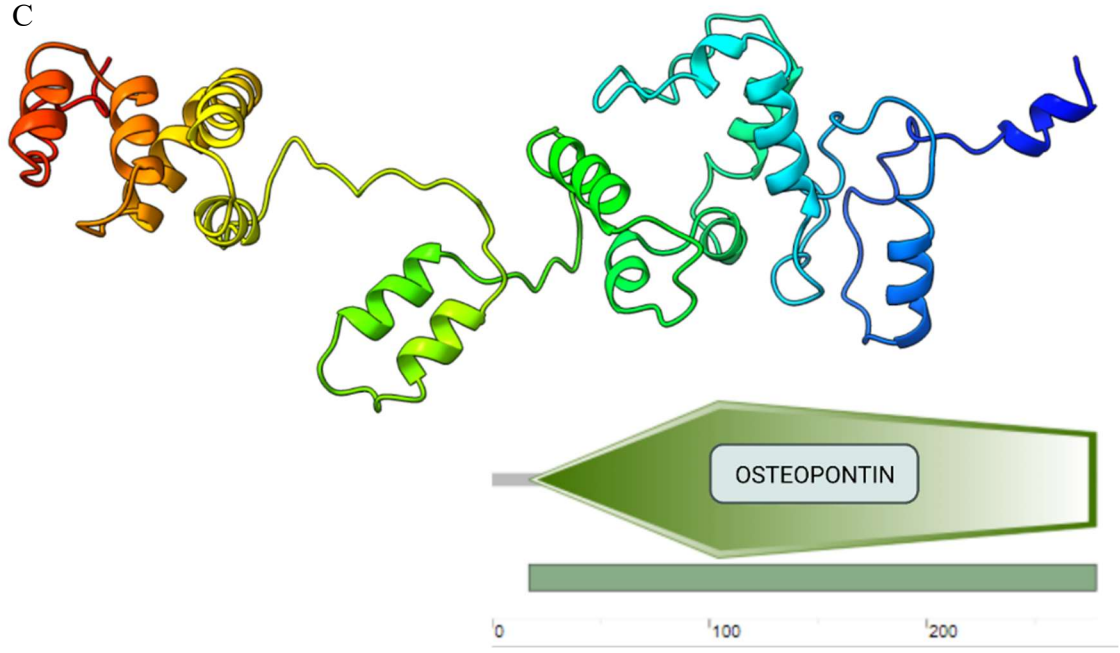


Figure 5.7. Structural characterization of bovine SPP1. A) MS spectra of SPP1 phospho peptide identified by LC–MS/MS; (B) SPP1 sequence showing the phosphorylation amino acids. C) Structure of phosphorylated SPP1 protein showing sheets and helices.

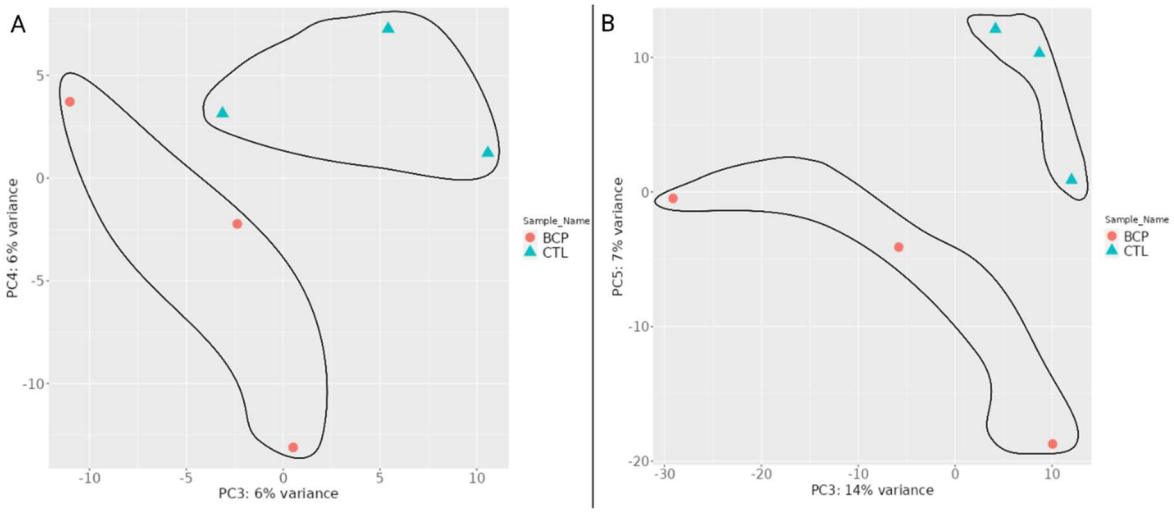


Figure S5.1. PCA analysis of A) proteins and B) phosphopeptides that were differentially affected by β-caryophyllene (BCP; n = 3/treatment) in bovine monocyte-derived macrophages (BMDM) that were challenged with lipopolysaccharide.

CHAPTER 6: DOES FEEDING STARCH CONTRIBUTE TO THE RISK OF SYSTEMIC INFLAMMATION IN DAIRY CATTLE?

ABSTRACT

In the high-producing dairy cow, providing an adequate supply of digestible energy is essential. One strategy to meet this need is to provide fermentable starch from cereal grains or silages like corn, barley, or wheat. Unfortunately, excess dietary starch increases the risk of rumen acidosis. Rumen acidosis challenge models using high-grain diets, particularly with wheat and barley, have demonstrated that a sudden change in starch concentration or digestibility leads to the breakdown of the rumen epithelial barrier. As a result, increases in circulating lipopolysaccharide (LPS: a marker for bacterial translocation) and acute phase proteins (APP) have been observed. Feeding increasing amounts of starch in chronic feeding studies does not appear to consistently modulate inflammation in early lactation cows who already experience inflammation. In mid and late lactation cows, increasing starch above 30% may increase APP, but the response is inconsistent and has not been investigated using different grains or differently processed starch sources. Abomasal starch infusion experiments indicate that increasing intestinal starch supply consistently reduces fecal pH but does not lead to an APP response or changes in gut integrity. Increasing intestinal starch supply increases fecal butyrate concentrations and butyrate has had positive effects on gut health and integrity in other species and experimental models. More chronic feeding experiments investigating how starch concentrations, sources, processing methods, and interactions affect inflammation and gut integrity are needed. There is a paucity of data investigating the role that carbohydrate concentrations and sources play on ruminant hindgut health, integrity, function, structure, or

microbiome. Currently, data indicate that feeding diets with less than 30% starch to lactating dairy cows does not contribute to systemic inflammation.

Key words: acidosis, gut health, carbohydrates,

INTRODUCTION

Starch, and the cereal grains that typically supply it, play an important role in dairy cattle nutrition. Starch is a highly digestible energy source which may increase milk yield (Sánchez-Duarte et al., 2019), milk protein yield (Dias et al., 2018; McCaughern et al., 2020; Morris et al., 2020), and body tissue accretion (Boerman et al., 2015) compared to feeding NDF or specific FA. Furthermore, increasing the concentration of starch from ~20% to ≥28% of diet DM reduces ruminal (Salfer et al., 2018) and hindgut pH (Neubauer et al., 2020). Reducing digesta pH may be one factor contributing to diminished gut health and integrity. Increased luminal LPS concentration (Li et al., 2012; Li et al., 2016) and osmolality (Chibisa et al., 2016) when feeding starch may also be contributing factors.

This mini review summarizes current research investigating systemic inflammation in lactating dairy cows provided different dietary starch concentrations during grain challenges, feeding experiments, or hindgut infusions. We use 'grain challenge' when referring to experiments where grains are abruptly increased to ≥20% of diet DM for the purpose of causing ruminal acidosis. These grain challenges have resulted in an increase in circulating markers of inflammation such as lipopolysaccharide (LPS) and acute phase proteins (APP; Khafipour et al., 2009b). A meta-analysis evaluating acidosis grain challenge data indicated that >44% concentrates or <39% NDF in diets fed to dairy cattle risks inflammation and increased circulating APP (Zebeli et al., 2012). Furthermore, ruminal acidosis grain challenges break down the rumen epithelial barrier (Steele et al., 2011) which may lead to an inflammatory response due

to rumen bacteria or their components moving across the epithelium and interacting with circulating and tissue resident immune cells. Inflammation may also result from free ruminal LPS binding its receptor, toll like receptor 4 (TLR4), on the rumen epithelium, which has increased mRNA abundance in rumen tissue during acidosis (Pederzolli et al., 2018). Either may lead to the release of proinflammatory cytokines and eventual increases in circulating APP, but there is no consensus on the mode of action.

ROLE OF DIET IN INFLAMMATION

Experiments that involve feeding elevated starch concentrations, usually ≥28% of diet DM as starch, and measuring its effects on inflammation have increased in the last two decades. Investigators have observed increases in plasma LPS, serum amyloid A (SAA), haptoglobin (Hp), and LPS binding protein (LBP) during these rumen acidosis grain-challenge experiments (Gozho et al., 2007; Khafipour et al., 2009b; Li et al., 2016). One experiment reported increases in serum Hp from non-detectable concentrations to 600 μg/mL in grain-challenged cows (Khafipour et al., 2009c). Interestingly, feeding pelleted alfalfa hay induced similar reductions in rumen pH and increases in ruminal LPS and VFA, but did not increase plasma LPS or APP (Khafipour et al., 2009a; Khafipour et al., 2009c); this indicates that inflammation does not likely stem from reduced rumen pH and increased rumen LPS concentration alone. Other factors such as diet adaptation or gut microbiome may play a role in this diet-induced inflammation (Plaizier et al., 2017). We do not know whether the cause of the APP responses observed during acidosis challenge studies originated from the rumen or intestine, although the rumen epithelium clearly degrades during severe ruminal acidosis (41% dietary starch; Steele et al., 2011). Abeyta et al. (2022) also observed increased APP in plasma during a severe acidosis challenge (2.75% BW as

corn; ~21 kg). When rumen fluid from these grain-challenged donor cows was abomasally infused into non-grain challenged recipients, they did not observe an increase in APP.

Data suggest that rumen adaptation plays a role in the APP response. In the non-grain challenge model (Khafipour et al., 2009a; Khafipour et al., 2009c), where alfalfa hay was gradually replaced by pelleted alfalfa hay over 6 weeks (22% dietary starch), there was no systemic inflammatory response, whereas abrupt diet change in the grain challenge experiments led to an APP response (Khafipour et al., 2009b). Gott et al. (2015) found that a high starch diet with corn grain (~29% starch) did not increase SAA, while an acidosis induction diet which provided corn and wheat grain (~32% starch) suddenly fed for two days after cows had been on a control diet (~24% starch) resulted in increased SAA. Sudden dietary changes may be more to blame for the APP response than chronically elevated dietary starch. Others have also observed no significant difference or reductions in circulating Hp when increasing dietary starch (Table 6.1; Ertl et al., 2015; Dias et al., 2018; McCaughern et al., 2020; Haisan et al., 2021).

HOW IS THE MICROBIOME INVOLVED

Increasing dietary starch reduces measurements of bacterial diversity (Deusch et al., 2017; Plaizier et al., 2017; Neubauer et al., 2020). Also, grain vs. pelleted-alfalfa acidosis induction results in different microbial population shifts. Khafipour et al. (2009c) investigated the microbial population in the rumen of dairy cows who were subject to either grain or alfalfa acidosis challenges. They observed a 64-fold increase in ruminal *E. coli* during the grain challenge experiment. They also discovered that ruminal *E. coli* concentrations were the greatest predictor of severity of acidosis which was determined by serum Hp, rumen pH, and free rumen LPS. Grain-based acidosis shifted the *E. coli* populations to strains with greater virulence than during alfalfa-based challenges (Khafipour et al., 2011). The genes associated with virulence

were related to adhesion of *E. coli* to epithelial cells. Increases in strains of *E. coli* with greater adhesion capabilities during grain-based acidosis challenges may be related to the increased inflammation observed in grain vs. non-grain challenges. Increased adhesion capability is problematic during acidosis because non-keratinized cells which are exposed to the rumen contents are more susceptible to adhesion by microbes like *E. coli* (Gálfi et al., 1998). By both increasing *E. coli* and increasing the sloughing of the stratum corneum during acidosis (Steele et al., 2011), grain-based acidosis challenges may present more risk of inflammation compared to the chronic feeding models.

A more thorough investigation of the digestive tract microbiome was conducted by Plaizier et al. (2017). They observed increased *E. coli* in cecal and fecal contents of cows subjected to grain acidosis challenges. It seems that increasing starch in the rumen or its flow to the hindgut may increase problematic microbial populations, like *E. coli.* This alteration in gut bacteria may increase the risk of inflammation when feeding high-starch diets, but investigation in a greater diversity of diets is warranted.

DOES THE SOURCE OF STARCH MATTER?

Increases in APP may occur when starch concentrations are ≥30% of diet DM and when barley is the primary grain being fed. Late lactation cows fed 32% starch diets (barley grain and barley silage as starch sources) had elevated serum Hp (1,500 μg/mL). Peak lactation cows fed 45% of diet DM as barley grain (45% NFC, 25% NDF, 30% forage) had reduced rumen pH, increased ruminal LPS, and increased SAA, but milk yield increased by 4 kg/d nonetheless while milk fat yield was reduced (Emmanuel et al., 2008; Zebeli and Ametaj, 2009). Pan et al. (2017) increased dietary starch from 20% to 33% of the diet DM by increasing corn grain and observed reduced rumen pH and a 10-fold increase in ruminal LPS. Plasma IL-1β and IL-6 increased by

50% and 20%, respectively. Rumen epithelial protein abundance of TLR4 and pro-inflammatory cytokines were increased, pointing to the possibility that the rumen was the source of increased cytokines in plasma. Similar to Emmanuel et al. (2008), milk yield increased by 2 kg/d, while component yields only slightly increased due to reductions in component concentrations with the high starch diets. Studies investigating the interactions of starch concentrations with starch source (i.e. corn) or processing methods (i.e. ensiling, steam flaking, cracking, rolling) are needed as most data stems from wheat or barley-based diets.

FEEDING STARCH TO FRESH COWS

Few studies have examined periparturient cow APP responses to increasing dietary starch concentrations, but there is little evidence of enhanced inflammation. Knoblock et al. (2019) observed similar APP concentrations in post-partum cows fed 22% or 28% starch diets. McCarthy et al. (2015b) fed 21% or 26% starch diets and greater starch increased plasma Hp from 700 to 1,000 μg/mL. Increasing starch also increased plasma glucose and insulin while reducing plasma NEFA and BHB concentrations. Increases in monocyte function (Yasui et al., 2016) and improved energy balance (McCarthy et al., 2015a) may demonstrate improved health and resilience of the transition dairy cow fed additional starch. Albornoz et al. (2020) investigated both starch concentration (22% vs 28%) and starch processing method (dry ground vs. high moisture) and observed greater reactive oxygen and nitrogen species when feeding high moisture corn. They also observed increased Hp when increasing dietary starch in the high moisture corn diets which may indicate that enhancing rumen fermentability when feeding greater concentrations of starch is problematic. In contrast, Haisan et al. (2021) found that high starch diets (32%) post-partum resulted in lower Hp and SAA. Feeding greater dietary starch to transition dairy cows does not consistently alter APP (Figure 6.1).

HINDGUT STARCH CHALLENGES: ABOMASAL INFUSIONS

Abomasally infusing starch has not resulted in inflammation or gut barrier integrity loss even though it reduces fecal pH. Abeyta et al. (2019a) and Abeyta et al. (2019b) infused 4 kg/d of starch into the abomasum and fecal pH decreased from 6.8 to 5.9 and 7.2 to 5.8, respectively. van Gastelen et al. (2021a) and van Gastelen et al. (2021b) infused up 3 kg of corn starch and 3 kg of ground corn, respectively, directly into the abomasum and fecal pH dropped from 6.5 to 5.15 and 6.8 to 6.0. Even though fecal pH was reduced, SAA and Hp were unchanged as a result of starch infusion. van Gastelen et al. (2021a) infused cows with Co-EDTA (indigestible marker to estimate gut permeability) and did not observe any changes in gut permeability. These data indicate that increasing intestinal starch supply, independent of other factors, does not result in systemic inflammation.

Infusing starch abomasally dramatically increased fecal butyrate (van Gastelen et al., 2021a; van Gastelen et al., 2021b). Ruminal butyrate concentrations increase during ruminal acidosis as well (Khafipour et al., 2009b). Butyrate is an endogenous ligand for the hydroxycarboxylic acid receptor 2 (HCA2) which is involved in inflammatory signaling and tight junction formation (Plöger et al., 2012; Li et al., 2021). Butyrate produced during hindgut starch fermentation may provide benefits to the gut (Figure 6.2). Butyrate increased mRNA abundance of IL-10 (anti-inflammatory cytokine) in intestinal dendritic cells and macrophages which, when incubated with naive $CD4^{+}CD25^{-}$ T cells, promoted development of regulatory T-cells which suppress inflammatory signaling. Butyrate also increased IL-18 secretion in colon epithelial cells (Singh et al., 2014) which promotes a cell-mediated T cell response which may promote inflammation. Butyrate modulates intestinal barrier function and integrity by increasing tight junction protein expression (Peng et al., 2009; Yan and Ajuwon, 2017). Supplementing

butyrate to calves promotes rumen and hindgut development (Górka et al., 2018), but the authors acknowledge that little is known about the role of butyrate in the ruminant hindgut.

These data are compelling but there are gaps in our understanding of the effects of starch on gut health. For example, increased fecal butyrate indicates that butyrate concentration in the distal colon is increased; we have yet to determine whether butyrate is increased in other areas of the hindgut. Furthermore, feeding additional starch may affect mucosal barriers that provide further protection for the hindgut tissue (Kim and Ho, 2010; Steele et al., 2016), but this has not been investigated in dairy cattle.

CONCLUSIONS

The potential effects of increasing dietary starch on systemic inflammation has become widely discussed in the dairy industry. During grain challenges where diets are suddenly changed and forages are replaced by highly fermentable grains, acidosis and systemic inflammation occur. Extremely high concentrations of starch (≥30% of the diet DM) in barley or wheat-based diets may also lead to increased APP, but data indicate that sudden diet changes contribute to inflammation more than chronically elevated dietary starch. Altering dietary starch (20-32% of diet DM) for transition cows does not consistently affect APP. Starch infusion experiments demonstrate that increasing the supply of starch in the small intestine does not cause inflammation even though fecal pH is reduced. Increasing intestinal starch supply increases fecal butyrate concentrations, which may provide benefits in the gut, but further investigation is needed. Evaluation of the gut microbiome and hindgut mucosal layers may yield important insights into the effects of starch on gut health and inflammation.

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APPENDIX

Table 6.1. Experiments where lactating dairy cows were fed different dietary starch concentrations in chronic feeding regimens and inflammatory biomarkers were assessed.

Study	Starch Source	Starch concentration	DIM[1]	Key responses to additional starch[2]
Albornoz et al., 2020	Corn	22%, 28%	1	↑RONS when fed high moisture corn
Dias et al., 2018	Corn	23%, 29%	176 ± 18	↔ Hp ↓ rumen pH ↑Milk protein yield
Emmanuel et al., 2008	Barley	NR[3] (35%, 38%, 42%, 45% NFC)	60-140	↑Rumen LPS ↑SAA
Ertl et al., 2015	Corn, Wheat, Oats	7%, 12%	108 ± 90	↔ Hp
Gott et al., 2015	Corn, Wheat	24%, 28%, 32%	140 ± 16	↑SAA when fed acidosis induction diet ↔ SAA when fed high starch diet
Haisan et al., 2021	Corn, Barley	25%, 32%	-28 ± 3	↓Hp and SAA
McCarthy et al., 2015b	Corn	21%, 26%	1	↑Hp ↓NEFA and BHB
McCaughern et al., 2020	Corn, Wheat	15%, 22%	33 ± 2.5	↔ Hp ↑Ceruloplasmin ↓ rumen pH ↑Milk protein yield
Tayyab et al., 2022	Corn, Wheat	9%, 22%, 22%, 32%	61 ± 0.2	↑Hp ↔ mean rumen pH

[1]DIM at the start of the study

[2]BHB = β-hydroxybutyrate, NEFA = non-esterified fatty acid, RONS = reactive oxygen and nitrogen species, SAA = serum amyloid A, Hp = haptoglobin, ↔ = no change, ↑ = significant increase, ↓ = significant decrease.

Table 6.1. (cont'd)

NR = not reported, NFC = non-fiber carbohydrates.

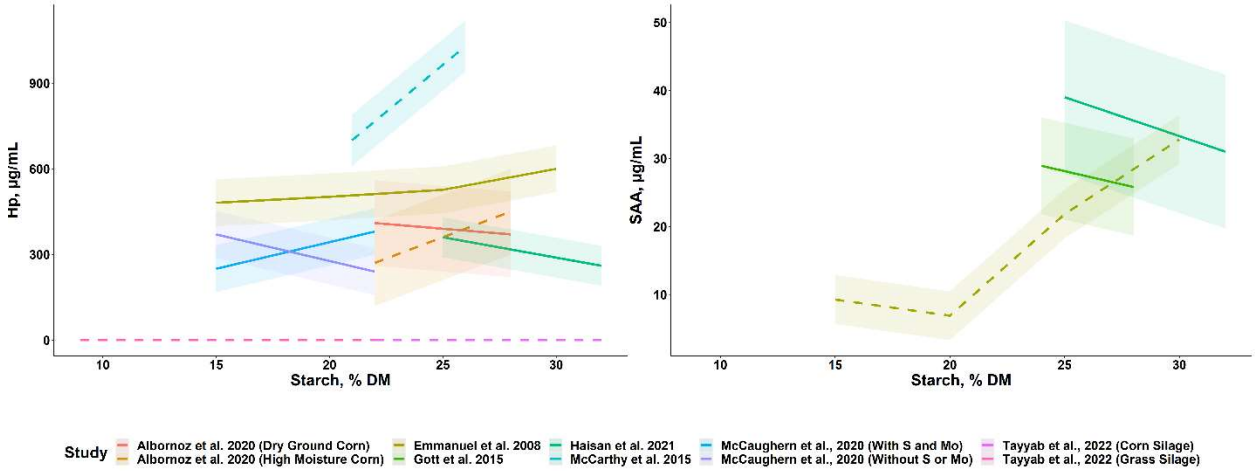

Figure 6.1. Summary of plasma haptoglobin (Hp) and serum amyloid A (SAA) means reported in chronic starch feeding experiments where lactating dairy cows were fed varying concentrations of dietary starch. Dashed lines indicate statistical significance detected in the experiment, whereas solid lines represent insignificant effects of starch concentration. Outliers for Hp (>1.5 ug/mL) were excluded from the graphic (1 study). Albornoz et al. (2020), Haisan et al. (2021), and McCarthy et al. (2015) publications reported experiments using periparturient dairy cows; others used cows ranging from 30 to 150 DIM.

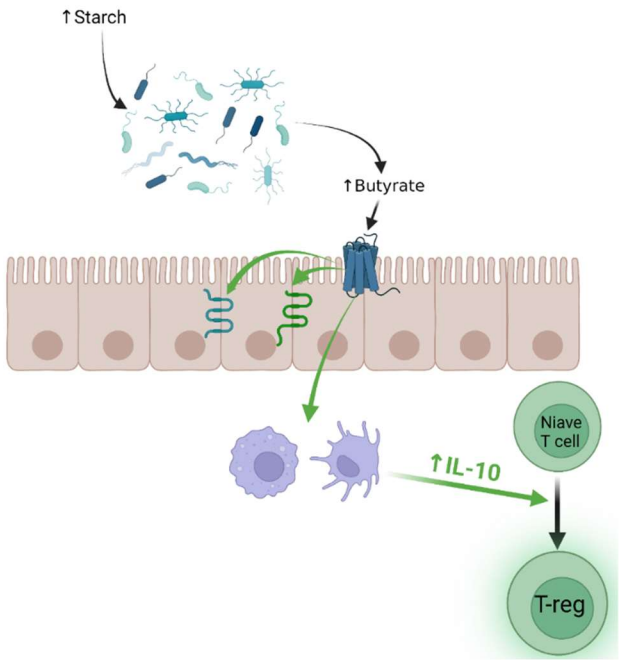

Figure 6.2. Possible effects of butyrate in the hindgut of dairy cows resulting from increased starch supply to the large intestine. Butyrate can increase tight junction mRNA and protein abundance in model species and cells. It also promotes IL-10 expression (anti-inflammatory) in dendritic cells and macrophages which helps to promote T-regulatory cell development (inflammation-suppressing cell type). These effects may help to mitigate the risk of intestinal inflammation.

CHAPTER 7: THE EFFECTS OF FEEDING α-AMYLASE ENHANCED CORN SILAGE WITH DIFFERENT DIETARY STARCH CONCENTRATIONS TO LACTATING DAIRY COWS ON MILK PRODUCTION, NUTRIENT DIGESTIBILITY, AND BLOOD METABOLITES

ABSTRACT

Corn silage is one of the most common ingredients fed to dairy cattle. Advancement of corn silage genetics has improved nutrient digestibility and dairy cow lactation performance in the past. A corn silage hybrid with enhanced endogenous α-amylase activity (Enogen, Syngenta Seeds LLC) may improve milk production efficiency and nutrient digestibility when fed to lactating dairy cows. Furthermore, evaluating how Enogen silage interacts with different dietary starch content is important because the ruminal environment is influenced by the amount of rumen fermentable organic matter consumed. To evaluate the effects of Enogen corn silage and dietary starch content, we conducted an 8-wk randomized complete block experiment (2-week covariate period, 6-week experimental period) with a 2 × 2 factorial treatment arrangement using 44 cows (n = 11/treatment; 28 multiparous, 16 primiparous; 151 ± 42 days in milk; 668 ± 63.6 kg of body weight). Treatment factors were Enogen corn silage (ENO) or control (CON) corn silage included at 40% of diet dry matter and 25% (LO) or 30% dietary starch (HI). The corn silage used in CON treatment was a similar hybrid as in ENO but without enhanced α-amylase activity. The experimental period began 41 d after silage harvest. Feed intake and milk yield data were collected daily, plasma metabolites and fecal pH were measured weekly, and digestibility was measured during the first and final weeks of the experimental period. Data were analyzed using a linear mixed model approach with repeated measures for all variables except for body condition score change and body weight change. Corn silage, starch, week, and their interactions were

included as fixed effects; baseline covariates and their interactions with corn silage and starch were also tested. Block and cow served as the random effects. Plasma glucose, insulin, haptoglobin, and serum amyloid A concentrations were unaffected by treatment. Fecal pH was greater for cows fed ENO vs. CON. Dry matter, crude protein, neutral detergent fiber, and starch digestibility were all greater for ENO than CON during wk 1, but differences were less by wk 6. The HI treatments depressed neutral detergent fiber digestibility compared to LO. Dry matter intake (DMI) was not affected by corn silage but was affected by the interaction of starch and wk; in wk 1, DMI was similar but by wk 6, cows fed HI had 1.8 ± 0.93 kg/d less DMI than LO cows. Milk, energy corrected milk, and milk protein yields were 1.7 ± 0.94 kg/d, 1.3 ± 0.70 kg/d, and 65 ± 27 g/d greater for HI than LO, respectively. In conclusion, ENO increased digestibility but it did not affect milk yield, component yield or DMI. Increasing dietary starch content improved milk production and feed efficiency without affecting markers of inflammation or metabolism.

Key words: forage, acute phase protein, carbohydrate, feed efficiency

INTRODUCTION

Corn silage is arguably the most important ingredient in North American dairy cattle diets. In the late 1990's nutritionists responded that corn silage was fed to 40-80% of lactating dairy cattle depending on region of the United States (Mowrey and Spain, 1999), and a similar survey found that 91% of lactating dairy cow diets and 82% of dry cow diets in the United States contain corn silage (Kellogg et al., 2001). Area harvested for corn silage in the United States has increased from 2.5 million hectares in 2018 to 2.7 million hectares in 2020, providing 1.3 million metric tons of corn silage in 2020 (USDA, 2021). Clearly, improving corn silage nutritive value could have substantial effects on dairy production in the United States.

An engineered corn trait providing enhanced endogenous α-amylase activity in the endosperm (Cueva et al., 2021b), which was originally designed for ethanol production, has increased feed efficiency when fed to beef cattle as dry rolled corn (Volk et al., 2021). When fed to dairy cattle as corn silage, there is evidence that it may increase milk yield by 2 to 3 kg/d, though it had less impact when evaluated on an ECM basis (Rebelo et al., 2020; Cueva et al., 2021b). Further research on this trait in different dietary formulations will be needed to elucidate whether it consistently improves the milk yield and feed efficiency of lactating dairy cows.

What has yet to be investigated is whether α-amylase enhanced corn silage (Enogen, Syngenta Seeds LLC) interacts with the concentration of starch fed in the total mixed ration. One previous experiment investigating exogenous amylase fed at different dietary starch contents found that in low starch diets (~25% dietary starch) the exogenous amylase enzyme increased NDF digestibility (Weiss et al., 2011) but did not affect milk yield. It may be that in diets with >25% dietary starch that addition of amylase is unwarranted but this has not been investigated with α-amylase enhanced corn silage. Furthermore, increasing the dietary starch content from 21% to 31% of the diet by replacing forages with ground or high moisture corn increased DMI, reduced total chewing time, reduced ruminal pH, and increased milk yield but reduced milk fat yield in peak lactation cows (Oba and Allen, 2003a). Feeding increased amounts of starch also tends to promote body tissue accretion when replacing other energy sources such as fat and fiber (Reynolds et al., 2001; Boerman et al., 2015).

Starch from ensiled sources like silage or high moisture corn increases in digestibility as ensiling time increases (Ferraretto et al., 2015). Previous research with Enogen silage investigated impacts after more than 200 days of fermentation, when starch digestibility is typically quite high (Cueva et al., 2021b). Such a long fermentation may mask the benefits of

enhanced starch digestibility resulting from increased enzyme activity immediately post-harvest. Investigations of Enogen silage early after harvest may yield different results than those after longer fermentations. Also, improved silage fermentation may improve diet adaptation when feeds and diets are abruptly changed; Enogen may improve adaptation of lactating cows to a new diet.

An emerging area of research is investigating the effects of diet fermentability on systemic inflammation of lactating dairy cows. Ruminal grain challenge experiments have observed that bouts of ruminal acidosis may lead to systemic inflammation (Li et al., 2011). Khafipour et al. (2009b) observed that increasing fermentable starch supply of a diet with wheat and barley pellets resulted in a systemic inflammatory response likely related to ruminal or hindgut acidosis. Evaluation of systemic inflammation in chronic feeding studies is less common. Intestinal starch infusions designed to cause hindgut acidosis demonstrated that fecal pH is consistently decreased, yet neither systemic inflammatory responses nor evidence of impaired gut function were observed (van Gastelen et al., 2021a; van Gastelen et al., 2021b). These data demonstrate that increasing starch flow to the small intestine may not cause inflammation but increasing amounts of fermentable grain fed to dairy cattle may cause inflammation stemming from ruminal acidosis. It also may be that sudden dietary changes lead to inflammation more than a chronically high-starch diet (Gott et al., 2015).

Therefore, the objectives of our experiment were to evaluate the effects of Enogen corn silage that had been ensiled for 41 days fed with different dietary starch contents on milk yield, component yield, plasma biomarkers of inflammation and metabolism, fecal pH, and total-tract nutrient digestibility. Our hypothesis was that the enhanced amylase activity of Enogen silage would increase starch digestibility in the rumen which would increase total tract starch

digestibility, milk yield, and milk component yields. We also hypothesized that increasing dietary starch content would increase milk production and would not affect circulating plasma acute phase protein concentrations.

MATERIALS AND METHODS

Cows, Treatments, and Design

This experiment was approved by the Michigan State University Institutional Animal Care and Use Committee. The 44 cows (28 multiparous, 16 primiparous) enrolled in this experiment were housed in individual tie stalls at the Michigan State Dairy Cattle Teaching and Research Center. Cows were fed once daily for ad libitum consumption (~10% refusal rate) at 0800 h and had ad libitum access to water. Cows were milked three times daily in a milking parlor at 0445, 1245, and 2045 h. Cows were let outside for exercise for approximately 1 h prior to each milking. At enrollment, cows averaged 151 ± 42.4 DIM with a range from 78 DIM to 225 DIM, 668 ± 63.6 kg of body weight (BW), and 45 ± 7.3 kg/d of milk with 3.8 ± 0.51% fat and 3.1 ± 0.24 % protein.

The experimental treatments were arranged in a 2 × 2 factorial layout. The first factor was corn silage, and the second factor was dietary starch content. The corn silage varieties were Enogen (ENO) or the control silage (CON), which was an isoline hybrid that did not have the inserted α-amylase gene. Both corn silage varieties were included at 40% of the diet DM. Dietary starch contents were formulated at either 25% of diet DM (LO) or 30% of diet DM (HI) by replacing soybean hulls with dry ground corn. These factors created four treatment combinations which were as follows: CON-LO, CON-HI, ENO-LO, and ENO-HI. Additionally, diets were composed of alfalfa silage, cottonseed and grain mixes. The diets were balanced to meet nutrient

requirements of dairy cows producing 50 kg/d of milk with 3.9% fat and 3.1% protein using Ag Modeling and Training Systems software (AMTS LLC., Groton, NY).

Silage plots (~6 hectares each) were side by side and managed identically. The Enogen silage was E110F4 (Syngenta), and the control variety was G11V76 (GoldenHarvest). Both varieties were planted at 70,074 seeds/hectare on May 6, 2021, and harvested using a self-propelled Claas forage harvester equipped with a kernel processor on September 10, 2021. Silages were ensiled in bags with a *Lactobacillus buchnerii* 40788 inoculant which also included *Pediococcus pentosaceus* (Crop-N-Rich Stage 2 100, VitaPlus). Bags were sealed for 41 d before feeding began.

The experiment was a randomized complete block design with cows blocked by DIM and milk yield. Treatments were randomly assigned to cows within each block (n = 11 cows per treatment) using a random number generator (Microsoft Excel). The experiment had a 2-wk covariate period where cows were fed a diet with 26% dietary starch content, 30.8% NDF, 17% CP, and a brown mid-rib corn silage hybrid. The 2-wk covariate period was followed by a 6-wk experimental period. Using variance from a previous experiment (Ylioja et al., 2018), at $\alpha = 0.05$ and a power of test (1-β) of 0.90, this design was expected to be sufficient to detect a change in milk yield of 3 kg/d. Covariate period summary statistics for cows enrolled on each treatment are listed in Table 7.1.

Data Collection and Sample Analysis

Feed intake was measured daily throughout the experiment. Milk samples were collected at all milkings during the last 4 days of the covariate period for use as parameters in statistical analysis. Milk samples were taken at all three milkings one day per week throughout the

experimental period. Individual milk samples were analyzed by Central Star Cooperative (Grand Ledge, MI) for milk fat, milk true protein, and MUN concentrations using Fourier transform infrared spectroscopy and SCC by flow cytometry (Bentley FTM/FCS, Bentley Instruments Inc.). Daily milk yield was collected by an automatic milk yield recording system in the milking parlor (BouMatic).

Body weight was measured 3 times per week after the 1245 h milking. Body condition score was measured on a 1-5 scale (Wildman et al., 1982) each week, independently, by 2 trained investigators.

Blood samples were collected at 0700 h, ~1 h prior to feed delivery, on d 0 and weekly thereafter throughout the experimental period. Blood was collected by coccygeal venipuncture with tubes containing K_2EDTA. Blood samples were immediately placed on ice and transported to the lab where they were centrifuged for 15 min at 1,500 × *g* and 4 °C to collect plasma. Two aliquots of plasma were kept at -20°C for later analysis. Plasma was analyzed for glucose by enzymatic methods (kit #997-03001; Fujifilm, Osaka, Japan), insulin using a bovine specific ELISA (no. 10–1201–01; Mercodia AB, Uppsala, Sweden), haptoglobin (Hp) using a bovine specific ELISA (kit # HAPT-11; Life Diagnostics, West Chester, PA), and serum amyloid A (SAA) using a multispecies ELISA (kit # TP 802; Tridelta Development Ltd., Maynooth Kidare, Ireland). The inter-assay CV were 2.0, 3.1, 5.7, and 12.0 for glucose, insulin, Hp, and SAA, respectively. Intra-assay CV were 6.2, 6.1, 6.0, and 7.3 for glucose, insulin, Hp, and SAA, respectively.

The experiment had two apparent total-tract digestibility measurement periods to investigate how digestibility of the diets may have changed as ensiling time of the silages increased. Feed ingredients and diet refusal samples were collected from d 4 to d 8 and from d 39

to d 42 of the experimental period. Feed and refusal samples were composited within period. Also, fecal samples (~300 g) were caught during defecation or collected from rectum collected every 9 h over d 5-8 and d 38-42 and composited by cow within measurement period. Feed ingredient samples were sent to Cumberland Valley Analytical Services (CVAS) and analyzed for DM (AOAC International, 2000), CP (Leco FP-528 N Combustion Analyzer, Leco Corp., St. Joseph, MO), NDF (Van Soest et al., 1991), aNDFom (Mertens, 2002), in vitro NDF digestibility (Goering and Van Soest, 1970), ADF (method 973.18; AOAC International, 2000), starch (Hall, 2009), 7 h in vitro starch digestibility (Goering and Van Soest, 1970; Richards et al., 1995), sugar (Dubois et al., 1956), crude fat (2003.05; AOAC International, 2006), fatty acids (Sukhija and Palmquist, 1988), and minerals (2003.05; AOAC International, 2006). Both fecal and refusal samples were analyzed for DM, aNDFom, NDF, starch, and CP. We used undigested NDF after 240 h of in vitro fermentation (uNDF240) as the internal marker to estimate fecal output. Then apparent total tract nutrient digestibility by dividing nutrient digested (nutrient intake-nutrient in feces) by nutrient intake. This was done to calculate digestibility during both wk 1 and wk 6.

Additional samples of both control and Enogen silages were collected 3 times per week during the experimental period and frozen at -20 °C. Each sample was analyzed for fermentation metabolites and silage pH at CVAS. Briefly, a thawed corn silage sample (~25 g) was diluted with 200 mL of deionized water overnight. The mixture was blended and strained through 20 µm filter paper. The extract was then analyzed for pH (Mettler DL12 Titrator, Mettler-Toledo, Inc.), ammonia (Labconco Rapidstill II, Labconco), lactic acid (YSI 2700 Select Biochemistry Analyzer), VFA, and other silage fermentation metabolites (Perkin Elmer AutoSystem gas chromatograph).

Additional fecal samples were collected one time each week approximately 12 h after feeding for determination of fecal pH using methods described by Branstad et al. (2017). 12 h after feeding was selected because this is when fecal pH reached a minimum in a grain challenge model (Sulzberger et al., 2016). Briefly, fecal pH was immediately after sample collection measured by mixing feces (~50 g) with water at a 1:1 ratio by weight. The feces and water were homogenized with a blender (#58148A; Hamilton Beach) and strained through cheesecloth. The pH of the liquid was measured using a handheld pH probe (PH100; Extech Instruments) calibrated according to manufacturer instructions (pH 4.0 and 7.0).

Statistical Analysis

The data were analyzed with a linear mixed model approach using the GLIMMIX procedures of SAS (9.4; SAS Institute, Cary NC). Models included the fixed effects of corn silage, starch, and week along with their two- and three-way interactions. The model also included fixed effects parity (primiparous vs. multiparous) and pre-treatment covariate along with their interactions with starch and corn silage. Block and cow were included as a random effect. Corn silage, starch, week, and their interactions were always included in the model. Parity, covariate, and their interactions with starch, corn silage, and week remained in the model if $P \leq 0.10$. Model hierarchy was always maintained. Weekly averages for milk yield and DMI were calculated and used for analysis. Weekly milk yield, component yields, plasma metabolites, DMI, digestibility (wk 1 & wk 6), and fecal pH were evaluated as repeated measures within cow. The covariance structures were selected based on the structure that yielded the lowest AIC for each respective variable. We evaluated compound symmetry, autoregressive, autoregressive heterogenous, and Toeplitz covariance structures for each variable. Change in BW per day (ΔBW) and change in BCS per week (ΔBCS) were determined by conducting a simple linear

regression for each cow. The slope coefficient from these regressions were analyzed. Statistical differences were declared at $P \leq 0.05$ and tendencies at $P \leq 0.10$. All models were visually evaluated to ensure normality of residuals. If the normality assumption was violated, the variable was log-transformed, and the model was re-fit. Furthermore, if an observation had a Studentized residual ≥ 4 or ≤ -4, the observation was removed and the model was re-fit.

RESULTS

Ingredient and diet nutrient composition

The control and Enogen silages had similar nutrient composition (Table 7.2). 30-h in vitro NDF digestibility was 45% for control and 52% for Enogen silage and 7-h in vitro starch digestibility was 67% and 74% for control and Enogen, respectively, during wk 1. During wk 6, in vitro digestibilities were similar for control and Enogen silages. The LO and HI concentrate mixes differed in the amount of NDF and starch by design (Table 7.2). Diets were similar in nutrient composition except for starch and NDF concentrations (Table 7.3). Targeted starch concentrations of 25% and 30% for LO and HI treatments, respectively, matched well with measured values.

Silage fermentation analysis

Weekly silage samples collected during the 6-week experimental period contained 6.5 ± 1.49% total VFA (DM basis; mean ± SD) for control silage and 7.0 ± 2.10% VFA for Enogen silage (Figure 7.1). Acetic acid was 1.2 ± 0.75% of DM and 1.6 ± 0.78% of DM for control and Enogen silages, respectively. Lactic acid was 5.3 ± 1.49% for control and 5.3 ± 1.72% for Enogen silage. Silage pH was 3.91 ± 0.089 for control and 3.93 ± 0.094 for Enogen. 7-h in vitro starch digestibility was 66.2 ± 6.32 and 65.5 ± 4.33% of starch for control and Enogen silage, respectively.

Metabolism and inflammation

Plasma glucose, insulin, haptoglobin, and serum amyloid A concentrations were unaffected by corn silage variety or starch concentrations (Table 7.4). Each biomarker was affected by week but no interactions of week with corn silage or starch were observed.

Nutrient intake, digestibility, and fecal pH

A starch × week interaction was observed for DMI ($P = 0.02$; Table 7.5). By wk 6, cows consuming HI ate 1.8 ± 0.93 kg/d less than cows consuming LO ($P = 0.05$; Figure 7.2). The starch × week interaction also affected NDF, starch, and CP intake ($P < 0.01$). NDF intake was greater for LO in both periods ($P < 0.01$). During wk 1, HI cows consumed 1.4 ± 0.27 kg/d more starch than LO cows ($P < 0.01$) but by wk 6, HI cows were only eating 0.6 ± 0.27 kg/d more starch than LO cows. Intake of CP during wk 1 was similar across groups ($P = 0.73$) but by wk 6, cows receiving LO consumed 0.6 ± 0.17 kg/d more CP than cows receiving HI ($P < 0.01$).

Dry matter digestibility (DMD) was affected by starch × week ($P < 0.01$) and corn silage × week ($P < 0.01$) interactions. In both wk 1 and 6, HI and LO resulted in similar DMD ($P > 0.80$). During wk 1, ENO cows had 3.4 ± 0.38% units greater DMD ($P < 0.01$), but by wk 6, DMD was similar across corn silages ($P = 0.22$; Figure 7.3). NDF digestibility (NDFD) was affected by a week × corn silage × starch interaction. During wk 1, ENO-LO had the greatest NDFD (48.7 ± 0.95%; $P < 0.01$), followed by ENO-HI and CON-LO (42.2 ± 0.95%, 41.5 ± 0.95%; $P = 0.94$), whereas CON-HI had the least NDFD in wk 1 (33.3 ± 0.90%; $P < 0.01$). During wk 6, CON-LO and ENO-LO had similar NDFD ($P = 0.18$; 45.4 ± 0.95%, 47.5 ± 0.95%), and CON-HI and ENO-HI also had similar NDFD ($P = 0.38$; 38.6% ± 1.0%, 41.4% ± 0.95%). Cows fed LO had greater NDFD than cows fed HI during both wk 1 and wk 6 ($P < 0.01$). Starch digestibility was affected by starch × week ($P = 0.04$) and corn silage × week ($P <$

0.01) interactions. Within week, HI and LO starch digestibility did not differ ($P > 0.23$). Starch digestibility for LO during wk 1 was greater than starch digestibility for HI during wk 6 ($P = 0.04$). During wk 1, ENO tended to have 0.4 ± 0.02% units greater starch digestibility than CON ($P = 0.07$), but during wk 6 the starch digestibility was similar for CON and ENO ($P = 0.41$). CP digestibility was also affected by the starch × week ($P < 0.01$) and corn silage × week ($P < 0.01$) interactions. In wk 1, HI and LO had similar CP digestibility ($P = 0.31$) but during wk 6, LO had 1.9 ± 0.79% units greater CP digestibility than HI ($P = 0.01$). During wk 1, ENO had 4.0 ± 0.73% units greater CP digestibility than CON ($P < 0.01$) but during wk 6 they were similar ($P = 0.91$).

Fecal pH (Table 7.5) was not affected by starch ($P = 0.75$) but it tended to be affected by corn silage ($P = 0.06$), with an increase of 0.07 ± 0.033 units for ENO compared to CON.

Bodyweight and BCS

The change in BW was greater for LO than HI ($P = 0.05$; Table 7.5), whereas corn silage had no effect on ΔBW. Change in body condition score (ΔBCS) was not affected by corn silage, starch, or the interaction ($P > 0.46$).

Milk yield and milk composition

Milk yield tended to be greater for HI than LO ($P = 0.10$; Table 7.6) but was not affected by corn silage ($P = 0.38$). Energy-corrected milk yield also tended to be greater for HI than LO ($P = 0.07$) and was not affected by corn silage ($P = 0.21$). Feed efficiency (FE), calculated as ECM/DMI, tended to be greater for HI than LO ($P = 0.07$) and tended to be affected by the interaction of corn silage × week ($P = 0.09$), such that cows receiving ENO had reduced FE during wk 6 compared to wk 1 ($P <0.01$) while cows fed CON had similar FE in both wk 1 and

wk 6 ($P = 0.75$). Within week, cows consuming CON or ENO had similar FE ($P > 0.65$) in both wk 1 and 6.

Milk fat concentration tended to be affected by corn silage × starch interaction ($P = 0.06$) but after Tukey adjustments none of the treatments differed from each other ($P \geq 0.27$). Fat yield was only affected by week ($P < 0.01$). Milk protein concentration tended to be affected by starch × corn silage × week interaction ($P = 0.10$). CON-LO tended to have greater milk protein content than ENO-HI and CON-HI during wk 6 ($P < 0.08$). Milk protein yield was affected by the interaction of starch × week ($P = 0.01$) such that during wk 3 ($P < 0.01$), 4 ($P = 0.04$), and 5 ($P = 0.04$), protein yield was greater for cows fed HI than cows fed LO by 142 ± 41.6, 89 ± 41.8, and 92 ± 44.6 g/d, respectively (Figure 7.4). Milk lactose concentration was affected by corn silage × week ($P = 0.06$) and starch × week ($P = 0.04$) interactions. During wk 2 and 3, CON tended to increase lactose content, while during wk 6, ENO tended to increase lactose content ($P = 0.09$). The starch × week interaction was such that HI had greater lactose content than LO during wk 5 and 6 ($P < 0.05$). Lactose yield was affected by starch ($P < 0.01$); HI cows yielded 104 ± 3.6 g/d more lactose than LO. Milk urea nitrogen was affected by a starch × corn silage × week interaction ($P = 0.02$). During wk 1, CON-LO tended to have greater MUN than ENO-LO ($P = 0.08$) and CON-LO had greater MUN than CON-HI and ENO-LO during wk 3 ($P < 0.03$). In wk 6, ENO-LO and CON-LO had greater MUN than CON-HI ($P < 0.01$). Furthermore, CON-LO and ENO-LO had greater MUN than ENO-HI during wk 6 ($P < 0.01$). Somatic cell score was not affected by treatments ($P > 0.15$).

DISCUSSION

Investigations of α-amylase enhanced corn in finishing beef cattle have revealed slight increases in gain:feed ratio when fed to as dry rolled corn but not when fed as high moisture corn

(Volk et al., 2021). To date, Cueva et al. (2021b) and Rebelo et al. (2020) have published the only two experiments investigating this trait in corn silage for dairy cattle diets. Previous results in dairy cattle are equivocal, as Enogen silage has increased milk yield but not energy-corrected milk yield, slightly reduced methane intensity and slightly increased FE (Rebelo et al., 2020; Cueva et al., 2021). However, neither experiment evaluated Enogen silage when fed with different contents of dietary starch. This may be important, as increased ruminal starch digestibility puts cows at increased risk of developing subacute ruminal acidosis, especially in diets with greater levels of starch (Humer et al., 2018).

Both silage sources had silage pH < 4.0 at the start of feeding, which is considered an indicator of adequate fermentation (Kung et al., 2018). In vitro starch digestibility (7-h) was also similar for both hybrids. Previous research has reported similar pH between α-amylase enhanced and control silages (Hellings et al., 2019; Cueva et al., 2021b).

Metabolism and inflammation

We did not observe any changes in plasma glucose or insulin concentrations. Previous research found that increasing dietary starch content by removing grass hay and increasing corn silage increased plasma glucose in early lactation (~30 DIM) but not later lactation (~190 DIM) cows (Piccioli-Cappelli et al., 2014). Insulin increased in response to greater starch supply in both early and late lactation cows in that experiment. Similarly, Boerman et al. (2015) increased starch from 16 to 32% of the diet DM by removing alfalfa silage and increasing high moisture corn and observed similar circulating glucose concentrations but increased insulin for cows fed greater starch. More modest increases in starch (27 vs. 21%) provided by dry ground corn did not affect circulating glucose concentration (Sánchez-Duarte et al., 2019). Providing additional starch (high moisture corn, corn silage) in place of forage fiber can increase glucose supply,

likely from increased propionate production in the rumen, although it does not consistently increase regulated plasma glucose concentrations. When starch replaces non-forage fiber, such as soybean hulls as we did in our experiment, the effect seems less dramatic, which aligns with the phenomenon that feeding non-forage fiber yields more propionate than forage NDF (Clark and Armentano, 1997; Mullins et al., 2010; Sullivan et al., 2012).

Insulin promotes tissue accretion, which can promote body weight gain in cows fed diets with greater starch content (Reynolds et al., 2001; Piccioli-Cappelli et al., 2014; Boerman et al., 2015). In this study we did not observe increased insulin concentrations or BW in cows fed the HI diet; in fact, we observed the opposite, as ΔBW was greater for cows fed LO vs. HI. Weiss (2019) also observed greater ΔBW when reduced starch diets were fed; he hypothesized that metabolizable amino acid supply limited milk yield in reduced starch diets leading to energy partitioning to tissue. Feeding starch in place of fiber increases net portal appearance of total and essential amino acids and plasma concentrations of His, Pro, and Asn (Cantalapiedra-Hijar et al., 2014). Milk synthesis requires both energy and protein, and subtle deficiencies in AA supply that decrease expression of proteins involved in milk lactose or fatty acid synthesis may result in decreased milk energy secretion, reduced utilization of substrates from the bloodstream, and greater substrate supply to other tissues. The impact of starch concentration on BW in the current study may be a reflection of dietary impacts on metabolizable AA supply and downstream repartitioning of nutrients, albeit minor. Alternatively, given the lack of corresponding treatment effects on ΔBCS, rumen or gut fill may have been partially responsible for differences in ΔBW as LO had greater DMI and NDF intake than HI. When forage fiber content increases, weight of rumen contents also increased (Shaver et al., 1988), but in this case we increased dietary NDF by

replacing corn grain with non-forage fiber which may not accumulate in the rumen or hindgut as forage fiber does.

Inflammation has gained greater attention in dairy cows (Bradford et al., 2015) because greater concentrations of biomarkers of inflammation are associated with poorer health and performance (Bertoni et al., 2008). This phenomenon has been tied to diet formulation, as experimentally induced ruminal acidosis results in increased markers of inflammation (Khafipour et al., 2009b; Li et al., 2011). Using a meta-analytic approach, Zebeli et al. (2012) determined that cows consuming barley and wheat-based diets with > 44% of diet DM as concentrates or < 39% NDF may be at greater risk of inflammation. If these results were applicable to corn-based diets, it would present challenges for feeding high-producing cows because concentrate feeding is among the most efficient ways to provide digestible energy to dairy cows. Starch is particularly useful because it increases milk and milk protein yields as well as feed efficiency (Boerman et al., 2015; Morris et al., 2020). The cutoffs derived by Zebeli et al. (2012) suggest that each of our four diets were at risk of causing systemic inflammation (≤ 30% NDF and 48% concentrates). However, we found no evidence of inflammatory responses to feeding starch (from dry ground corn and corn silage) up to 30% of diet DM. Haptoglobin and SAA are widely used acute phase protein biomarkers of inflammation, and the concentrations reported herein are well below thresholds used to identify sub-clinical or clinical inflammatory conditions (Ceciliani et al., 2012; Quanz et al., 2022).

Even though grain challenge acidosis induction models lead to inflammation, our data demonstrate that chronically high starch diets do not necessarily result in inflammation. Further supporting this notion, Gott et al. (2015) fed cows a 28% starch diet or an acidosis induction diet in the same experiment. They observed that SAA was similar between the control (24% starch)

and 28% starch diets. When cows were fed a SARA induction diet (32% starch; wheat replaced wet corn gluten feed) for 2 days, plasma SAA concentrations doubled. We speculate that chronically high starch diets may not increase biomarkers of inflammation, but that sudden dietary changes that rapidly increase fermentable substrate in the rumen may result in inflammation. Further supporting this hypothesis, Khafipour et al. (2009a) induced ruminal acidosis by gradually increasing the supply of alfalfa pellets over 5 weeks, which did not result in an inflammatory response; in contrast, a sudden grain challenge model elicited an inflammatory response (Khafipour et al., 2009b). Additional research in a wider variety of diets with a broader set of measurements related to inflammation (APP, cytokines, gut histology and integrity measures) is warranted to determine what concentrations or sources of starch may result in systemic inflammation.

Nutrient intake and digestibility

Nutrient digestibility is affected by dietary nutrient concentrations and DMI (de Souza et al., 2018). It is well documented that feeding increased dietary starch content reduces the total tract NDFD of diets fed to dairy cattle (Ferraretto et al., 2013; Boerman et al., 2015; Dias et al., 2018). In the most recent revision of the nutrient requirements of dairy cattle (NASEM, 2021), NDFD is modeled as a function of DMI and dietary starch content. For each unit increase in dietary starch content above 26% of DM, the model estimates that NDFD will drop by 0.6 percentage units. Directionally, our results agree with this model, but we observed double the reduction in NDFD than predicted by NASEM (2021). It may be that the corn sources fed in our experiment were more rumen degradable than the average of the NASEM (2021) database, which may depress NDFD more than starch degraded post-ruminally. The committee acknowledged that ruminal starch degradability likely affects this relationship between NDFD

and dietary starch content. Ferraretto et al. (2013) observed this phenomenon in a meta-analysis as ensiled corn grain reduced NDFD more than dry or steam-flaked corn sources.

Increasing starch concentration has resulted in no change (Boerman et al., 2015), reduced (Morris et al., 2020), or even increased starch digestibility (Sánchez-Duarte et al., 2019). de Souza et al. (2018) reported from a meta-regression that each 1-unit increase in dietary starch content results in a 0.19-unit decrease in starch digestibility. They also observed regional differences; observations from Georgia exhibited a more negative relationship between starch concentration and digestibility than those from Ohio and Michigan. Dietary starch content effects on starch digestibility appear very reliant on the digestibility of the starch source being used (Ferraretto et al., 2013), such that increasing supply of highly fermentable sources like ensiled or very finely ground corn may be positive while increasing the amount of starch from less fermentable sources like whole, cracked, or coarsely ground corn may reduce starch digestibility.

We observed a slight increase (0.4 ± 0.02%) in starch digestibility for ENO vs. CON during wk 1, but during wk 6 both in vivo and in vitro starch digestibility were similar for CON and ENO. This may be because longer ensiling time increases starch digestibility (Ferraretto et al., 2015) which may be enough to wash out any additional effect of the enhanced α-amylase activity. Hellings et al. (2019) observed that starch digestibility of silage with the α-amylase trait was 20% units greater than its counterpart prior to ensiling, but after ensiling it was only 2% units greater. Cueva et al. (2021b) observed similar in vitro starch digestibility between α-amylase enhanced corn silage and the control silage; even though α-amylase activity remained 14 times greater in α-amylase enhanced silage after >200 days of ensiling (Cueva et al., 2021b), the ensiling process increases the digestibility of starch in corn silage and likely reduces the starch digestibility advantage of having additional α-amylase activity.

Exogenous α-amylase supplementation (as opposed to genetic integration of α-amylase in corn grain) demonstrates slightly increased total tract starch digestibility (Pech-Cervantes et al., 2022), but average starch digestibility was lower across studies (~95%) than we observed here. Exogenous amylase may enhance ruminal starch digestion, but hindgut starch digestion may largely compensate for differences in ruminal starch digestion (Nozière et al., 2014). Additionally, the rumen microbiota has native amylase activity, and the addition of Enogen silage or exogenous amylase does little to affect total ruminal amylase activity (Hristov et al., 2008; Cueva et al., 2021b). Enogen silage may improve starch digestibility early after harvest (< 45 d) but it seems this effect is diminished after at least 80 d of ensiling.

A recent meta-analysis observed that exogenous amylase does not consistently affect NDF digestibility (Pech-Cervantes et al., 2022), even though some individual experiments have observed substantial increases in NDFD when amylase was fed (Klingerman et al., 2009; Gencoglu et al., 2010; Weiss, 2019). Previous experiments with Enogen silage have not reported effects on NDFD (Rebelo et al., 2020; Cueva et al., 2021b), but in both cases silages were ensiled longer prior to feeding, which may have removed the advantage of Enogen corn silage that we observed in our experiment. The reasons for the enhanced total tract NDFD observed here, and previously with exogenous α-amylase, are not entirely clear. We, and Tricarico et al. (2008), speculate that α-amylase may increase ruminal concentrations of starch hydrolysis products like maltose, dextrin, and glucose, which increase ruminal fibrolytic capacity through increased bacterial populations or enhanced bacterial function. Cueva et al. (2021a) observed a reduction in *Rikinella* and *Bifidobacterium* when Enogen silage was fed demonstrating the rumen microbiome may play a role in the observed responses. Exogenous amylase may increase growth of *Butyvibrio fibrosolvens*, *Selenomonas ruminantium,* or *Megasphaera elsdenni* (Tricarico et al.,

2008). Metagenomic or metatranscriptomic methods may be useful in determining if exogenous amylase or Enogen silages alter the functional capacity of the rumen microbiome, especially capacity for NDF digestion.

Crude protein digestibility was also greater for ENO early after harvest with diminished effects over time; there was no effect of ENO on CP digestibility during wk 6. It may be that enhanced ruminal starch digestibility during wk 1 reduced the amount of starch entering the small intestine which limited hindgut microbial protein production and decreased microbial protein in feces. This is in line with the tendency for increased fecal pH when ENO was fed during our experiment. Exogenous α-amylase does not appear to affect CP digestibility (Pech-Cervantes et al., 2022).

Milk yield and milk composition

Even though we observed increased DM digestibility with ENO and similar DMI for each silage, there was not an increase in milk yield or ΔBW when ENO was fed. Previous research demonstrated that Enogen silage increased milk yield but not ECM (Rebelo et al., 2020). Cueva et al. (2021b) observed a 2 kg/d increase in milk yield ($P < 0.01$) and a 1.4 kg/d numerical increase in ECM ($P = 0.12$). Feed efficiency was similar for ENO and CON in our study, which is contrary to previous results where FE was increased by 3% (Cueva et al., 2021b); in that experiment, however, the Enogen silage had greater starch concentration than control, and greater starch concentration resulted in greater FE in our study. Greater dietary starch content does not always result in increased FE (Boerman et al., 2015; Dias et al., 2018), and is likely dependent on what starch is substituted for and whether it changes DMI. In our experiment, starch replaced non-forage fiber which had limited effects on gut fill, but when starch replaces forage fiber it would reduce the filling effect of the diet and lead to increased DMI, as observed

by Boerman et al. (2015) and Dann et al. (2015). It also may depend on ruminal starch degradability, as more rumen degradable starch sources depress intake as a result of the hypophagic effects of ruminal propionate (Allen and Bradford, 2012; Albornoz and Allen, 2018).

Even though increasing dietary starch contents to 30% of diet DM does not consistently affect gross measures of FE, it does often increase N use efficiency (Huhtanen and Hristov, 2009). In our study, increasing dietary starch content reduced MUN. It also increased milk protein yield despite decreased CP intake which demonstrates greater N use efficiency. Morris et al. (2020) fed starch in place of fat to late-lactation Jersey cows and observed that milk N as a proportion of N intake increased from 29% to 32%. Dias et al. (2018) and Oba and Allen (2003a) also observed increased milk protein concentration and yield with additional starch. Starch likely promotes microbial protein production in the rumen by providing more fermentable organic matter than fiber (Oba and Allen, 2003b); in turn, microbial protein can increase metabolizable AA supply for milk protein synthesis. Greater starch concentrations may also increase milk protein through insulin signaling, which has been shown to influence milk protein synthesis (Griinari et al., 1997; Menzies et al., 2009). We did not observe increased pre-feeding insulin concentrations in response to increased starch, it is possible that insulin may have been greater after meal bouts (Oba and Allen, 2003a) in cows fed HI diets.

Feeding diets with greater dietary starch content or increasing rumen fermentability of starch are risk factors for milk fat depression (Ramirez Ramirez et al., 2015). Milk fat depression is caused by the incomplete biohydrogenation of polyunsaturated fatty acids which leave the rumen and down-regulate genes related to milk fat synthesis in the mammary gland (Osorio et al., 2016). We observed a tendency for a silage × starch interaction for milk fat content; fat concentration was greater for cows fed CON-LO. However, milk fat yield was similar for all

treatments. Diets likely did not dramatically alter rumen metabolism and biohydrogenation to induce milk fat depression. Our diets were formulated for adequate fNDF, which aids in maintaining greater rumen pH and milk fat content (Li et al., 2020), and had low concentrations of polyunsaturated fatty acids (1.5% -1.6 % DM). Furthermore, the additional starch in HI was from dry ground corn, which is not as fermentable as ensiled corn (Ferraretto et al., 2013), minimizing risk of milk fat depression.

CONCLUSIONS

Feeding Enogen silage early after harvest increased total-tract nutrient digestibility, but the increment in nutrient digestibility diminished with time, which aligned with the in vitro digestibility results. However, ENO did not alter milk or milk component yields. Increasing dietary starch content reduced NDF digestibility but still resulted in increased milk and milk protein yields, likely from improved N utilization and increased energy supply. Increasing the concentration of starch to 30% of diet DM did not alter plasma acute phase protein concentrations, providing no evidence of systemic inflammation. Also, increasing dietary starch content did not reduce fecal pH, suggesting minimal effects on hindgut fermentation.

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APPENDIX

Table 7.1. Days in milk, body weight, BCS, milk yield, and milk component production of cows during the 2-week covariate period, grouped by subsequent treatment assignment.

Item	Treatment[1, 2]			
	CON		ENO	
	LO	HI	LO	HI
DIM	153 ± 45	149 ± 41	160 ± 43	155 ± 46
BW[3], kg	660 ± 71	651 ± 71	669 ± 64	695 ± 53
BCS	3.11 ± 0.38	3.16 ± 0.30	3.23 ± 0.24	3.52 ± 0.49
Milk yield, kg/d	46.1 ± 9.4	43.6 ± 5.4	46.3 ± 7.2	44.3 ± 6.4
Milk fat %	3.83 ± 0.43	3.66 ± 0.59	3.77 ± 0.58	3.85 ± 0.46
Milk fat yield, kg/d	1.74 ± 0.28	1.58 ± 0.25	1.72 ± 0.21	1.70 ± 0.32
Milk protein %	3.16 ± 0.26	3.03 ± 0.24	3.07 ± 0.22	3.18 ± 0.18
Milk protein yield, kg/d	1.44 ± 0.21	1.32 ± 0.15	1.42 ± 0.19	1.40 ± 0.18
Milk lactose %	4.89 ± 0.08	4.89 ± 0.10	4.91 ± 0.07	4.91 ± 0.10
Milk lactose yield, kg/d	2.25 ± 0.46	2.13 ± 0.26	2.27 ± 0.35	2.18 ± 0.33
SCC, x1,000 cells/mL	34 ± 41	32 ± 33	17 ± 13	48 ± 100
MUN, mg/dL	14.4 ± 1.9	14.6 ± 1.6	14.5 ± 1.5	14.4 ± 2.0

[1]Mean ± SD, n = 11/treatment.

[2]CON = control corn silage, ENO = α-amylase enzyme enhanced corn silage (Enogen, Syngenta Seeds LLC), LO = low starch (25% of diet DM), HI = high starch (30% of diet DM). Treatments assigned after the covariate period.

[3]BW = body weight

Table 7.2. Nutrient composition of corn silages, alfalfa haylage, whole cottonseed, high starch grain mix, and low starch grain mix used in an experiment feeding α-amylase enhanced corn silage with 25% or 30% dietary starch[1].

Item[2], % DM unless noted otherwise	Control corn silage[3]		Enogen corn silage[4]		Alfalfa haylage		Whole cottonseed		LO concentrate[5]		HI concentrate[6]	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
DM, % as-is	35.8	1.01	35.2	2.00	46.8	0.01	90.8	0.05	89.7	0.10	88.1	2.17
CP	7.4	0.71	7.8	0.07	18.3	0.57	23.3	3.25	25.9	0.28	24.6	1.06
Soluble protein	49.1	4.24	47.2	8.98	62.5	6.43	31.3	20.01	17.9	14.57	20.2	12.1
ADF	21.8	0.64	21.6	0.71	31.8	0.00	33.5	0.14	14.2	0.71	6.7	0.07
NDF	34.1	3.68	36.0	3.25	36.9	2.62	49.0	5.37	21.9	0.99	13.3	0.64
aNDFom	33.6	4.03	35.3	2.69	36.5	2.76	47.7	4.81	21.6	0.92	12.9	0.49
dNDF30, %NDF	51.5	8.82	53.7	2.4	49.5	1.5	29.7	9.36	84.6	0.99	75.5	0.51
dNDF240, %NDF	67.6	4.36	70.1	0.89	57.1	0.55	59.1	3.67	90.1	1.56	86.6	1.01
ADICP	0.5	0.07	0.6	0.02	1.4	0.02	1.8	0.85	0.6	0.15	0.4	0.05
NDICP	1.4	0.76	0.7	0.01	1.8	0.44	2.4	0.50	1.1	0.77	2.9	2.54
Lignin	2.6	0.18	3.0	0.29	5.9	0.43	11.0	0.63	1.4	0.88	0.8	0.13
Ethanol-soluble carbohydrate	1.0	0.00	2.3	1.41	4.5	0.78	4.0	1.41	5.2	1.77	5.6	0.14
Starch	37.0	0.07	37.8	1.77	1.9	0.14	0.6	0.35	24.4	2.05	34.4	2.76
7-h starch digestibility, % starch	70.4	3.54	73.0	1.48	-	-	-	-	-	-	-	-

Table 7.2. (cont'd)

Crude fat	2.6	0.16	2.8	0.22	2.7	0.13	16.9	0.71	2.1	0.18	3.8	2.04
Total fatty acids	2.0	0.31	2.3	0.18	1.7	0.16	16.2	0	2.4	0.13	3.9	2.31
Ash	3.1	0.58	3.2	0.28	9.8	0.79	4.7	1.32	11.0	0.60	11.4	1.60
Ca	0.2	0.06	0.3	0.06	1.8	0.24	0.2	0.02	1.9	0.23	2.0	0.45
P	0.2	0.00	0.2	0.01	0.3	0.05	0.7	0.03	0.6	0.01	0.6	0.01
Mg	0.1	0.02	0.2	0.02	0.3	0.03	0.4	0.02	0.7	0.27	0.6	0.07
K	0.8	0.04	0.7	0.01	2.0	0.21	1.3	0.03	1.4	0.13	1.4	0.04

[1]n = 2 composite feed samples.

[2]aNDFom = α-amylase treated NDF – NDF ash, dNDF30 = aNDFom digested after 30 h in vitro, dNDF240 = aNDFom digested after 240 h in vitro, ADICP = acid detergent insoluble CP, NDICP = neutral detergent insoluble CP.

[3]Isoline control corn silage.

[4]α-amylase enhanced corn silage (Enogen, Syngenta Seeds LLC).

[5]Low starch concentrate mix.

[6]High starch concentrate mix.

Table 7.3. Ingredient and nutrient composition diets containing either control or α-amylase enhanced corn silage with low (25%) or high (30%) concentrations of dietary starch.

Item	Treatment[1]			
	CON		ENO	
	LO	HI	LO	HI
Ingredient, % DM				
Control corn silage[2]	40.0	40.0	-	-
Enogen corn silage[3]	-	-	40.0	40.0
Alfalfa silage	12.5	12.5	12.5	12.5
Dry ground corn	13.9	20.8	13.9	20.8
Soybean meal	11.3	11.3	11.3	11.3
Soybean hulls	9.9	3.0	9.9	3.0
Cottonseed	3.4	3.4	3.4	3.4
Heat-treated soybean meal[4]	4.9	4.9	4.9	4.9
Mineral and vitamin premix[5]	3.7	3.7	3.7	3.7
Chemical composition[6], % DM unless noted otherwise				
DM	52.2 (0.57)	51.9 (0.03)	51.9 (1.15)	51.5 (0.62)
CP	17.43 (0.47)	16.8 (0.19)	17.7 (0.05)	17.0 (0.61)
ADF	20.0 (0.08)	16.2 (0.78)	19.6 (0.19)	16.0 (0.34)
NDF	29.5 (2.47)	25.8 (2.50)	30.2 (2.37)	26.5 (2.45)
aNDFom	29.0 (2.58)	25.3 (2.58)	29.7 (2.10)	25.9 (2.15)
Forage NDF	17.8 (2.14)	18.0 (2.34)	18.4 (1.79)	18.6 (1.99)
dNDF30, % NDF	65.3 (3.45)	61.1 (4.05)	66.3 (0.71)	62.0 (1.35)
dNDF240, % NDF	73.1 (1.30)	69.0 (1.63)	74.2 (0.28)	70.3 (0.17)
Starch	26.0 (0.84)	30.5 (3.29)	26.3 (1.39)	30.7 (1.84)
Total fatty acids	2.6 (0.08)	3.3 (1.14)	2.7 (0.22)	3.4 (1.08)
Ash	7.4 (0.58)	7.6 (0.99)	7.5 (0.42)	7.6 (0.82)
ROM	17.0 (2.77)	16.1 (3.20)	15.6 (1.47)	14.7 (1.90)

Table 7.3. (cont'd)

[1]CON = control corn silage, ENO = α-amylase enhanced corn silage, LO = low starch (25% diet DM), HI = high starch (30% of diet DM). n = 2 composite TMR samples

[2]Isoline corn silage without enhanced α-amylase

[3]α-amylase enhanced corn silage

[4]Amino Plus, Ag Processing Inc., Omaha, NE

[5]Mineral and vitamin premix composition, DM% basis: 9.41% dicalcium phosphate, 5.64% Mg oxide, 9.41% NaCl, 37.62% $CaCO_3$, 1.22% Se premix (600 ppm), 2.07% rumen-protected Met (Smartamine M; Adisseo), 34.14% sodium sesquicarbonate, 0.49% vitamin / trace mineral premix.

[6]Data presented as means (SD); n = 2 composite samples; aNDFom (α-amylase treated) = NDF – NDF ash, dNDF30 = aNDFom digested after 30 h in vitro, dNDF240 = aNDFom digested after 240 h in vitro, ROM (residual organic matter) = 100 − %CP − % TFA − %Ash − %Starch − %NDF.

Table 7.4. Plasma glucose, insulin, haptoglobin, and serum amyloid A of dairy cows fed a control (CON) or α-amylase enhanced corn silage (ENO) with low (25%) or high (30%) concentrations of dietary starch (*n* = 11 cows per treatment).

Item	Treatments				SEM	*P*-values						
	CON		ENO[1]			Corn silage	Starch	Week[2]	CS × Starch	CS × Week	Starch ×Week	CS × Starch × Week
	LO	HI	LO	HI								
Glucose, mg/dL	69.8	69.7	70.9	69.5	1.39	0.66	0.45	<0.01	0.55	0.19	0.86	0.79
Insulin, µg/L	0.26	0.23	0.24	0.25	0.024	0.99	0.48	<0.01	0.34	0.29	0.77	0.76
Haptoglobin, µg /mL	6.32	4.08	3.62	6.84	3.71	0.99	0.89	0.03	0.46	0.48	0.27	0.82
Serum amyloid A, µg/mL	10.61	8.96	8.87	14.04	4.64	0.67	0.63	0.05	0.31	0.61	0.20	0.42

[1]Enogen, Syngenta Seeds LLC

[2]Weely data during all 6 wk of experimental period

Table 7.5. Nutrient intake, nutrient digestibility, body weight change, body condition score, and fecal pH of dairy cows fed a control (CON) or α-amylase enhanced corn silage (ENO) with low (25%) or high (30%) concentrations of dietary starch (*n* = 11 cows per treatment).[1]

Item	Treatments				SEM	*P*-values						
	CON		ENO[2]			Corn silage	Starch	Week[3]	CS × Starch	CS × Week	Starch ×Week	CS × Starch × Week
	LO	HI	LO	HI								
Intake, kg/d												
DM	28.0	26.3	27.5	27.3	0.91	0.75	0.26	0.41	0.40	0.77	0.02	0.28
NDF	8.3	6.7	8.3	7.1	0.24	0.53	0.01	<0.01	0.41	0.53	<0.01	0.35
Starch	7.2	8.0	7.2	8.4	0.26	0.45	0.03	<0.01	0.48	0.12	<0.01	0.15
CP	4.9	4.4	4.9	4.6	0.16	0.47	0.03	0.20	0.55	0.12	<0.01	0.16
Digestibility, %												
DM	67.8	67.6	69.6	69.9	0.39	<0.01	0.82	0.17	0.56	<0.01	<0.01	0.80
NDF	43.4	35.9	48.1	41.8	0.95	<0.01	<0.01	<0.01	0.53	<0.01	<0.01	0.03
Starch	98.0	97.7	98.2	98.1	0.23	0.18	0.34	<0.01	0.67	<0.01	0.04	0.69
CP	66.8	67.1	69.7	68.4	0.79	0.01	0.39	<0.01	0.18	<0.01	<0.01	0.79
ΔBW, kg/d	0.49	0.35	0.43	0.23	0.094	0.27	0.05	-	0.74	-	-	-
ΔBCS, units/week	-0.009	-0.015	-0.006	-0.015	0.0097	0.84	0.46	-	0.87	-	-	-
Fecal pH	6.76	6.82	6.87	6.83	0.032	0.06	0.75	0.05	0.15	0.36	0.71	0.49

[1]Means presented as lsmeans across both wk 1 and wk 6 digestibility

[2]Enogen, Syngenta Seeds LLC

[3]Weely data during wk 1 and wk 6 except for fecal pH where data are from all 6 wk were collected.

Table 7.6. Milk yield, milk composition, and milk component yield of dairy cows fed a control (CON) or α-amylase enhanced corn silage (ENO) with low (25%) or high (30%) concentrations of dietary starch (*n* = 11 cows per treatment).

Item	Treatments				SEM	*P*-values						
	CON		ENO[1]			Corn Silage	Starch	Week[2]	CS × Starch	CS × Week	Starch ×Week	CS × Starch × Week
	LO	HI	LO	HI								
Milk yield, kg/d	38.6	40.6	38.2	39.5	0.91	0.38	0.10	<0.01	0.70	0.86	0.14	0.37
ECM, kg/d	41.3	42.7	40.5	41.7	0.77	0.21	0.07	<0.01	0.86	0.87	0.38	0.68
FE[3], ECM/DMI	1.47	1.58	1.49	1.54	0.042	0.79	0.07	<0.01	0.44	0.09	0.28	0.59
Milk fat, %	3.94	3.81	3.84	3.79	0.060	0.84	0.87	<0.01	0.06	0.11	0.98	0.34
Milk fat, kg/d	1.51	1.53	1.46	1.48	0.039	0.13	0.57	<0.01	0.95	0.76	0.44	0.70
Milk protein, %	3.33	3.28	3.28	3.31	0.028	0.64	0.76	<0.01	0.18	0.35	<0.01	0.10
Milk protein, kg/d	1.27	1.34	1.24	1.30	0.028	0.23	0.03	<0.01	0.93	0.23	0.01	0.18
Milk lactose, %	4.88	4.89	4.88	4.88	0.011	0.69	0.20	<0.01	0.49	0.06	0.04	0.49
Milk lactose, kg/d	1.87	2.00	1.86	1.94	0.039	0.20	<0.01	<0.01	0.49	0.79	0.51	0.64
MUN, mg/dL	13.7	12.4	12.6	12.3	0.28	0.05	<0.01	<0.01	0.08	0.01	<0.01	0.02
Somatic cell score[4]	2.80	2.71	3.32	2.85	0.260	0.15	0.28	<0.01	0.47	0.21	0.35	0.43

[1]Enogen, Syngenta Seeds LLC

[2]Weely data during all 6 wk of experimental period

[3]FE = feed efficiency

[4]SCS = $Log_2(SCC/10000) + 3$

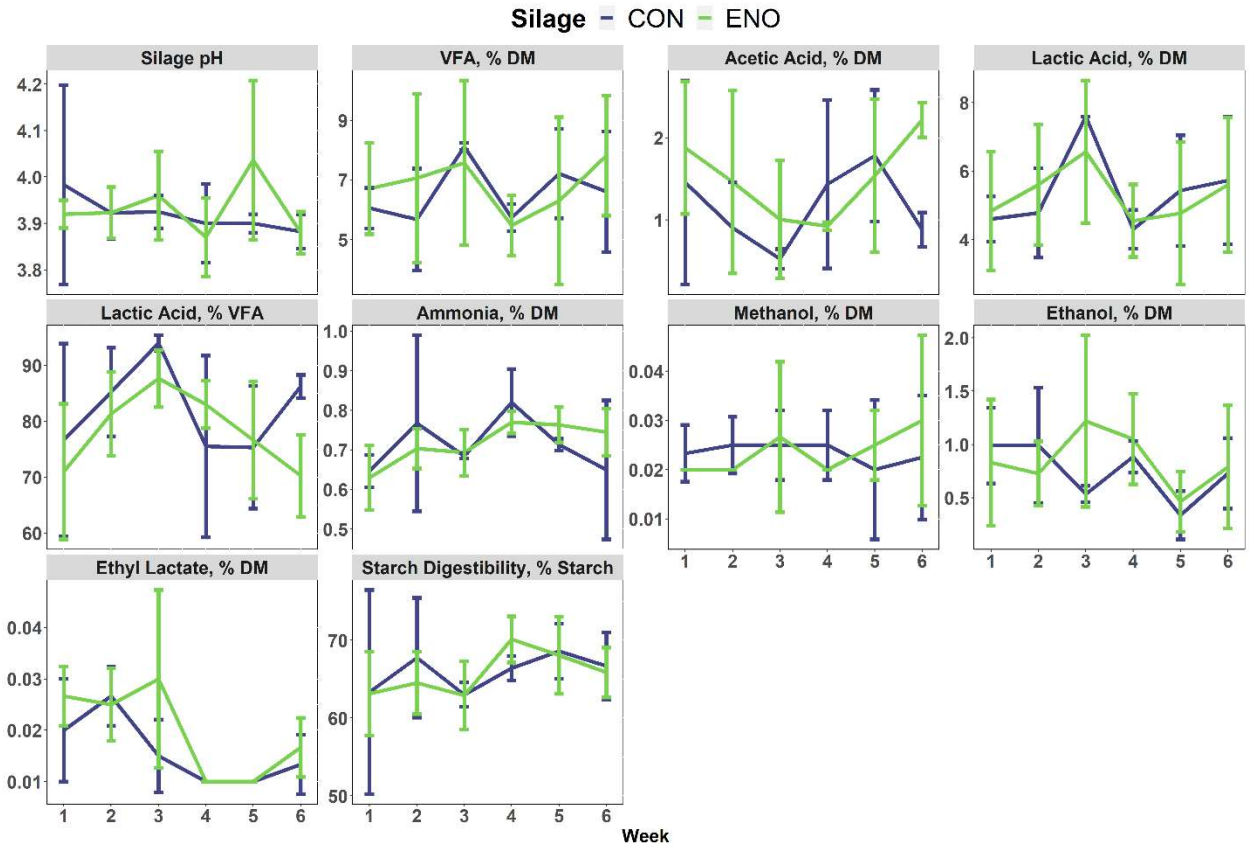

Figure 7.1. Silage fermentation analysis for silage (n =18) with enhanced endogenous α-amylase (ENO; Enogen, Syngenta Seeds LLC) or a control silage without the α-amylase gene (CON). Samples were collected 3 times per week from 41 d post-harvest until 83 d post-harvest.

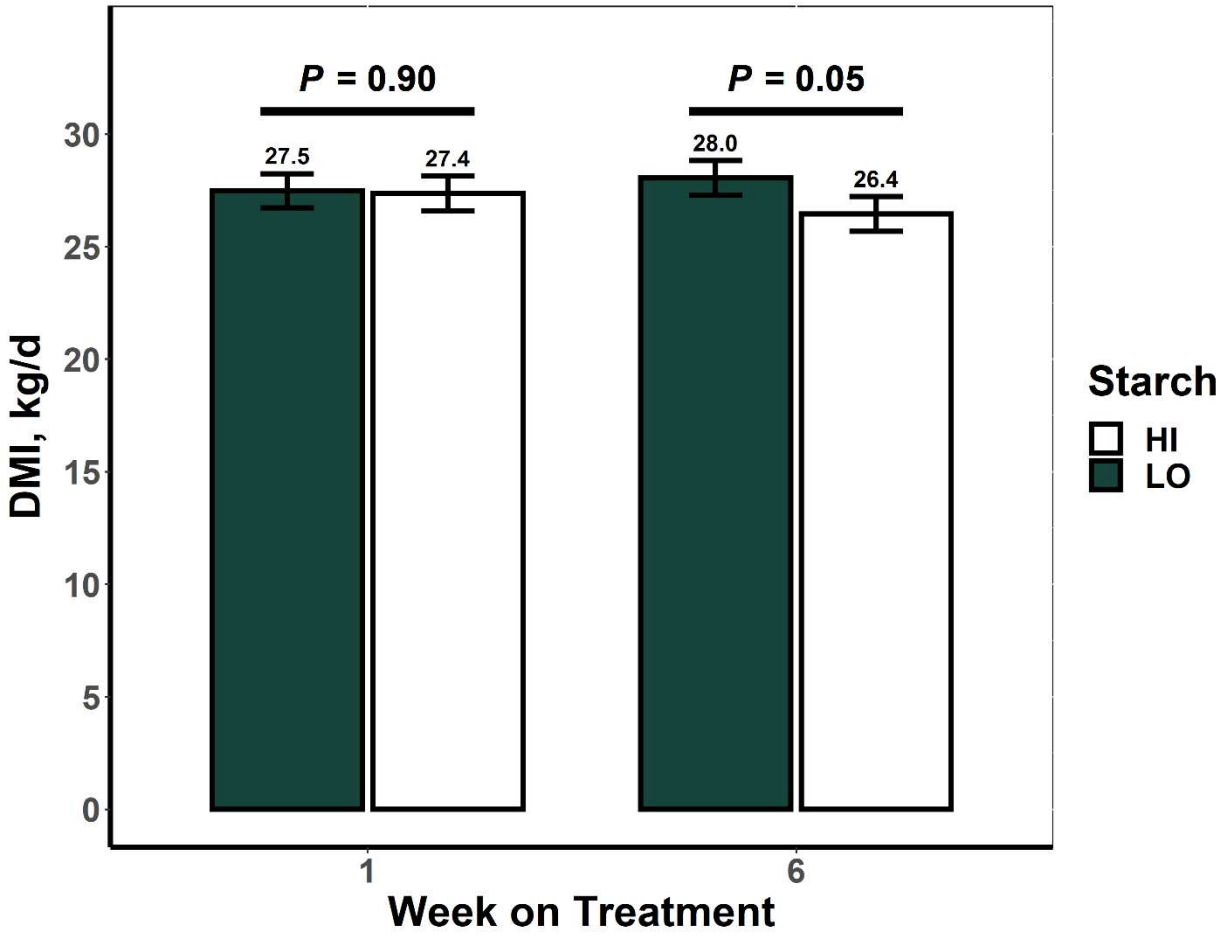

Figure 7.2. Dry matter intake during week 1 and week 6 of cows fed α-amylase enhanced corn silage (ENO; Enogen, Syngenta Seeds LLC) or a control silage without the α-amylase gene (CON) with low (LO; 25% of diet DM) or high (HI; 30% of diet DM) concentrations of dietary starch (n = 11 cows per treatment).

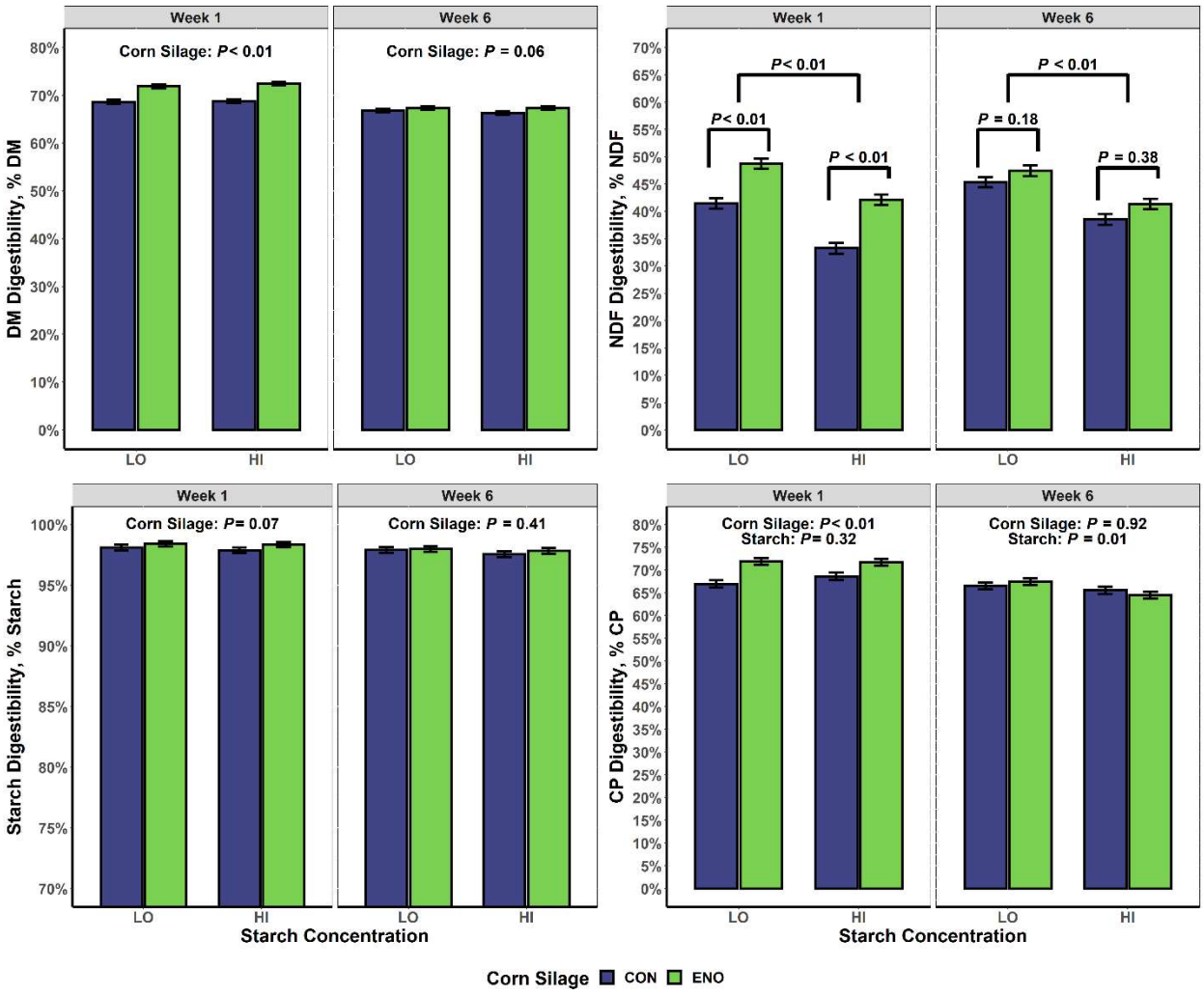

Figure 7.3. Apparent total tract DM, NDF, starch, and CP digestibility during week 1 and week 6 from cows fed α-amylase enhanced corn silage (ENO; Enogen, Syngenta Seeds LLC) or a control silage without the α-amylase gene (CON) with low (LO; 25% of diet DM) or high (HI; 30% of diet DM) concentrations of dietary starch (n = 11 cows per treatment).

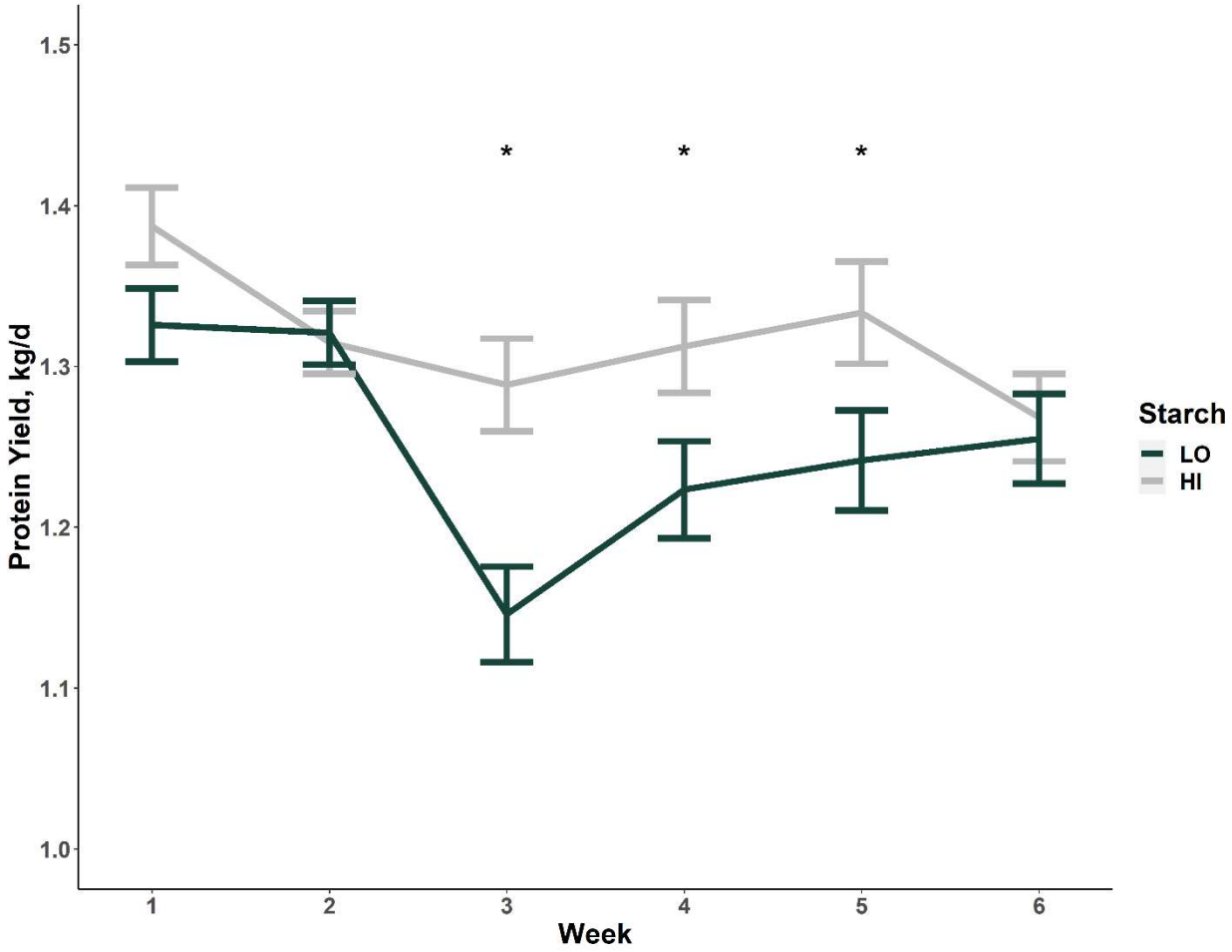

Figure 7.4. Milk protein yield from cows fed α-amylase enhanced corn silage (ENO; Enogen, Syngenta Seeds LLC) or a control silage without the α-amylase gene (CON) with low (LO; 25% of diet DM) or high (HI; 30% of diet DM) concentrations of dietary starch (n = 11 cows per treatment). Milk protein yield was greater for HI during weeks 3, 4, and 5 ($P < 0.05$).

CHAPTER 8: CONCLUSIONS

Within this dissertation, we have investigated and discussed a variety of nutritional strategies to reduce inflammation and improve cow health and efficiency throughout the entire lactation. Each chapter within this dissertation had concrete takeaways which will be useful in continued research or for field application on dairy farms.

Niacin supplementation has been studied for decades, but our research was the first to focus on the potential effects of niacin on the inflammatory response of dairy cattle to repeated intramammary challenges. In chapter 3, we investigated whether feeding a rumen protected niacin supplement affects systemic inflammation in late-lactation dairy cows. The supplement was bioavailable as the circulating nicotinic acid and nicotinamide were substantially increased in cows fed RPN. This was repeated in the experiment in Chapter 4 when niacin was supplemented on farm through the AMS. Also, our results confirmed previous data suggesting that rumen-protected niacin may improve milk quality. Even in these extremely healthy cows, SCS was substantially reduced prior to the 1st mastitis challenge. Furthermore, supplementing RPN to late-lactation dairy cows reduced systemic markers of inflammation. It reduced peak LBP during the first challenge and reduced Hp during both challenges. The fact that Hp was reduced during both the first and second mastitis challenges suggests that there was a carryover effect of RPN as it ceased being supplemented 2 weeks prior to the 2nd challenge. Some epigenetic programming may be taking place with RPN supplementation. Finally, our data also suggests that RPN may reduce circulating T-cell populations and increase the number of immature neutrophils after a challenge indicating the immunomodulatory capabilities in this experiment. The phenotype, such as the specific T-cell lineages (Th1, Th2, Th17, etc.) or activity of such cells, should be a point of further investigation.

Whether RPN would improve health and reduce disease on a commercial dairy farm was our next question. In chapter 4, we investigated whether supplementing RPN during the postpartum period would improve lactation performance, health outcomes, and fertility on a Michigan dairy farm. Feeding RPN did not affect plasma Hp in the postpartum period as we hypothesized or as was demonstrated in the late-lactation cows. This is likely a result of the timing of supplementation; RPN was only supplemented after calving and the anti-inflammatory effects would most likely be observed had the RPN been supplemented both prior to and after calving. Supplementation of anti-inflammatory feed additives should be supplemented before the suspected challenge period, such as calving. Interestingly, RPN did reduce insulin concentrations without affecting glucose concentrations suggesting that maybe insulin sensitivity was enhanced, or glucose metabolism was altered in these cows. Interestingly, BHB and free fatty acids were increased as a result of RPN which were contrary to our hypothesis. RPN also tended to increase milk yield in the immediate postpartum period. Additionally, RPN increased milk yield long after the supplementation period. Milk yield was greater for RPN by 9-13 wk of lactation until wk 40-42 of lactation in primi- and 2nd parity cows. Such a carryover effect of RPN had not been observed previously and investigation as to its cause is necessary. To our knowledge, our experiment is the only one where RPN was supplemented only postpartum. The observed increase in milk yield suggests that timing of supplementation must be more thoroughly investigated when considering feed additives and other B-vitamins, such as niacin.

In addition to niacin, we investigated a novel feed additive called β-caryophyllene (BCP). BCP interacts with the endocannabinoid system which is now well understood to affect both metabolism and inflammation in mammals. In our preliminary investigation, our objective was to identify the effects of BCP on primary bovine immune cells. In chapter 5, we observed that BCP

altered the proteome and phosphoproteome of these immune cells in such a way to suggest that BCP may be anti-inflammatory by reducing cytokine secretion, modulating immune cell phenotype, and affecting immune cell function. An *in vivo* experiment investigating BCP supplementation with lactating cows should be done to test whether these *in vitro* anti-inflammatory effects translate to the whole animal.

Our final 2 chapters investigated how feeding dietary starch affects health and production of lactating dairy cows. In our review (Chapter 6), we concluded that additional dietary starch does not appear to consistently alter the inflammatory status of lactating dairy cattle but there is scant data in this area. It does appear that diet adaptation and feed management may play a role in any diet induced inflammation or challenge. Further investigations of how diet affects health should focus on local effects on the gut epithelium as well as systemic markers of inflammation. Also, investigations in this area should investigate different gain sources, grain processing, forage concentrations, and forage types to determine what feeding strategies optimize gut health and feed efficiency. In chapter 7 we demonstrated that additional starch did not increase the circulating concentrations of inflammatory biomarkers which supported conclusions from the review in chapter 6. Also, increasing dietary starch increased milk protein yield and feed efficiency. The novel α-amylase enhanced corn silage that was investigated increased fiber digestibility and slightly increased starch digestibility, but these increases did not translate to increased milk or component yields.

These results demonstrate both the potential opportunities and risks involved with how feeding cows may affect their health and efficiency. We advanced our understanding of the effects of niacin suggesting it has potential to improve health and milk production of dairy cows depending on the timing of supplementation. We explored a new avenue to reduce inflammation

by targeting the endocannabinoid system; targeting this system through the provision of BCP has promise and deserves to be scrutinized in a variety of contexts. Finally, feeding starch does not appear to affect animal health and inflammation but more investigation is necessary. Specific focus should be on the gut epithelium, microbiome, and immune function locally within the epithelium.