

METABOLIC INFLAMMATION AND IMMUNOMODULATION IN DAIRY COWS

by

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B.S., Yangzhou University, 2009
M.S., University of Wisconsin-Madison, 2011

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Department of Animal Sciences and Industry
College of Agriculture

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Abstract

The transition period in dairy cows is characterized by dramatic increases in nutrient requirements for lactation and substantial metabolic stress. The disturbed metabolic balance, coupled with suppressed immune function, contributes to markedly elevated incidence of health disorders. Several lines of evidence suggest that increased inflammation is common during the transition period. Unlike the classical inflammation associated with acute infection, the postpartum inflammatory state is low-grade and often of metabolic origin. This metabolic inflammation plays a key role in numerous disorders; an improved understanding of inflammatory pathways in transition cows may improve our ability to predict and prevent disorders. To mimic metabolic inflammation, in Experiment 1, we administered low amounts of recombinant bovine tumor necrosis factor- α (rbTNF α), a pro-inflammatory cytokine, to early lactation cows, and evaluated whether rbTNF α affects milk production, metabolism, and health. We found that rbTNF α administration increased systemic inflammation, decreased feed intake and milk yield, and increased incidence of disorders. Conversely, preventing excessive inflammation has the potential to improve productivity and health of dairy cows. To identify nutritional strategies that could enhance metabolism and immunity, we evaluated the efficacy of several feed additives. In Experiment 2, we evaluated effects of chromium propionate, rumen-protected lysine and methionine, or both on metabolism and immunity in lactating dairy cows, and found that supplementation of these nutrients may enhance neutrophil function. In Experiment 3, we determined whether supplementation of yeast product to transition cows could enhance production, metabolism, and immunity, and found that yeast product modulated feeding behavior, metabolism, immunity, and uterine inflammation. Overall, a greater understanding of the role of metabolic inflammation in the transition period and the nutritional strategies that could modulate these signals may improve the production and health of dairy cows.

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Approved by:

Major Professor
Barry J. Bradford

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*Always pray to have eyes that see the best in people,
A heart that forgives the worst,
A mind that forgets the bad,
And a soul that never loses faith in God.*

Chapter 1 - Literature Review

Introduction

The transition from late gestation to early lactation is often the most problematic period in the production cycle of dairy cows. In an attempt to cope with dramatic nutrient and energy requirements for milk production, cows experience tremendous metabolic stress. The disturbed metabolic balance, coupled with suppressed immune function around parturition, contributes to markedly elevated incidence of metabolic disorders and infectious diseases during this time. Therefore, the transition period directly determines the lactational performance, health, and profitability of dairy cows; it is critical to develop effective nutritional strategies that could enhance the metabolism and immunity of transition cows.

Among other diseases, early-lactation cows experience particularly high rates of mastitis, metritis, displaced abomasum, ketosis, and fatty liver. Although these issues have been the focus of much research in recent decades, the etiology of these diseases is not completely understood. With the physiological stress and metabolic changes that accompany the processes of parturition and the initiation of lactation, increased inflammation is common during the postpartum period. Inflammation is known to play a major role in infectious diseases and recent research has suggested that it is involved in metabolic diseases as well. An improved understanding of the role of inflammatory pathways in early-lactation cows may improve our ability to prevent disorders.

This literature review will first describe the characteristics of nutrient metabolism and immune function in transition dairy cows. It will then explore the roles of “classical” and “metabolic” inflammation in diseases, particularly the impact of inflammation on the

metabolism, health, and production of transition cows. Finally, it will discuss several nutritional strategies that could potentially modulate the metabolism and immunity in dairy cows.

Characteristics of Transition Cows

1. Lipid Metabolism

The transition from late gestation to early lactation is characterized by dramatic nutrient and energy requirements for milk synthesis and secretion, substantial metabolic stress, and endocrine changes. In the meantime, feed intake is often depressed, leading to negative energy balance status (Grummer, 1995). In responses to the energy deficit, cows mobilize adipose tissue, resulting in dramatic elevation of non-esterified fatty acid (NEFA) concentrations in the blood. The liver takes up the high flux of blood NEFA and often exceeds its capacity to completely oxidize NEFA to CO₂, resulting in partial oxidation to form ketones or esterification to form triglyceride (TG) within hepatocytes (Grummer, 2008). Excessive production and accumulation of ketones often lead to ketosis, which is characterized by elevated blood concentrations of β -hydroxybutyrate (BHBA), depressed appetite, and decreased milk production (Oetzel, 2007). Furthermore, because ruminants are inefficient at exporting TG out of the liver, excessive TG accumulation or fatty liver occurs. Ketosis and fatty liver affect up to 50% of dairy cows, compromising health and production (Bobe et al., 2004; Oetzel, 2007). These health issues are also associated with increased incidence of multiple other disorders or diseases, including displaced abomasum, mastitis, and metritis, which together create a costly epidemic of transition cow disorders (Bobe et al., 2004; Drackley, 1999). Although lipid metabolism in transition cows

has been extensively studied, the mechanisms underlying the development of these metabolic disorders are not fully understood.

2. Glucose Metabolism

In response to metabolic demands of lactation, the requirements for glucose increase dramatically during the transition period. For example, estimated glucose demand is approximately 1 kg/d during the last 21 d of gestation, but increases dramatically after parturition to approximately 2.5 kg/d at 21 d postpartum (Overton, 1998). Most of this increased glucose requirement must be met via hepatic gluconeogenesis, which is of great importance in ruminants at all times, providing the majority of necessary glucose (Young, 1977). Simultaneously, as part of the homeorhetic adaptation, glucose oxidation by peripheral tissues is decreased (Bennink et al., 1972) to direct glucose to the mammary gland for lactose synthesis. The primary substrate to support gluconeogenesis is propionate from ruminal fermentation; yet because early-lactation cows often experience inadequate feed intake, meeting glucose needs can be a tremendous challenge. Besides propionate, other substrates contributing to gluconeogenesis include lactate from Cori cycling, amino acids from protein catabolism or net absorption, and glycerol released from adipose tissue mobilization (Seal and Reynolds, 1993). The contributions to net liver glucose release have been estimated to be from 50 to 60% for propionate, 20 to 30% for amino acids, and only 2 to 4% for glycerol during the transition period (Reynolds et al., 2003). Cows adapt to early lactation by increasing the capacity and efficiency of glucose synthesis from multiple substrates. For example, compared with d 21 prepartum, propionate conversion to glucose by liver slices was increased by 19 and 29% at d 1 and 21 postpartum, respectively; and

hepatic capacity to convert [1-¹⁴C] alanine to glucose was approximately doubled immediately postpartum (Overton et al., 1998).

Metabolic disorders, particularly fatty liver and ketosis, have been associated with decreased gluconeogenesis (Cadorniga-Valino et al., 1997; Veenhuizen et al., 1991), but there is no clear mechanism explaining this association. Based on the evidence that inflammatory cytokines, such as tumor necrosis factor α (TNF α), can promote liver fat accumulation (Feldstein et al., 2004) and alter gluconeogenesis (da Rocha et al., 2013), we hypothesized that inflammatory signals may link fatty liver to decreased glucose production in dairy cows. In fact, our previous research found that TNF α infusion induced liver TG accumulation and reduced liver gluconeogenic gene expression in late-lactation cows (Bradford et al., 2009), but the effect of TNF α administration on liver glucose production per se was not assessed. If inflammatory cytokines impair glucose production in early-lactation cows or those with naturally occurring fatty liver, the resulting glucose deficiency would likely further increase adipose mobilization and exacerbate metabolic disorders.

3. Immunosuppression

Infectious diseases reduce profitability through decreased production efficiency and increased morbidity and mortality. Mastitis has been reported to reduce whole-lactation milk yield by almost 600 kg (Rajala-Schultz et al., 1999), and is also an important risk factor for involuntary culling (Gröhn et al., 1988). Risk of infections of the mammary gland (mastitis) or uterus (metritis) increases dramatically during the transition period. Although the etiologies of infectious and metabolic disorders differ, there was a significant association between their occurrences. For example, milk fever increases incidence of clinical mastitis by over 5-fold

(Curtis et al., 1985), suggesting that there are potential interactions between transition cow metabolism and immune function.

The increased incidence and severity of diseases in the transition period are partially due to suppressed immune function, or immunosuppression. This immune dysfunction is broad in scope and affects multiple functions of various immune cell types. When *E. coli* were administered into the mammary gland, transition cows experienced more rapid bacterial growth and greater bacterial concentrations compared with mid-lactation cows (Shuster et al., 1996). Among other immune cells, neutrophils are the first line of immune defense, and decreased neutrophil function is a main characteristic of impaired immunity in transition cows. Upon maturation in bone marrow, neutrophils are released into circulation and perform key functions for successful immune surveillance and defense against pathogens, including margination, migration, phagocytosis, respiratory burst, and degranulation (Burton and Erskine, 2003). Each of these steps must work rapidly and effectively to clear infections. For example, impaired margination and migration may partially explain neutrophil dysfunction in early-lactation cows. Circulating neutrophils use adhesion molecules, such as L-selectin, for vascular margination. Kimura et al. (1999) reported that expression of L-selectin on neutrophils was dramatically decreased at calving, correlating well with the surge of blood cortisol, a potent immunosuppressive hormone. This reduced L-selectin expression would inhibit neutrophil margination and depress neutrophil recruitment into mammary glands of newly calved cows, increasing the risk of mastitis. Other studies have reported that compared with blood neutrophils isolated from mid-lactation or late-lactation cows, neutrophils in early-lactation cows have decreased ability to generate reactive oxygen species following phagocytosis of opsonized particles (Mehrzhad et al., 2001; Cai et al., 1994). Taken together, impaired neutrophil trafficking,

phagocytosis, and killing may directly contribute to immunosuppression and increased disease incidence in transition cows (Kehrli et al., 1989).

The cause of immunosuppression in the transition period is not completely understood, but is related to endocrine and metabolic factors. For example, elevated glucocorticoids around parturition are partially responsible for immunosuppression (Burton et al., 1995). Also, changes in estradiol and progesterone concentrations prior to calving may affect immune function (Weber et al., 2001). Kimura and colleagues (1999) reported that compared with mastectomized cows, the presence of the mammary gland and its subsequent metabolic demands slowed recovery of neutrophil function postpartum, suggesting that metabolic factors associated with lactation exacerbated immunosuppression. Negative energy balance may also contribute to immune dysfunction. In fact, markers associated with energy deficiency, including NEFA and ketones, have been implicated in impaired immunity. Concentrations of NEFA similar to that reported in transition cows, decreased neutrophil respiratory burst activity *in vitro* (Ster et al., 2012). Field studies also suggest that elevated NEFA and ketones around parturition increase the risk of clinical mastitis, displaced abomasum, metritis, and retained placenta (Moyes et al., 2009; Ospina et al., 2010).

Inflammation

1. Classical Inflammation

Underlying many physiological and pathological processes, inflammation is an adaptive response that is triggered by adverse stimuli and conditions, such as infection and tissue injury (Medzhitov, 2008). Classical inflammation is defined by the signs of redness, swelling, heat, and

pain. In response to inflammatory stimuli, the body often increases the release or expression of inflammatory mediators that are critical for both vascular changes and leukocyte infiltration (Newton and Dixit, 2012). Some of the key mediators released by activated immune cells include cytokines, chemokines, adhesion molecules, eicosanoids, and plasma proteins. These molecules form complex regulatory networks and promote increased blood flow to the infected tissue and immune cell infiltration and activation, and they also stimulate systemic responses, including increased body temperature, increased heart rate, and decreased appetite (Dantzer and Kelley, 2007). Cytokines such as TNF α , interleukin (IL) 1 β , and IL-6 are produced by many cell types, especially by macrophages and mast cells. They play important roles in the inflammatory response, including activation of the endothelium and leukocytes and induction of the acute-phase response (Bannerman, 2009). Eicosanoids represent another class of inflammatory mediators. They are fatty acid-derived molecules exerting complex control over inflammation, and can either enhance (such as certain types of prostaglandins, prostacyclins, thromboxanes, and leukotrienes) or resolve (such as resolvins, protectins, and maresins) inflammatory response depending on their types and timing of expression (Sordillo et al., 2009).

In dairy cows, the most costly inflammatory disease is mastitis, which commonly results from microbial infection of the mammary gland (Bannerman et al., 2009). If the infection is caused by Gram-negative bacteria, lipopolysaccharide (LPS) released from the bacteria outer membrane is the main pathogen component initiating inflammatory responses (Hogan and Smith, 2003). At the initiation of immune response, the ligation of LPS to toll-like receptor (TLR) 4 initiates a signal transduction pathway that activates a complex cascade of inflammatory mediators released by host immune and epithelial cells (Ballou, 2012; Erskine, 1995). The TLR4 pathways are known to eventually activate the transcription factor nuclear factor- κ B (NF- κ B),

which is considered the master regulator of inflammation controlling the expression of hundreds of immune-associated genes (Ghosh and Hayden, 2008). Activated NF- κ B subsequently enters the nucleus and bind to target promoters, orchestrating the synthesis of a whole battery of inflammatory genes (Akira and Takeda, 2004). In mastitis, the production of pro-inflammatory cytokines, including IL-1 β , IL-6, IL-8, and TNF α , are increased by LPS in an NF- κ B-dependent manner (Schukken et al., 2011). These cytokines, together with other inflammatory mediators, elicit the migration of leukocytes (primarily neutrophils) to the site of infection (i.e. mammary gland). Once they reach the afflicted tissue, neutrophils become activated and release the toxic contents of their granules, including reactive oxygen species, reactive nitrogen species, proteinase, and elastase. These highly potent effectors are important to facilitate the clearance of pathogens, but they may also promote the breakdown of blood-milk barrier and induce mammary epithelial tissue damage (Schukken et al., 2011). The reduced number and activity of secretory cells consequently contribute to decreased milk synthesis and secretion (Schukken et al., 2011).

Cytokines can also directly elicit pro-inflammatory signals by binding to their receptors. For example, in response to TNF α , TNF receptor-1 (TNFR1) and TNFR2 are activated, leading to the activation of several distinct signaling pathways, including NF- κ B, activator protein 1 (AP-1), and Caspases pathways (Baud and Karin, 2001). In the initiation of NF- κ B signaling, TNFR-associated death domain (TRADD) binds to TNFR1, and recruits TNF receptor-associated factor 2 (TRAF2) and receptor-interacting kinase (RIP). TRAF2 in turn recruits and eventually activates inhibitor of NF- κ B kinase (IKK). In nonstimulated cells, NF- κ B is bound to an inhibitory protein I κ B α and sequestered in the cytoplasm. In the activation process, I κ B α is phosphorylated by IKK and then degraded; subsequently, the liberated NF- κ B is translocated to the nucleus to mediate the transcription of a vast array of proteins involved in cell survival,

proliferation, and inflammatory responses (Wajant et al., 2003; Häcker and Karin, 2006). Stimulation of TNFR1 can also lead to a strong activation of the apoptotic pathway, and there is increasing evidence that the NF- κ B and the apoptotic pathway are tightly connected (Wajant et al., 2003). Furthermore, TNF α can induce strong activation of the stress-related c-Jun N-terminal kinase (JNK) group and the p38- mitogen-activated protein kinase (p38-MAPK). These pathways are also involved in cell differentiation, proliferation, apoptosis, stress, and inflammation (Wajant et al., 2003). The precise pathways of TNF α signaling have not been described in bovine diseases.

In addition to mastitis, infection of the uterus is also common in the early-lactation period. Essentially all cows have bacterial contamination of the uterus within 3 wk after calving, and the majority of them have at least one form of pathology of the reproductive tract (LeBlanc et al., 2011). Metritis is a severe inflammatory reaction (often occurs with the first week of lactation) involving all layers of the uterus, resulting in systemic signs of sickness, including fever, watery foul-smelling uterine discharge, and low milk production (LeBlanc, 2008). Endometritis is the inflammation of uterus surface epithelium without systemic illness, occurring later than 3 wk postpartum. Histologically, endometritis is characterized by disruption of the endometrial epithelium, inflammatory cell infiltration, and vascular congestion (LeBlanc, 2008).

Although a controlled inflammatory process normally leads to recovery from infection, a dysregulated (such as septic shock) or chronic inflammatory condition can also be detrimental. Therefore, in an ideal acute inflammatory response, a rapid resolution phase following the elimination of the infectious agents is necessary (Medzhitov, 2008).

2. Metabolic Inflammation

Although inflammation is part of the protective host response to infection and injury, inappropriate inflammation leads to many adverse outcomes. Indeed, systemic low-grade chronic inflammation occurs in a wide variety of diseases, including obesity and type 2 diabetes. The chronic inflammation associated with metabolic disorders is also referred to as metabolic inflammation (Hotamisligil, 2006). In contrast to classical inflammation that is induced by infection and injury, metabolic inflammation seems to be associated with tissue malfunction. In light of the world epidemic of obesity, extensive research has been conducted to explore the role of metabolic inflammation in this disorder. Therefore, I will use obesity as an example to describe the characteristics of metabolic inflammation. In obesity, metabolic inflammation is orchestrated by metabolic cells in response to excess nutrients and energy, and the hallmarks are as follows (Gregor and Hotamisligil, 2011).

First, this inflammatory response is induced by nutrient signals and orchestrated by metabolic tissues (such as adipocytes, liver, pancreas, and brain), and in turn impairs metabolic homeostasis. Research demonstrated that an array of inflammatory cytokines, including $\text{TNF}\alpha$, IL-6, IL- 1β , and others are increased in obese adipose tissues (Hotamisligil et al., 1995; Shoelson et al., 2006). Second, the response is low-grade; in contrast to the acute inflammation observed in infection, the elevated inflammatory responses in metabolic tissues are often modest and local. Third, it creates an environment with modified composition of immune cells promoting a proinflammatory status. For example, one key feature of obesity is increased infiltration of macrophage into the adipose tissues. Compared with the lean state, there is a 5-fold increase in adipose tissue macrophage content in the obese state, with macrophages constituting up to 50% of the cells in obese adipose tissue (Weisberg et al., 2006). In addition, macrophages shift from

an anti-inflammatory M2 type to a proinflammatory M1 type, which in turn decrease insulin sensitivity (Olefsky and Glass, 2010). Emerging evidence suggests that the profiles of other immune cells, including B and T lymphocytes, mast cells, and natural killer T cells, are also altered in obese adipose tissues (Johnson et al., 2012; Liu et al., 2009; Ohmura et al., 2010), creating an environment favorable to immune activation. These data indicate that multiple types of immune cells are involved in the response to the metabolic stress, together leading to metabolic pathophysiology. Fourth, it is a chronic state without clear resolution. In contrast to acute inflammation where rapid response and resolution occur, metabolic inflammation appears to develop gradually and remain unresolved.

It is known that in classical inflammatory responses, pathogens often engage a cell signaling pathway to initiate an immune response. How is metabolic inflammation initiated? Although the initiating factor of this inflammation is not completely clear, metabolic cells seem to be capable of engaging inflammatory signaling in response to abnormal signals (Gregor and Hotamisligil, 2011). For example, excess nutrients in obesity can engage the pathogen-sensing or immune response pathways, such as TLR and NF- κ B signaling, to produce cytokines in response to metabolic stress signals (Gregor and Hotamisligil, 2011). Specifically, the roles of glucotoxicity, lipotoxicity, stress kinases, and endoplasmic reticulum stress in shaping the immune response in adipose tissue were all highlighted in recent research (Johnson et al., 2012).

In summary, metabolic inflammation represents a low-grade chronic inflammatory response initiated by excess nutrients in metabolic cells. This response eventually activates multiple types of immune cells and leads to an unresolved tissue inflammatory response. This inflammatory response often disrupts metabolism by mechanisms such as inhibiting insulin action. Nevertheless, inflammation is an adaptive response that attempts to restore functionality

and homeostasis. In the case of transient abnormal conditions, a successful acute inflammatory response often provides benefits; however, if the abnormal conditions are sustained, the response can become maladaptive.

3. Inflammatory Status in Transition Cows

With the physiological stress and metabolic changes that accompany the processes of parturition and the initiation of lactation, increased inflammation is common during the postpartum period. Unlike the inflammation associated with acute infection, the postpartum inflammatory state is often low-grade without the classical signs of inflammation, consistent with the concept of metabolic inflammation (Hotamisligil, 2006). A recent study (Sabedra, 2012) using 161 transition cows found that serum acute phase protein haptoglobin was elevated around parturition, even in cows that were apparently healthy; cows that experienced diseases or calving difficulties had significantly greater concentrations compared with healthy animals. In addition to increased haptoglobin in the serum, evidence suggests that the mRNA or protein abundance of haptoglobin was also increased in liver and adipose tissue in the first week of lactation (Saremi et al., 2012), indicating elevated inflammatory signals postpartum are often systemic and not limited to a specific focal organ.

The association between inflammation and bovine metabolic diseases is well established. Ametaj et al. (2005) reported that plasma concentrations of inflammatory markers, including haptoglobin and serum amyloid A, were elevated in cows that developed fatty liver; plasma TNF α concentration prior to parturition was positively related to postpartum liver lipid concentration. Similarly, Ohtsuka et al. (2001) observed increased serum TNF α activity in cows with moderate to severe fatty liver. To further investigate causality, several studies have

evaluated direct effects of inflammation on metabolic function. Oral administration of interferon- α daily during the last 2 wk prepartum increased markers of inflammation and plasma ketone concentrations postpartum (Trevisi et al., 2009). Subcutaneous injection of low dose TNF α for 7 days increased liver TG content by 2-fold in late-lactation dairy cows (Bradford et al., 2009). The changes in the patterns of gene expression in the liver were also consistent with the increase in fatty acid uptake and storage, and a decrease in fatty acid oxidation. These data indicate that inflammation disrupts metabolism, and may be a mechanism underlying the development of fatty liver.

Inflammation may negatively impact production. By measuring a panel of inflammatory markers and separating transition cows into quartiles for degree of inflammation, Bertoni et al. (2008) reported cows in the highest quartile had significantly lower (by 20%) milk yields than those in the lowest quartile throughout the first month of lactation. Similarly, postpartum plasma concentrations of haptoglobin greater than 1.1 g/L were associated with 947 kg decrease in 305-day mature equivalent milk yield (Huzzey et al., 2012). These data seem to be consistent with the concept that in response to inflammation, which is often associated with decreased energy intake, cows divert resources away from milk production and toward survival (Ballou, 2012). On the other hand, several studies showed that anti-inflammatory treatments improved long-term production. Trevisi and Bertoni (2008) found that aspirin treatment over the first 5 d postpartum increased milk yield through the first 60 d of lactation, with a 13% increase in peak milk yield. Our laboratory recently reported that administration of sodium salicylate (123 g/cow daily) via individual water bowls during the first 7 d postpartum increased 305-d milk yield by 2,469 kg (21% increase) and milk fat by 130 kg (30% increase) in ≥ 3 rd parity cows compared with the same parity controls (Farney et al., 2013).

Despite the potential harmful effects of inflammation, certain amounts of inflammation may be necessary to facilitate the adaptation of metabolic and physiological changes in the transition period. Perhaps the most well known aspect of inflammation is its role in clearance of pathogens and resolution of infections. Given that the incidence of infections increases dramatically in early lactation, activation of inflammatory pathways may help cows fight off diseases. One key mechanism to support copious milk synthesis and secretion in dairy cows is insulin resistance, which diverts the nutrients away from peripheral adipose tissue and muscle to the mammary gland (Bell, 1995). The mechanisms initiating this adaptive insulin resistance in early lactation are not clear. Because inflammation of muscle and adipose tissue is tightly linked to insulin resistance in many metabolic scenarios (Odegaard and Chawla, 2013), it is possible that endogenous inflammation in early lactation is needed to promote insulin resistance as nutrient demands for the mammary gland increase. Another key role of inflammation in the transition cow is to facilitate calving. Parturition is characterized by a massive influx of macrophages and neutrophils into the myometrium; this proinflammatory environment promotes the dilation of the cervix, contraction of the uterus, rupturing the fetal membranes, and expulsion of the placenta (Challis et al., 2009).

Taken together, inflammation plays a crucial role in mammalian physiological and pathological functions. An increased inflammatory state is common in transition dairy cows. This mild inflammation may support the cow's ability to fight against infection, redistribute glucose for lactation, and facilitate the process of parturition. On the other hand, excessive inflammation may decrease feed intake, exacerbate metabolic imbalances, promote health disorders, and impair lactation. Therefore, to limit disorders and improve production, it is

necessary to identify and promote a healthy degree of inflammation while minimize the pathological aspects of inflammation in transition cows.

Nutritional Strategies to Modulate Metabolism and Immunity

1. Chromium

Chromium (Cr) is an essential nutrient that modulates metabolism. Supplementation of Cr in cattle increased milk production (Hayirli et al., 2001; McNamara and Valdez, 2005; Smith et al., 2005), improved glucose clearance and insulin sensitivity (Bunting et al., 1994; Spears et al., 2012; Sumner et al., 2007), reduced lipolysis (Besong, 1996; Bryan et al., 2004), altered adipose tissue metabolism (McNamara and Valdez, 2005), and modulated immune response (Burton et al., 1993; Burton, 1995; Burton et al., 1996). Most studies evaluating effects of Cr supplementation on dairy cattle metabolism have been conducted in the transition period. Hayirli et al. (2001) reported that supplementing cows with 0, 0.03, 0.06, and 0.12 mg/kg BW^{0.75} of Cr as chromium-L-methionine (Cr-Met) from 21 d prepartum to 28 d postpartum did not affect prepartum plasma glucose or glucagon concentrations, but increased insulin and decreased NEFA. After parturition, none of these measures was affected by Cr except for decreased plasma insulin. Smith et al. (2008) reported that supplementation of 0.03 mg/kg BW^{0.75} of Cr as Cr-Met increased prepartum plasma glucose and glucagon concentrations, and tended to decrease prepartum plasma NEFA concentrations compared with either 0 or 0.06 mg of Cr/kg of BW^{0.75}; these metabolites were unaffected by Cr postpartum. By feeding Cr-propionate (CrPr) to dairy cows from 21 d prepartum to 35 d postpartum, McNamara and Valdez (2005) increased dry matter intake and milk yield in early lactation. Interestingly, they also observed that CrPr

dramatically increased adipose tissue lipogenesis compared to that of controls in the postpartum period. Collectively, these results indicate that CrPr may improve production by modulating metabolism of lactating cows.

One important characteristic of Cr is its modulatory effect on insulin sensitivity. It is established that Cr supplementation alleviates insulin resistance, enhances glucose tolerance, and improves metabolism in obese or type 2 diabetic subjects (Cefalu et al., 2010; Kim et al., 2011a; Wang and Cefalu, 2010). Although the mode of action is not precisely known, Cr has been shown to enhance insulin binding, insulin receptor abundance, and pancreatic β -cell sensitivity (Wang and Cefalu, 2010). In dairy cows, there has been interest in effects of Cr supplementation on insulin sensitivity in the transition period (Sano et al., 1993), when insulin resistance typically occurs (Bell and Bauman, 1997). Subiyatno et al. (1996) found that supplementation of 0.5 mg Cr/kg dry matter beginning 6 wk prepartum increased insulin sensitivity in primiparous but not multiparous dairy cows at 2 wk before parturition. Hayirli et al. (2001) reported that Cr-Met supplementation from 21 d prepartum to 28 d postpartum improved glucose tolerance and insulin responsiveness measured at 28 d postpartum in multiparous dairy cows. Therefore, by modulating insulin action, Cr may have significant implications for health and production of dairy cows.

Several studies have highlighted the role of Cr in bovine immunity. Burton et al. (1993) reported that Cr supplementation at 0.5 mg Cr/kg dry matter from 6 wk prepartum through 16 wk postpartum increased antibody responses to ovalbumin administration and mitogen-stimulated blastogenesis of blood monocytes in lactating cows. In addition, TNF α and IL-2 production from mitogen-stimulated monocytes was significantly decreased for cows supplemented with Cr compared with controls, particularly around peak lactation (Burton et al., 1996). Together, these

results suggest that supplemental Cr may modulate immune function and metabolism of dairy cows.

2. Yeast Product

Yeasts are unicellular fungi known for the ability to ferment sugars for the production of ethanol. The most commercially significant yeasts are *Saccharomyces cerevisiae*. These organisms have long been used to ferment starch to produce alcoholic beverages and in the baking industry to expand, raise, or dough (Schneiter, 2004). In humans, yeast is often taken as a nutritional supplement because it is an excellent source of protein and vitamins (Schneiter, 2004). In ruminants, yeast (*Saccharomyces cerevisiae*) or yeast cultures have been added to diets in an attempt to improve ruminal fermentation, feed intake, and milk yield. Dann et al. (2000) reported that yeast culture supplementation at 60 g/d from 21 d prepartum to 140 d postpartum improved dry matter intake during both the last 7 d prepartum (9.8 vs. 7.7 kg) and the first 42 d of lactation (13.7 vs. 11.9 kg) in Jersey cows. In addition, compared with control animals, cows supplemented with yeast culture reached peak milk production more quickly. The effects of yeast product supplementation on intake, production, and rumen fermentation characteristics were inconsistent among different studies. Desnoyers et al. (2009) conducted a quantitative meta-analysis using 157 experiments to assess the responses to yeast supplementation. They found that yeast product supplementation increased rumen pH (+0.03), total-tract organic matter digestibility (+0.8%), dry matter intake (DMI, +0.44 g/kg of body weight), milk yield (+1.2 g/kg of body weight), and tended to increase milk fat content (+0.05%). These results indicate that yeast supplementation may modulate the metabolism and improve the production of dairy cows.

Recent findings suggest yeast products may enhance immune function. Kim et al. (2011b) reported that neonatal calves fed hydrolyzed yeast had enhanced acute phase response, antibody production, and health scores when challenged with live vaccines, compared with control. Such responses are often attributed either to improved energy status via effects on digestive function or on activation of the immune system through cell sensing of yeast components in the gut. One overlooked aspect of the response to yeast is the potential to specifically influence mucosal immunity. Mucosal surfaces of the intestinal, respiratory, and urinary tracts are the most common route of entry of microbial pathogens into the host. Epithelial cells that line mucosal surfaces are important mechanical barriers to prevent most microbes found in the environment, including commensal microbes, from entering epithelial cells (Kagnoff and Eckmann, 1997). Indeed, production and secretion of immunoglobulin A (IgA) into the gut lumen provide the “first line of defense” against enteric pathogens by preventing colonization of the mucosal surface (Winner et al., 1991). A recent study (Zanello et al., 2011) found that yeast modulated gene expression involved in inflammation and immune cell activation in porcine intestinal epithelial cells. Specifically, yeast decreased the expression of pro-inflammatory transcripts and proteins (including IL-6 and IL-8) induced by *E. coli*.

In summary, interest in yeast products for dairy cows has expanded beyond a focus on altering ruminal fermentation and improving production. There are intriguing data suggesting that dietary yeast can improve energy status and immune function, particularly during physiological challenges. This raises the possibility that yeast supplementation can be used to address the most critical aspects of cow health during the transition period.

3. Bioactive Fatty Acids

Bioactive fatty acids are important for the normal physiological functions and health of humans and domestic animals. As a class of long-chain fatty acids, omega-3 fatty acids include α -linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), which are known to suppress inflammatory pathways. A number of recent studies have evaluated the use of flaxseed or fish oil-derived products to increase dietary supply of omega-3 fatty acids. Lessard et al. (2003) reported that feeding transition cows with flaxseed (a source of α -linolenic acid) increased serum omega-3 fatty acid concentration; on d 5 postpartum, the lymphocyte proliferative response of cows fed flaxseed was reduced compared with cows received omega-6 fatty acid supplements, suggesting an anti-inflammatory effect was achieved. On the other hand, Thatcher and colleagues have attempted to promote immune function in the transition period by supplementing calcium salts of omega-6 fatty acids instead of omega-3 fatty acids. Increasing the ratio of omega-6 to omega-3 fatty acids increased the production of hydrogen peroxide and phagocytosis of bacteria by neutrophils, and also increased plasma concentrations of acute phase proteins (Silvestre et al., 2011). Although an increased neutrophil function may improve the ability of immune system to fight off infection, elevated acute phase protein markers may also indicate metabolic inflammation, which could possibly impair metabolic function (Bertoni et al., 2008; Bradford et al., 2009). Therefore, further studies are needed to investigate if such an approach would benefit the clinical outcomes of transition cows.

In addition to dietary fatty acid supplementation, the profiles of blood NEFA may also greatly impact immunity and metabolism. Increasing evidence suggests that transition cows experience not only elevated blood NEFA concentrations but also modified NEFA compositions. At the time around parturition, the saturated fatty acids (e.g. palmitate and stearate) and the

monounsaturated oleic acid are the predominant lipids in plasma NEFA, whereas there is a decrease in EPA and DHA (Sordillo and Raphael, 2013). These NEFA compositional changes can directly influence the fatty acid profiles of immune cells (Contreras et al., 2010), which may in turn alter immune function. For example, saturated fatty acids can enhance proinflammatory pathways through the activation of NF- κ B (Kennedy et al., 2009). Conversely, EPA and DHA possess potent anti-inflammatory effects by activating G protein-coupled receptor 120 (Oh et al., 2010). In addition, changes in NEFA composition could affect immunity by modifying the profile of eicosanoids produced. In general, eicosanoids generated from omega-6 fatty acids tend to promote inflammation, and those derived from omega-3 fatty acids tend to promote the resolution of inflammation (Serhan, 2009). Therefore, strategies that can modify blood lipid concentrations and composition, such as bioactive fatty acid supplementation, may also modulate the immunity and metabolism of dairy cows.

Conclusions

The transition period in dairy cows is characterized by dramatic nutrient and energy requirements for lactation, substantial metabolic stress, and negative energy balance. In an attempt to adapt to the metabolic demands, cows increase lipid mobilization and hepatic gluconeogenesis. Inadequate adaptation is often associated with metabolic disorders, such as fatty liver and ketosis. Partially because of the disturbed endocrine and metabolic balance, the immune function in transition dairy cows is suppressed, leading to increased incidence of infectious diseases. Therefore, effective nutritional strategies that could enhance the metabolism and immunity of transition cows may improve the production and health of these animals.

With the metabolic stress and changes that accompany the processes of calving and the initiation of lactation, increased inflammation is common during the postpartum period.

Inflammation plays a major role in infectious diseases and has been suggested to be involved in metabolic diseases as well. An improved understanding of the role of inflammatory pathways in early lactation cows may improve our ability to prevent disorders.

The overall objectives of this dissertation were 1) to explore the impact of low-grade metabolic inflammation on metabolism, health, and production in early-lactation cows, and 2) to investigate nutritional strategies that could potentially modulate immunity and metabolism. In Experiment 1 (**Chapter 2**), we tested whether exogenous TNF α administration promotes inflammation, impairs glucose and lipid metabolism, and affects milk production and health in early-lactation dairy cows. In Experiment 2 (**Chapter 3**), we evaluated the effects of supplemental chromium propionate and amino acids on nutrient metabolism and neutrophil activation in lactating dairy cows. In Experiment 3 (**Chapter 4 and 5**), we determined the effects of yeast product supplementation on milk production, feeding behavior, metabolism, immunity, and uterine inflammation in transition dairy cows. A greater understanding of the role of inflammation in the transition period and the nutritional strategies that could modulate immunity and metabolism may improve the health and production of dairy cows.

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Chapter 2 - TNF α altered inflammatory responses, impaired health and productivity, but did not affect glucose or lipid metabolism in early-lactation dairy cows

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Abstract

Inflammation may be a major contributing factor to peripartum metabolic disorders in dairy cattle. We tested whether administering an inflammatory cytokine, recombinant bovine tumor necrosis factor- α (rbTNF α), affects milk production, metabolism, and health during this period. Thirty-three Holstein cows (9 primiparous and 24 multiparous) were randomly assigned to 1 of 3 treatments at parturition. Treatments were 0 (Control), 1.5, or 3.0 $\mu\text{g/kg}$ body weight rbTNF α , which were administered once daily by subcutaneous injection for the first 7 days of lactation. Statistical contrasts were used to evaluate the treatment and dose effects of rbTNF α administration. Plasma TNF α concentrations at 16 h post-administration tended to be increased ($P < 0.10$) by rbTNF α administration, but no dose effect ($P > 0.10$) was detected; rbTNF α treatments increased ($P < 0.01$) concentrations of plasma haptoglobin. Most plasma eicosanoids were not affected ($P > 0.10$) by rbTNF α administration, but 6 out of 16 measured eicosanoids changed ($P < 0.05$) over the first week of lactation, reflecting elevated inflammatory mediators in the days immediately following parturition. Dry matter and water intake, milk yield, and milk fat and protein yields were all decreased ($P < 0.05$) by rbTNF α treatments by 15 to 18%. Concentrations of plasma glucose, insulin, β -hydroxybutyrate, non-esterified fatty acids, triglyceride, 3-methylhistidine, and liver triglyceride were unaffected ($P > 0.10$) by rbTNF α treatment. Glucose turnover rate was unaffected ($P = 0.18$) by rbTNF α administration. The higher dose of rbTNF α tended to increase the risk of cows developing one or more health disorders ($P = 0.08$). Taken together, these results indicate that administration of rbTNF α daily for the first 7 days of lactation altered inflammatory responses, impaired milk production and health, but did not significantly affect liver triglyceride accumulation or nutrient metabolism in dairy cows.

Key words: Inflammation; tumor necrosis factor; gluconeogenesis; cattle; metabolic disease

Introduction

The periparturient period in dairy cows is characterized by substantial metabolic stress, endocrine changes, depressed feed intake, and negative energy balance (Grummer, 1995). In response to these changes, dairy cows mobilize adipose tissue triglyceride (TG), leading to elevated non-esterified fatty acid (NEFA) concentrations in the blood. The high flux of blood NEFA to the liver often exceeds the capacity of the liver to completely oxidize NEFA to CO₂, resulting in partial oxidation to form ketones or esterification to form TG within hepatocytes (Grummer, 2008). Excessive production of ketones often leads to ketosis, which is characterized by elevated blood concentrations of β -hydroxybutyrate (BHBA), depressed appetite, and decreased milk production (Oetzel, 2007). Furthermore, because ruminants are inefficient at exporting TG from the liver, excessive TG accumulation or fatty liver occurs. Ketosis and fatty liver affect up to 50% of dairy cows, compromising production, health, and reproduction (Oetzel, 2007; Bobe et al., 2004). Gluconeogenesis is of great importance at all times in ruminants, providing up to 90% of the necessary glucose (Young, 1977). Meeting glucose needs can be a tremendous metabolic challenge for early-lactation dairy cows, which often experience inadequate feed intake. Although lipid and glucose metabolism in periparturient cows has been extensively studied, the mechanisms underlying the development of these metabolic disorders are not fully understood.

Inflammatory mediators play critical roles in immunity and metabolism, and recent research has suggested that inflammation is involved in metabolic disorders as well. For example, obesity is associated with a chronic low-grade inflammatory state in multiple metabolic tissues including adipose, liver, muscle, pancreas, and brain (Gregor and Hotamisligil, 2011). Compared with lean controls, obese mice had increased levels of adipose tissue tumor necrosis

factor- α (TNF α), a potent cytokine capable of triggering inflammatory responses (Hotamisligil et al., 1995). In dairy cows, fatty liver was associated with increased plasma inflammation markers, including haptoglobin and serum amyloid A (Ametaj et al., 2008). Although a recent study reported that plasma concentrations of TNF α in periparturient cows were decreased postpartum compared with prepartum (Schoenberg et al., 2011), elevated activity of serum TNF α was observed in cows with moderate to severe fatty liver (Ohtsuka et al., 2001). In light of these findings, we hypothesized that chronic low-grade inflammation may be a mechanism underlying bovine fatty liver. In fact, we found that low-level administration of the recombinant bovine TNF α (rbTNF α) for 7 d promoted liver inflammation and TG accumulation (Bradford et al., 2009). However, late-lactation animals were used in that work, and it is unclear if this model is applicable to cows in the first week of lactation, when fatty liver naturally occurs. Beyond direct promotion of fatty liver, hepatic inflammation may also affect glucose production. Bradford et al. (2009) reported that rbTNF α administration reduced liver gluconeogenic gene expression in late-lactation cows. If inflammatory cytokines impair glucose production in early-lactation animals, the resulting hypoglycemia would likely increase adipose TG mobilization and metabolic disorders.

We hypothesized that metabolic stress in early-lactation cows is exacerbated by inflammatory challenge, thereby adversely affecting production and health. Therefore, the objective of this study was to determine whether exogenous rbTNF α administration promotes inflammation and TG accumulation, impairs gluconeogenesis, alters lipid metabolism, and affects milk production and health status in early-lactation dairy cows. A greater understanding of the pathological impact of inflammatory pathways in these animals may improve our ability to prevent metabolic disorders and increase production.

Materials and Methods

The Kansas State University Institutional Animal Care and Use Committee approved all experimental procedures.

Design and Treatments

A recombinant expression vector encoding the secreted form of bovine TNF α (Entrez Protein Accession AAB84086.1, region 77–233) was expressed in *Escherichia coli* and purified by a commercial laboratory (GenScript Corp., Piscataway, NJ). Purity of the isolated rbTNF α was verified by SDS-PAGE, and endotoxin was removed using polymyxin B until contamination was less than < 1 EU/ μ g protein. Thirty-three Holstein cows (9 primiparous and 24 multiparous; body condition score: 3.26 ± 0.31 , body weight 741 ± 83 kg; mean \pm SD) were randomly assigned to 1 of 3 treatments at parturition. Treatments were balanced within parity, and dystocia did not differ by treatment (calving difficulty scores: 1.36, 1.18, and 1.18 ± 0.5 for Control, 1.5 μ g/kg and 3.0 μ g/kg TNF, respectively; 1 to 5 scale, with 1 = no difficulty). Treatments (dose per d) were no rbTNF α (Control; 10% glycerol in saline), 1.5 μ g rbTNF α /kg body weight in saline with 10% glycerol, and 3.0 μ g rbTNF α /kg body weight in saline with 10% glycerol. These doses are slightly less and slightly more, respectively, than the 2.0 μ g/kg dose that was previously shown to alter hepatic nutrient metabolism in lactating cows (Ohtsuka et al., 2001). Glycerol was included in the solution to improve solubility of the rbTNF α protein. Cows were injected subcutaneously once daily (1600 h) for the first 7 d of lactation. Cows were milked 3 times daily (0400, 1200, and 2000 h) in a milking parlor and fed twice daily (0800 and 1600 h) for *ad*

libitum intake of a diet formulated to meet National Research Council (NRC, 2001) nutrient requirements. Ingredient and nutrient composition of the diet are shown in Table 1.

Sample and Data Collection and Analysis

Feed Intake, Milk Production, Energy Balance, and Health Monitoring

During the 7-d experimental period, intake of feed and water and milk yield were recorded daily. Milk samples were collected from d 4 to 7 of treatment at each of the 3 milkings for analysis of milk components. Samples were analyzed for concentrations of fat, true protein, lactose (B-2000 Infrared Analyzer; Bentley Instruments Inc., Chaska, MN), urea nitrogen (MUN spectrophotometer, Bentley Instruments Inc.), and somatic cells (SCC 500, Bentley Instruments Inc., Heart of America DHIA, Manhattan, KS). Somatic cell linear score was calculated as described by Shook (1993): $\log_2(\text{somatic cell count}/100) + 3$. Energy balance was calculated for each cow using the following equation from NRC (2001): $\text{energy balance} = \text{net energy intake} - (\text{net energy of maintenance} + \text{net energy of lactation})$. $\text{Net energy intake} = \text{dry matter intake} \times \text{net energy density of the diet}$ (NRC, 2001); $\text{net energy of maintenance} = 0.08 \times \text{body weight}^{0.75}$; $\text{net energy of lactation} = [(0.0929 \times \text{fat } \%) + (0.0547 \times \text{protein } \%) + (0.0395 \times \text{lactose } \%)] \times \text{milk yield}$. In all equations, energy is expressed in Mcal and mass in kg.

Cows were monitored daily for health status. Ketosis was recorded when urine acetoacetate exceeded 80 mg/dL on any day or 40 mg/dL for 2 consecutive days (Ketostix; Bayer Corp. Diagnostics Division, Elkhart, IN). Fever was diagnosed when a cow had a rectal temperature greater than 39.4°C. Other disorders or diseases, including mastitis, metritis, and milk fever, were diagnosed according to the guidelines by Kelton et al. (1998).

Plasma Metabolites and Hormones

Blood samples were collected from coccygeal vessels daily (0800 h) in 2 tubes, one containing potassium EDTA and the other containing potassium oxalate with sodium fluoride as a glycolytic inhibitor (Vacutainer; Becton Dickinson, Franklin Lakes, NJ). Blood was centrifuged at $2,000 \times g$ for 10 min immediately after sample collection, and plasma was frozen at -20°C until further analysis. Plasma samples collected daily during the 7-d treatment period were analyzed for NEFA (NEFA-HR; Wako Chemicals USA Inc., Richmond, VA), glucose (kit #439-90901; Wako Chemicals USA Inc.), BHBA (kit #H7587-58; Pointe Scientific Inc., Canton, MI), and TG (#10010303; Cayman Chemical, Ann Arbor, MI). Plasma samples collected on d 0, 3, 5, and 7 of treatment were analyzed for TNF α (an ELISA method described by Farney et al., 2011), insulin (ELISA kit #10-1201-01; Mercodia AB, Uppsala, Sweden), and haptoglobin (ELISA kit #2410-7; Life Diagnostics, West Chester, PA). Plasma samples collected on d 7 of treatment were analyzed for 3-methylhistidine. Samples were prepared by mixing 500 μL of plasma with 500 μL of Seraprep (Pickering Labs, Mountain View, CA) and frozen overnight. The deproteinized plasma was then thawed, vortexed, and centrifuged at $17,000 \times g$ for 10 min. The resulting supernatant was analyzed for 3-methylhistidine using Li^+ cation exchange chromatography and detection by fluorimetry following post column derivitization with o-phthalaldehyde. The HPLC column and all reagents and eluents were purchased from Pickering Labs (Mountain View, CA). Column flow rate was 0.375 mL/min and column temperature was maintained at 36°C . Injection volume was 10 μL . Eluent 1 (Li357) was run for 5 min then eluent 2 (Li750) for 15 min and eluent 3 (RG003) for 5 min. The column was then re-equilibrated back to eluent 1 for 20 min. Post column derivitization was performed using a post column mixing T at a flow rate of 0.375 mL/min using 950 mL OPA diluent (OD104) containing 0.3 g o-

phthalaldehyde, 2.0 g Thiofluor, and 3 mL 30% Brij 35. The fluorimeter excitation was set to 330 nm and emission to 465 nm.

Plasma Eicosanoids

Plasma samples collected on d 0, 1, 3, and 5 of treatment were analyzed for eicosanoids as previously described (Farney et al., 2011). Plasma samples (500 μ L) were mixed with 1 mL ice cold methanol, 6 μ L antioxidant/reducing agent containing EDTA, butylhydroxytoluene, triphenylphosphine, and indomethacin (4 μ L/mL), 200 μ L of a mixture of internal standards, and 1 μ L of formic acid. Sample mixtures were centrifuged at 4°C for 10 min at $3,000 \times g$, and the supernatant was used for solid-phase extraction using Strata-X SPE columns (Phenomenex Inc., Torrance, CA). Eicosanoids were isolated using 2 distinct ultra-high–pressure liquid chromatography (UPLC) and mass spectrometry (UPLC-MS) methods. Both methods used reverse-phase liquid chromatography on an Acquity UPLC BEH C18 1.7- μ m column (2.1 \times 100 mm; Waters Corp., Milford, MA) at a flow rate of 0.6 mL/min at 35°C and a single-quadrupole H-class Acquity SQD mass spectrometer in electrospray negative ionization mode (Waters Corp.). The electrospray voltage was –3 kV, and the turbo ion spray source temperature was 450°C. Nitrogen was used as the drying agent. For each method, a 10- μ L sample was injected 3 consecutive times using a 10- μ L injection loop. An isocratic mobile phase consisting of acetonitrile/water/formic acid (45/55/0.01; vol/vol/vol) with an analysis time of 15 min was used to measure leukotriene B₄ (LTB₄), thromboxane B₂ (TXB₂), prostaglandin E₂ (PGE₂), prostaglandin F₂ (PGF₂), lipoxin A₄, resolvin D₁, and resolvin D₂. The second method used an isocratic mobile phase of acetonitrile/methanol/water/formic acid (47.4/15.8/26.8/0.01; vol/vol/vol/vol) and an analysis time of 10 min to analyze 9-hydroxyoctadecadienoic acid

(HODE), 13-HODE, 9-oxo-octadecadienoic acid (oxoODE), 13-oxoODE, 5-hydroxyeicosatetraenoic acid (HETE), 15-HETE, 7-maresin 1, leukotriene D₄ (LTD₄), and protectin.

Quantitation of eicosanoid concentrations was performed with Waters Empower 2 software (Waters Corp.). A linear calibration curve with 5 points ($R^2 > 0.99$) was generated for each eicosanoid with standards and internal standards purchased from Cayman Chemical Co. (Ann Arbor, MI). The curves ranged from 0.0024 to 2.38 ng/ μ L. Empower 2 identified the sample peak by matching its retention time with the standard. A response was calculated for each matched peak by dividing the sample peak's response by its internal standard's response. This response was multiplied by the concentration of the internal standard for each analyte. The concentration of each analyte was calculated using the response peak and injection volume.

Glucose Turnover Rate

After daily blood sampling on d 5 of treatment, jugular vein catheters were placed and at least 18 h of recovery were allowed prior to sample collection through the catheters. On treatment d 7, cows were given a glucose bolus containing U-¹³C-glucose (99 atom %, Sigma-Aldrich Co., St. Louis, MO) through jugular catheters (Schulze et al., 1991). Jugular blood samples were collected 10 min prior to infusion and at 10, 20, 30, 40, 50, 60, 90, and 120 min post-infusion. Each cow received approximately 1 g of U-¹³C-glucose in 50 mL of sterile saline; syringes were weighed immediately before and after infusion to determine the exact amount administered. Catheters were flushed with a sterile solution of 3.5% sodium citrate after the labeled glucose bolus (20 mL) and after each blood sample collection (5 mL). Plasma glucose concentration was quantified to verify that steady-state conditions were met.

Plasma samples collected for the glucose turnover assay were analyzed for U-¹³C-glucose enrichment (Metabolic Solutions, Inc., Nashua, NH). Glucose was extracted and converted to aldonitrile pentaacetate derivative (Tserng and Kalhan, 1983), and negative chemical ionization GC/MS (Hewlett-Packard 5890; Agilent Technologies, Santa Clara, CA) was used to analyze derivatized samples. The isotopic composition of the glucose was determined by monitoring unlabeled (M+0: m/z = 328) versus U-¹³C-labeled (M+6: m/z = 334) glucose derivatives. This approach, as opposed to oxidation of glucose and measurement of CO₂ enrichment, ensures that results are not biased by carbon recycling via the Cori cycle. Turnover rate of plasma glucose was calculated from the disappearance curve for U-¹³C-glucose. Enrichment of plasma glucose for each animal was fitted to an exponential decay curve according to the following equation: $E_t = E_0 \times e^{-kt}$, where t = time relative to infusion (min), E_t = enrichment of plasma glucose (U-¹³C-glucose: unlabeled glucose ratio) at time t, E_0 = enrichment at time t = 0, and k = rate constant (min⁻¹). After using the best-fit equations to determine k and E_0 , the total glucose pool was calculated by the following equation: $G = M \div E_0$, where G = total glucose pool (g) and M = mass of tracer infused (g). Plasma glucose turnover rate (GTR, g/min) was calculated according to the equation $GTR = G \times k$. Samples collected 10 min prior to infusion of U-¹³C-glucose were also analyzed to verify the lack of natural occurrence of the M+6 isotopomer.

Liver TG

At the end of treatment d 7, liver samples were collected by percutaneous biopsy as described by Morey et al. (2011). For analysis of TG content, approximately 20 mg of liver was placed into 500 µL of chilled phosphate buffered saline (pH 7.4) and homogenized. The homogenate was centrifuged at 2,000 × g for 10 min at 4°C, and 100 µL of the supernatant was

then removed for free glycerol and total protein analyses. Triglyceride content was measured using a method adapted from Starke et al. (2010). The remaining liver homogenate was incubated with 100 μ L of lipase (porcine pancreatic lipase; MP Biomedicals, Solon, OH) for 16 h at 37°C, and glycerol content was then determined by an enzymatic glycerol phosphate oxidase method (#F6428, Sigma-Aldrich Co.). Triglyceride content was calculated based on the difference between glycerol concentrations before and after lipase digestion. Total protein content of the original homogenate was analyzed by a Coomassie blue (Bradford, 1976) colorimetric method (kit #23236; Thermo Scientific Pierce, Rockford, IL). To avoid potential bias introduced by differences in moisture content of liver samples, liver TG concentration was normalized by protein concentration, which is unaltered in fatty liver (Fronk et al., 1980).

Transcript Abundance

Total RNA was extracted from liver tissue using a commercial kit (RNeasy Lipid Tissue Mini Kit; Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. Two micrograms of total RNA was used as the template for the reverse transcriptase reaction using random primers (High-Capacity cDNA RT Kit; Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed in duplicate on 96-well plates with 5% of the cDNA product in the presence of 200 nM gene-specific forward and reverse primers with real-time SYBR green fluorescent detection using SYBR Green Premix reagent (7500 Fast Real-Time PCR System, Applied Biosystems). Primers were designed (www.ncbi.nlm.nih.gov/tools/primer-blast/) using GenBank sequences (Table 2). Data were recorded and analyzed with Sequence Detector software (Applied Biosystems). All sample values were normalized against ribosomal protein subunit 9 (*RPS9*) values (Mamedova et al., 2010), and relative gene expression was

quantified by using the $2^{-\Delta Ct}$ method. Treatment did not influence the Ct value for *RPS9* ($P = 0.56$), suggesting that it served as a valid control gene.

Western Blot

Relative protein abundance of I κ B α , c-Jun, phosphorylated c-Jun, and TNF α in liver samples was determined by Western blot. Liver samples (~20 mg) were homogenized at 4°C in RIPA lysis buffer containing a broad-spectrum protease inhibitor cocktail (Protease Inhibitor Cocktail I; EMD Millipore, Billerica, MA) and a phosphatase inhibitor (PhosphoStop; Santa Cruz Biotechnology, Santa Cruz, CA). The homogenate was centrifuged at $15,000 \times g$ for 10 min at 4°C, and total protein concentration of the supernatant was measured (Bradford, 1976). Forty micrograms of total protein from liver tissue was heated at 90°C for 5 min, vortexed, and loaded onto a 4 to 12% Tris-HCl gel for electrophoresis. Samples were separated by SDS-PAGE and dry-transferred onto nitrocellulose membranes (iBlot; Invitrogen, Carlsbad, CA). Membranes were blocked for 2 h in blocking buffer (5% dry milk in Tris-HCl buffer, pH 7.5, with 0.05% Tween 20). After incubation with blocking buffer, the membranes were washed 3 times for 5 min each with washing buffer (phosphate-buffered saline, pH 7.5, containing 0.05% Tween 20). Membranes were then incubated with primary antibodies (diluted 1:250) from Santa Cruz Biotechnology against I κ B α (catalog #sc-847), c-Jun (#sc-44), and phosphorylated c-Jun (#sc-822) overnight at 4°C. After washing, secondary antibody diluted 1:10,000 was incubated for 1 h at room temperature. Antibody incubation and detection for TNF α was carried out as previously described (Bradford et al., 2009). Immunodetection was performed by chemiluminescence (West-Dura; Thermo Scientific, Waltham, MA), and bands were quantified by scanning densitometry (ChemiDoc-It Imaging System; UVP Inc., Upland, CA).

Calculations and Statistical Analyses

One cow that received 3 µg/kg rbTNFα developed a severe fever on d 6 of treatment, and the d 7 treatment was not given. Consequently, the liver biopsy and glucose turnover test were not conducted for this cow, but all other data were retained through d 6. Plasma glucose data indicated that 2 cows did not meet steady-state conditions during the glucose turnover test; these cows were not used for turnover rate analysis. In total, data from 33, 32, and 30 cows were used for production responses and plasma analyses, liver tissue analyses, and glucose turnover analysis, respectively. Data were analyzed using the MIXED Procedure of SAS (version 9.2; SAS Institute Inc., Cary, NC) to assess the fixed effects of treatment, parity, time, and 2- and 3-way interactions; cow was included as a random effect. Data were log-transformed for analysis when necessary (including plasma concentrations of TNFα and 3-methylhistidine, and hepatic transcript abundance of *AGPAT1*, *PCK1*, and *CPT1a*) to achieve a normal distribution of residuals, and data presented are back-transformed in these cases. Repeated measures over time were modeled with either autoregressive or heterogeneous autoregressive covariance structures, depending on which analysis had the lowest Bayesian Information Criterion value. Denominator degrees of freedom were estimated using the Kenward-Rogers method. Values were deemed outliers and omitted from analysis when Studentized residuals were > 3.5 or < -3.5. Interactions were investigated when $P < 0.10$ using the slice option, and slices were declared significant at $P < 0.05$. Contrasts were used to evaluate Control vs. rbTNFα treatments and 1.5 µg/kg vs. 3.0 µg/kg rbTNFα doses. Health disorders were analyzed using JMP (version 8.0; SAS Institute Inc., Cary, NC). A nominal logistic analysis was first run for each health disorder to assess the fixed effects of treatment, parity, and treatment × parity interaction. For those disorders with an

interaction $P < 0.10$, Fisher's exact test was used to assess rbTNF α and dose contrasts within each parity group. For all other disorders, Fisher's exact test was used to test these contrasts across all animals. Significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P < 0.10$.

Results

Inflammatory Signals

Compared with Control, plasma TNF α concentrations tended to be increased ($P = 0.09$) by rbTNF α treatments, but 1.5 $\mu\text{g/kg}$ did not differ ($P = 0.19$) from 3 $\mu\text{g/kg}$ rbTNF (Figure 1A). Plasma haptoglobin concentrations were greater ($P < 0.01$, Figure 1B) in rbTNF α treatments than Control, but did not differ between 1.5 and 3 $\mu\text{g/kg}$ rbTNF ($P = 0.68$). Across the 3 groups, haptoglobin levels were comparable ($P = 0.94$) at parturition, but increased during the first week of lactation in response to rbTNF α . On d 5 and 7 of treatment, rbTNF α treatments increased haptoglobin concentration by ~2.5- and ~3.5-fold, respectively, compared with Control.

Plasma eicosanoid results are shown in Table 3. Most eicosanoids were unaffected ($P > 0.10$) by treatments; out of the 16 measured eicosanoids, only 9-oxoODE was decreased ($P = 0.01$) by rbTNF α administration. The pro-inflammatory class as a composite was unaffected by treatments; however, the anti-inflammatory class tended ($P = 0.08$) to be decreased by the 3 $\mu\text{g/kg}$ compared with 1.5 $\mu\text{g/kg}$ rbTNF α dose (Figure 2), though rbTNF α treatment did not differ from Control ($P = 0.22$).

Plasma eicosanoids with significant ($P < 0.05$) day effects are presented in Table 4. Plasma concentrations of TXB₂, 15-HETE, and 9-HODE were elevated around parturition and decreased during the first week of lactation. In contrast, resolvins D₂ and 7-maresin 1 increased

gradually after parturition. The pro-inflammatory class decreased ($P = 0.02$) during the first week of lactation, whereas the anti-inflammatory class ($P < 0.01$) increased after parturition.

As shown in Figure 3A, hepatic transcript abundance of *TNF α* was increased ($P = 0.02$) by 3 $\mu\text{g/kg}$ compared with 1.5 $\mu\text{g/kg}$ rbTNF α , but overall, rbTNF α treatments did not differ from Control ($P = 0.73$). There were no treatment effects ($P > 0.10$) for protein abundance of TNF α , c-Jun, or relative c-Jun phosphorylation (Figure 3B). There was a tendency for a treatment \times parity interaction ($P = 0.07$) for I κ B α , reflecting increased ($P = 0.04$) I κ B α abundance by rbTNF α treatment in primiparous cows (data not shown). Compared with primiparous cows, I κ B α was decreased ($P = 0.02$), TNF α was increased ($P = 0.04$), and relative c-Jun phosphorylation was decreased ($P = 0.04$) in multiparous cows (Figure 3C).

Energetics

Dry matter and water intake were decreased ($P < 0.05$) by rbTNF α treatments, but no dose effect ($P > 0.10$) was observed (Table 5). Cows were in negative energy balance during the experimental period, and the energy balance values were not affected ($P > 0.10$) by treatments. Milk yield was decreased ($P = 0.03$) by rbTNF α treatments, with no dose effect ($P = 0.76$). Similar to milk yield, milk energy output was decreased ($P < 0.01$) by rbTNF α administration. Milk fat percentage was decreased ($P = 0.02$) by 3.0 $\mu\text{g/kg}$ rbTNF α compared with the 1.5 $\mu\text{g/kg}$ dose, but overall, rbTNF α treatments did not differ from Control ($P = 0.55$). Nevertheless, milk fat yield was decreased ($P = 0.01$) in cows treated with rbTNF α , and there was a tendency for decreased ($P = 0.07$) milk fat yield for the 3.0 $\mu\text{g/kg}$ rbTNF dose compared with 1.5 $\mu\text{g/kg}$. Milk protein and lactose content were unaffected by treatment ($P > 0.10$), but yields of both were

decreased ($P < 0.01$) by rbTNF α administration. Milk urea nitrogen concentration and somatic cell linear score were not affected ($P > 0.10$) by treatments.

Metabolism

Plasma concentrations of glucose, insulin, BHBA, NEFA, TG, and 3-methylhistidine, and liver TG were not affected ($P > 0.10$, Table 6) by rbTNF α treatment. Glucose turnover rate (Table 6) was unaffected ($P = 0.18$) by rbTNF α treatments. When expressed relative to dry matter intake (daily glucose turnover rate/day 7 dry matter intake), 3 $\mu\text{g/kg}$ rbTNF α was greater ($P < 0.01$) than other treatments.

As shown in Figure 4, the mRNA abundance of mitochondrial carnitine palmitoyltransferase 1a (*CPT1a*) was increased ($P < 0.01$) by rbTNF α treatments, with 3 $\mu\text{g/kg}$ greater ($P = 0.04$) than 1.5 $\mu\text{g/kg}$ rbTNF. No treatment effects ($P > 0.10$) were detected for apolipoprotein B (*ApoB*), 1-acylglycerol-3-phosphate O-acyltransferase 1 (*AGPAT1*), phosphoenolpyruvate carboxykinase 1 (*PCK1*), or pyruvate carboxylase (*PC*).

Health Disorders

Incidence of health disorders is shown in Table 7. There was a tendency for increased ($P = 0.08$) risk of experiencing 1 or more health disorders for 3 $\mu\text{g/kg}$ compared with 1.5 $\mu\text{g/kg}$ rbTNF α , although the overall rbTNF α effect was not significant ($P = 0.25$). Ketosis incidence reported in Table 7 was based on detection of urine ketones; of the cows identified by this method, 5 cows (1 control, 2 on each of the rbTNF α treatments) had plasma BHBA

concentrations that exceeded 3 mM on at least one day, identifying them as clinical cases, whereas the 2 others exceeded the 1.4 mM threshold for subclinical ketosis (Oetzel, 2007).

Discussion

Our previous research demonstrated that administration of 2 µg/kg body weight rbTNFα daily for 7 d was adequate to mimic a chronic low-grade inflammatory state without causing acute systemic inflammation in late-lactation cows (Bradford et al., 2009). Therefore, in the present study, we administered 1.5 and 3 µg/kg rbTNFα in an attempt to characterize a similar low-grade inflammation in early-lactation cows, which experience dramatic energy demands of milk production and tremendous metabolic stress compared with late-lactation animals. Although blood samples were collected 16 h after the previous injection, we still observed a tendency for elevated plasma TNFα concentrations in treated cows, indicating a sustained chronic inflammation status in cows receiving rbTNFα. Moreover, 3 µg/kg rbTNFα administration increased hepatic transcript abundance of *TNFα*, further supporting that treatment enhanced pro-inflammatory responses.

We also evaluated plasma concentrations of haptoglobin and eicosanoids, and the protein abundance of key inflammatory mediators in the liver. Haptoglobin is an acute phase protein primarily released by the liver in inflammation (Hachenberg et al., 2007). Our finding that rbTNFα increased haptoglobin agrees with a previous study that daily administration of 2.5 µg/kg rbTNFα for 7 d dramatically elevated plasma haptoglobin in lactating cows (Kushibiki et al., 2003). Interestingly, haptoglobin elevation has been associated with bovine fatty liver (Bobe et al., 2004), suggesting that liver TG accumulation may be associated with inflammation. However, recent research indicated that excess NEFA influx to the liver, rather than hepatic TG

accumulation *per se*, causes liver damage through promoting lipotoxicity, oxidative stress, and inflammatory reactions (Neuschwander-Tetri, 2010). In the present study, neither NEFA nor liver TG were affected by treatments, suggesting that rbTNF α increased haptoglobin independent of altered lipid mobilization.

Eicosanoids are a family of signaling molecules produced by oxidation of 20-carbon essential fatty acids. They exert complex control over inflammation, and can either enhance (such as certain types of prostaglandins, thromboxanes, and leukotrienes) or resolve (such as resolvins, protectins, and maresins) inflammatory responses depending on their types and the timing of production (Sordillo et al., 2009). The increased lipolysis and elevated plasma NEFA in early lactation may increase the supply of fatty acid substrates for eicosanoid production, thereby affecting the duration and magnitude of inflammation. In the present study, 6 out of the 16 measured plasma eicosanoids were significantly altered over time, but few treatment effects were detected for eicosanoids. One interesting finding was that the highest dose of rbTNF α tended to suppress the anti-inflammatory eicosanoid class, primarily reflecting decreased concentrations of 7-maresin 1, protectin, and 9-oxoODE. We are unaware of evidence in the literature of cytokines inhibiting release of anti-inflammatory eicosanoids, but such an effect is consistent with the pro-inflammatory effects of TNF α (Hotamisligil et al., 1995). We cannot rule out the possibility that decreases in anti-inflammatory eicosanoids may have been driven by an unknown mediator rather than a direct effect of TNF α . Still, it is intriguing that rbTNF α administration apparently did more to suppress the production of resolving lipid mediators than to enhance the production of inflammatory lipids, and this result should inform choices about measures of interest in future studies.

We found that concentrations of pro-inflammatory eicosanoids, including certain types of prostaglandins and thromboxanes, were elevated around parturition and decreased during the first week of lactation, whereas pro-resolving metabolites, such as resolvin D₂ and 7-maresin 1, increased after parturition. Consistent with the results of the above-mentioned eicosanoids, the composite pro-inflammatory class was elevated at parturition and decreased thereafter, whereas the anti-inflammatory class changed in the opposite direction. Although the inflammatory state of postpartum dairy cows has been well documented (Bionaz et al., 2007), these are the first data to document an increase in anti-inflammatory eicosanoids during the resolution of inflammation as lactation proceeds. The causative role of eicosanoids in this resolution phase should be a fruitful area for further investigation.

Nuclear factor- κ B (NF- κ B) is a key transcription factor that plays crucial roles in inflammation (Karin and Ben-Neriah, 2000). In nonstimulated cells, NF- κ B is bound to inhibitory I κ B proteins and sequestered in the cytoplasm. Activation of NF- κ B primarily occurs via the phosphorylation, ubiquitination, and degradation of I κ B proteins; subsequently, the liberated NF- κ B is translocated to the nucleus to drive expression of inflammatory genes (Häcker and Karin, 2006). One of the major I κ B proteins in inhibiting NF- κ B activation is I κ B α , the abundance of which can be used to estimate anti-inflammatory status. In this study, hepatic I κ B α abundance was significantly increased by rbTNF α in primiparous cows. This observation is unexpected, because TNF α is a potent activator of I κ B α degradation (Karin and Ben-Neriah, 2000), and suggests the involvement of compensatory anti-inflammatory adaptations in response to rbTNF α .

We also investigated the inflammatory mediator c-Jun, which is a key component of the pro-inflammatory transcription factor activator protein-1. The transcriptional activity of c-

Jun is increased by phosphorylation of serine residues in response to various stimuli, including inflammation (Lee et al., 2001). As for the NF- κ B pathway, we found no evidence of hepatic c-Jun activation in response to rbTNF α . Collectively, rbTNF α treatment did not significantly alter the hepatic protein abundance of inflammatory mediators, but nevertheless greatly increased the plasma concentration of haptoglobin, an acute phase protein induced primarily by inflammatory signals in the liver (Nakagawa-Tosa et al., 1995). It is possible that haptoglobin was primarily induced by transcription factors other than NF- κ B or activator protein-1.

In this study, feed intake and milk production were decreased by rbTNF α treatments by 18 and 15%, respectively. TNF α is a key mediator causing anorexigenic responses in various diseases, and many of the effects of TNF α are dependent on its actions in the hypothalamus (Romanatto et al., 2007). Interestingly, our previous research in late-lactation cows showed that 2 μ g/kg rbTNF α treatment daily for 7 d also decreased feed intake by 18%, but did not significantly alter milk production (Bradford et al., 2009). Kushibiki et al. (2003) reported that daily administration of 2.5 μ g/kg rbTNF α for 7 d dramatically decreased feed intake (by ~34%) and milk yield (by ~15%). As expected, cows were in negative energy balance during the immediate postpartum period, indicating that their energy expenditure, driven largely by lactation requirements, greatly exceeded their energy intake. However, energy balance values were not affected by rbTNF α administration because the magnitude of decreased milk production was comparable to that of decreased feed intake.

Administration of rbTNF α did not alter plasma markers of lipid metabolism or promote fatty liver. As a lipolytic mediator in adipose tissue (Zhang et al., 2002), previous studies found that rbTNF α administration at 2.5 μ g/kg significantly increased plasma NEFA concentrations in mid-lactation cows (Kushibiki et al., 2003) and heifers (Kushibiki et al., 2000). We also

previously reported that 7-d rbTNF α administration at 2 μ g/kg daily increased liver TG by ~2-fold (Bradford et al., 2009). It is possible that because plasma NEFA and BHBA concentrations, as well as liver TG content, were already dramatically elevated in response to negative energy balance in the periparturient period, the doses of rbTNF α used in this study were insufficient to promote further alterations in lipid metabolism. On the transcriptional level, our previous work (Bradford et al., 2009) in late-lactation cows showed that 2 μ g/kg rbTNF α administration daily for 7 d increased the transcript abundance of *AGPAT1* (which catalyzes the esterification of a fatty acyl-CoA to the sn-2 position of the glycerol backbone in TG synthesis), tended to decrease *CPT1a* (which catalyzes transport of fatty acids into mitochondria for oxidation), and did not affect *ApoB* (which is a key component of very low-density lipoprotein that mediates hepatic TG exportation). Here, we did not find treatment effects for *AGPAT1* or *ApoB*, and found that rbTNF α increased *CPT1a* abundance dose-dependently. Collectively, rbTNF α did not promote the pathways favoring hepatic lipid accumulation in early-lactation cows.

Lactating cows rely heavily on hepatic gluconeogenesis to meet glucose requirements because the majority of dietary carbohydrates are fermented in the rumen. Under steady-state conditions, the rate of glucose appearance equals the rate of its disappearance, and the calculated glucose turnover rate approximately equals gluconeogenesis in ruminants, because net portal appearance of glucose is negligible (Young, 1977). In this study, there were no treatment effects on plasma glucose or insulin, abundance of key gluconeogenic transcripts, or plasma glucose turnover rate, indicating that low-grade inflammation did not affect hepatic gluconeogenesis in early-lactation cows. Previous data regarding TNF α effects on gluconeogenesis have been inconsistent. Short-term recombinant human TNF α administration at 10 μ g/kg, but not 3.5 μ g/kg, significantly increased glucose production (likely from glycogenolysis) and metabolic clearance

rate in dogs; coupled with decreased fatty acid flux, the authors concluded that TNF α given in a high dose causes a shift toward carbohydrate as an energy substrate (Sakurai et al., 1993). In a subsequent study, Sakurai et al. (1996) infused 2.5 $\mu\text{g/kg}$ TNF α in dogs for 2 h and did not observe a change in glucose production.

The primary substrate for gluconeogenesis in ruminants is propionate, a short-chain fatty acid derived from ruminal fermentation; as a result, liver glucose production in cows is often a function of energy intake (Reynolds et al., 2003). To assess whether gluconeogenesis was affected by substrate supply, we expressed glucose production relative to feed intake. Interestingly, this analysis revealed that the 3 $\mu\text{g/kg}$ rbTNF α treatment increased the apparent efficiency of gluconeogenesis from dietary substrates, primarily because of the low feed intake for that treatment. Given that propionate is used for glucose production at >90% efficiency in ruminants (Steinhour and Bauman, 1988), increased metabolic efficiency was not likely the reason for this response. When requirements are greater than nutrient intake, such as in early lactation, the contributions of lactate, glycerol, and amino acids from body tissues to gluconeogenesis increase markedly (Bell, 1995). Glycerol is released primarily from adipose TG mobilization, which was likely unaffected by treatments (based on unaltered plasma NEFA). In an attempt to evaluate whether 3 $\mu\text{g/kg}$ rbTNF α -treated cows had greater amino acid supply from protein mobilization, we measured concentrations of plasma 3-methylhistidine, a marker of muscle protein breakdown that has been shown to increase rapidly during the periparturient period and peak at 1 week after parturition (Blum et al., 1985). Treatment effects on plasma 3-methylhistidine were not significant; however, the 3 $\mu\text{g/kg}$ rbTNF α treatment mean was numerically 20% greater than the other treatments. It seems likely that a combination of slightly

enhanced muscle proteolysis and decreased amino acid use for milk protein synthesis resulted in greater supply of amino acids for gluconeogenic substrate in the 3 µg/kg rbTNFα treatment.

Despite the lack of dramatic alterations in metabolism, the higher dose of rbTNFα tended to impair the health of postpartum cows. Health disorders affect considerable numbers of periparturient dairy cows, compromising animal welfare, milk production, and fertility. Ketosis is considered the most important metabolic disease in the U.S. dairy industry (Oetzel, 2007), and ketotic cows are also at increased risk for other disorders, such as displaced abomasum and metritis (Duffield et al., 2009). Ketosis may increase the risk of infectious disorders through negative effects of ketone bodies on immune cell function (Grinberg et al., 2008). In this study, rbTNFα increased the incidence of ketosis by 3-fold in the first week of lactation, accounting for much of the increase in total disease incidence. There are numerous mechanisms by which rbTNFα may have promoted subclinical ketosis, but poor feed intake in early lactation is a key risk factor for ketosis (Grummer, 1995), and it is likely that the suppression of intake by rbTNFα played a role in this study. Decreased feed intake did not decrease energy balance or increase lipolysis in this study, but low intake affects more than just energy status; decreased protein, mineral, vitamin, or lipid intake may have adversely affected health.

Conclusions

In conclusion, rbTNFα treatment during the first 7 d of lactation decreased feed intake, reduced milk production, and tended to impair health status, but did not significantly alter glucose or lipid metabolism. These data indicate that in response to low-grade inflammation, early-lactation cows compensate for the reduced intake by suppressing milk production rather than further compromising their energy balance or systemic metabolism. This coordinated

physiological response to inflammatory mediators can be considered a biological tradeoff between lactational performance and survival (Ballou, 2012). However, because effects on feed intake and milk yield occurred simultaneously, it is unclear whether one or the other was a secondary response. Further studies are needed to investigate if inflammation directly alters mammary or neurological function. Nevertheless, this study indicates that preventing excessive inflammation in early lactation has the potential to improve productivity and health of dairy cows.

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Table 2-1 Ingredient and nutrient composition of the diet.

Item	Value
Ingredient, % of dry matter	
Corn silage	22.2
WCGF ¹	30.3
Alfalfa hay	20.4
Cottonseed	4.9
Corn grain	9.2
Sorghum grain	4.0
Micronutrient premix ²	9.0
Nutrient, % of dry matter	
Dry matter (% as-fed)	58.6
Crude protein	17.3
Acid detergent fiber	17.8
Neutral detergent fiber	31.8
Ether extract	5.0
Ash	9.1
Net energy for lactation ³ (Mcal/kg dry matter)	1.72

¹ Wet corn gluten feed (Sweet Bran; Cargill Inc., Blair, NE).

² Premix consisted of 54.9% expeller soybean meal, 14.3% limestone, 10.2% sodium bicarbonate, 10.0% calcium salts of long-chain fatty acids (Megalac-R; Arm & Hammer Animal Nutrition, Princeton, NJ), 2.6% Diamond V XP yeast (Diamond V Mills, Inc., Cedar Rapids, IA), 2.0%

magnesium oxide, 1.6% potassium carbonate, 1.6% salt, 1.6% Vitamin E premix (44 IU/g), 0.6% 4-Plex (Zinpro Corp., Eden Prairie, MN; consists of zinc 2.58%, manganese 1.48%, copper 0.90%, cobalt 0.18%, methionine 8.21%, and lysine 3.80%), 0.3% selenium premix (0.06% Se), 0.1% Vitamin A premix (30 kIU/g), 0.1% Vitamin D premix (30 kIU/g), 0.1% Rumensin 90 (Elanco Animal Health, Greenfield, IN), and 0.1% Zinpro 100 (Zinpro Corp., Eden Prairie, MN; consists of 10% zinc and 30% methionine).

³ Estimated according to NRC (2001).

Table 2-2 Primers used for real-time PCR gene expression analysis.

Gene ¹	Sequences of primers (5' to 3')	Accession number ²	Amplicon region	Median Ct	Efficiency ³
<i>AGPAT1</i>	Forward, GGAGTCATCTTCATTGACCGGA Reverse, GCCCTCAGGAAAAACCCA	NM_177518.1	737-847	29.3	99%
<i>ApoB</i>	Forward, TCCTTGATTCCACATGCAGCT Reverse, GGTGTGCAAAGGATGCGTTAG	XM_003582812.1	11567-11666	20.6	94%
<i>CPT1a</i>	Forward, CTTCCCATTCGCACTTTC Reverse, CCATGTCCTTGTAATGAGCCA	XM_002699420.2	1950-2033	22.6	93%
<i>PC</i>	Forward, CTTCAAGGACTTCACTGCCACC Reverse, GCCAAGGCTTTGATGTGCA	NM_177946.4	3160-3276	21.4	98%
<i>PCK1</i>	Forward, CGAGAGCAAAGAGATACGGTGC Reverse, TGACATACATGGTGCGACCCT	NM_174737.2	427-542	18.5	98%
<i>RPS9</i>	Forward, GAACAAACGTGAGGTCTGGAGG Reverse, TTACCTTCGAACAGACGCCG	NM_001101152.2	170-261	19.2	92%
<i>TNFα</i>	Forward, AAGTAACAAGCCGGTAGCCCA	NM_173966.3	448- 538	27.9	103%

Reverse, CTTCCAGCTTCACACCGTTG

¹ *AGPAT1*: 1-acylglycerol-3-phosphate O-acyltransferase 1; *ApoB*: apolipoprotein B; *CPT1a*: mitochondrial carnitine palmitoyltransferase 1A; *PC*: pyruvate carboxylase; *PCK1*: phosphoenolpyruvate carboxykinase 1; *RPS9*: ribosomal protein S 9; *TNF α* : tumor necrosis factor α .

² From NCBI Entrez Nucleotide Database (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide>).

³ The coefficients of determination (R^2) for all genes were greater than 0.98.

Table 2-3 Concentrations of plasma eicosanoids in early-lactation dairy cows during the experimental period. Eicosanoids were measured on day 0, 1, 3, and 5 of treatment. Values are least squares means \pm SEM, n = 10-11.

Eicosanoids ¹ , ng/mL	Treatments ²			SEM	P-value ³	
	Control	1.5 μ g/kg	3.0 μ g/kg		C vs. T ⁴	Dose ⁵
PGE ₂	1.07	1.26	1.21	0.14	0.16	0.88
PGF ₂	1.55	1.61	1.56	0.075	0.36	0.61
LTB ₄ [*]	0.43	0.43	0.28	0.101	0.87	0.49
LTD ₄	335	305	275	120	0.68	0.74
TXB ₂	33.2	25.5	36.9	6.6	0.86	0.31
9-HODE	30.4	31.7	27.4	3.6	0.89	0.64
13-HODE	28.5	28.7	26.7	3.5	0.60	0.48
5-HETE	94.9	96.1	65.3	18.2	0.26	0.25
15-HETE	4.49	4.50	4.75	0.69	0.64	0.73
Pro-inflammatory class ⁶	482	426	384	95	0.40	0.63
Resolvin D ₁	0.172	0.294	0.241	0.053	0.14	0.59
Resolvin D ₂	19.3	16.6	8.6	4.7	0.22	0.26
Lipoxin A ₄	1.84	1.38	1.29	0.33	0.31	0.90
7-maresin 1	472	430	302	69	0.22	< 0.10
Protectin	89.9	83.6	49.2	18.7	0.30	0.06
9-oxoODE	54.1	46.0	46.0	3.2	0.01	0.86
13-oxoODE	26.4	27.1	22.5	2.8	0.34	0.43

Anti-inflammatory class ⁷	689	636	469	96	0.22	0.08
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¹ LT: leukotriene; PG: prostaglandin; TX: thromboxane; HODE: hydroxyoctadecadienoic acid; HETE: hydroxyeicosatetraenoic acid; oxoODE: octadecadienoic acid.

² Treatments: cows were given 0, 1.5, or 3.0 µg/kg body weight rbTNFα injections daily for the first 7 days of lactation, respectively.

³ No treatment by day interactions were significant except as noted.

⁴ Contrast between Control and rbTNFα treatments.

⁵ Contrast between 1.5 and 3.0 µg/kg rbTNFα treatments.

⁶ Pro-inflammatory class is the sum all of PGE₂, PGF₂, LTB₄, LTD₄, TXB₂, 9-HODE, 13-HODE, 5-HETE, and 15-HETE concentrations (Sordillo et al., 2009).

⁷ Anti-inflammatory class is the sum of resolvin D₁ and D₂, protectin, lipoxin A₄, 7-maresin 1, 9-oxoODE, and 13-oxoODE concentrations (Sordillo et al., 2009).

* A significant treatment × time interaction was detected ($P < 0.04$), but treatment contrasts were not significant on any individual day.

Table 2-4 Concentrations (across treatments) of plasma eicosanoids in early-lactation dairy cows with significant ($P < 0.05$) day effects during the experimental period. Values are least squares means \pm SEM, $n = 31$ -33.

Eicosanoids ¹ , ng/mL	Day ²				SEM	<i>P</i> -value
	0	1	3	5		
PGF ₂	1.67	1.44	1.41	1.78	0.11	0.02
TXB ₂	52	16	26	35	10	< 0.001
Resolvin D ₂	5.1	12.2	21.8	20.4	3.7	< 0.01
9-HODE	34.4	29.0	25.8	30.1	3.67	0.04
15-HETE	6.7	4.4	3.7	4.2	1.04	0.03
7-maresin 1	248	358	481	568	59	< 0.01
Pro-inflammatory class ³	531	369	374	460	70	0.02
Anti-inflammatory class ⁴	418	538	689	781	85	< 0.01

¹ PG: prostaglandin; TX: thromboxane; HODE: hydroxyoctadecadienoic acid; HETE: hydroxyeicosatetraenoic acid.

² Day of lactation. Plasma eicosanoids with significant ($P < 0.05$) day effects are presented.

³ Pro-inflammatory class is the sum of PGE₂, PGF₂, LTB₄, LTD₄, TXB₂, 9-HODE, 13-HODE, 5-HETE, and 15-HETE concentrations (Sordillo et al., 2009).

⁴ Anti-inflammatory class is the sum of resolvin D₁ and D₂, protectin, lipoxin A₄, 7-maresin 1, 9-oxoODE, and 13-oxoODE concentrations (Sordillo et al., 2009).

Table 2-5 Production responses in early-lactation dairy cows during the experimental period. Values are least squares means \pm SEM, n = 10-11.

Item	Treatments ¹			SEM	<i>P</i> -value ²	
	Control	1.5 μ g/kg	3.0 μ g/kg		C vs. T ³	Dose ⁴
Dry matter intake, kg/d	13.7	12.1	10.4	0.80	0.02	0.15
Water intake, L/d	79.8	71.9	66.5	3.9	0.04	0.34
Milk yield, kg/d	33.7	29.1	28.4	1.7	0.03	0.76
Milk fat, %	5.41	6.00	5.18	0.24	0.55	0.02
Milk fat, kg/d	2.08	1.87	1.59	0.11	0.01	0.07
Milk protein, %	3.51	3.57	3.37	0.11	0.75	0.19
Milk protein, kg/d	1.29	1.11	1.01	0.05	< 0.01	0.19
Milk lactose*, %	4.52	4.52	4.45	0.05	0.65	0.33
Milk lactose, kg/d	1.65	1.39	1.32	0.08	< 0.01	0.55
Milk energy output, Mcal/d	36.8	32.1	28.7	1.6	< 0.01	0.13
Energy balance, Mcal/d	-23.6	-21.6	-22.0	1.6	0.36	0.89
Milk urea nitrogen, mg/dL	11.1	12.7	12.3	0.75	0.13	0.75
Somatic cell linear score	3.27	2.49	3.13	0.51	0.50	0.39

¹ Treatments: cows were given 0, 1.5, or 3.0 μ g/kg body weight rbTNF α injections daily for the first 7 days of lactation, respectively.

² No treatment by day interactions were significant except as noted.

³ Contrast between Control and rbTNF α treatments.

⁴ Contrast between 1.5 and 3.0 μ g/kg rbTNF α treatments.

* There was a treatment by day interaction ($P = 0.04$) for milk lactose %.

Table 2-6 Plasma metabolites and hormones, liver triglycerides, and glucose turnover rate in early-lactation dairy cows. Values are least squares means \pm SEM, n = 10-11.

Item	Treatments ¹			SEM	P-value ²	
	Control	1.5 μ g/kg	3.0 μ g/kg		C vs. T ³	Dose ⁴
Plasma BHBA, μ M	996	1108	1186	154	0.40	0.72
Plasma glucose, mg/dL	50.8	51.9	51.7	2.1	0.68	0.94
Plasma insulin, ng/mL	0.28	0.31	0.32	0.07	0.35	0.74
Plasma NEFA, μ M	857	854	757	83	0.62	0.41
Plasma TG, mg/dL	13.5	12.9	12.4	1.1	0.52	0.73
Plasma 3-methylhistidine, μ M	10.2	10.0	12.2	1.7	0.58	0.28
Liver TG, mg/g protein	769	750	702	106	0.34	0.58
Glucose turnover rate, g/min	3.73	3.04	3.23	0.35	0.18	0.73
Glucose turnover rate, g/kg dry matter intake	389	335	480	29	0.32	<0.01

¹ Treatments: cows were given 0, 1.5, or 3.0 μ g/kg body weight rbTNF α injections daily for the first 7 days of lactation, respectively.

² No treatment by day interactions were significant.

³ Contrast between Control and rbTNF α treatments.

Table 2-7 Health disorders in early-lactation dairy cows during the experimental period.

Item ²	Treatments ¹			Total
	Control	1.5 µg/kg	3.0 µg/kg	
Ketosis	1	3	3	7
Subclinical mastitis	1	0	2	3
Respiratory distress	0	0	2	2
Metritis	1	0	0	1
Milk fever	0	0	1	1
Fever	0	0	2	2
≥ 1 event (fever excluded)	2	3	7	12

¹ Treatments: cows were given 0, 1.5, or 3.0 µg/kg body weight rbTNFα injections daily for the first 7 days of lactation, respectively. There were 11 cows at risk in each treatment.

² Ketosis was recorded when the urine ketone dipstick test (Ketostix; Bayer Corp. Diagnostics Division, Elkhart, IN) detected acetoacetate > 80 mg/dL on any day or > 40 mg/dL for 2 consecutive days. Fever designates that a cow had a rectal temperature greater than 39.4°C. Other health disorders were diagnosed according to the guidelines by Kelton et al. (1998). Fever was excluded from the summary data because of the possibility that it was a direct response to treatment rather than a sign of infection.

Figure 2-1 Plasma concentrations of TNF α and haptoglobin during 7 days of rbTNF α or Control administration. (A) Plasma TNF α tended to be increased by rbTNF α treatments ($P = 0.09$), but no difference was detected between 1.5 and 3.0 $\mu\text{g/kg}$ rbTNF α treatments ($P = 0.19$). (B) Haptoglobin differed between rbTNF α treatments and Control ($P = 0.01$), but not between 1.5 and 3.0 $\mu\text{g/kg}$ rbTNF α treatments ($P = 0.68$). Values are least squares means \pm SEM, $n = 10$ -11.

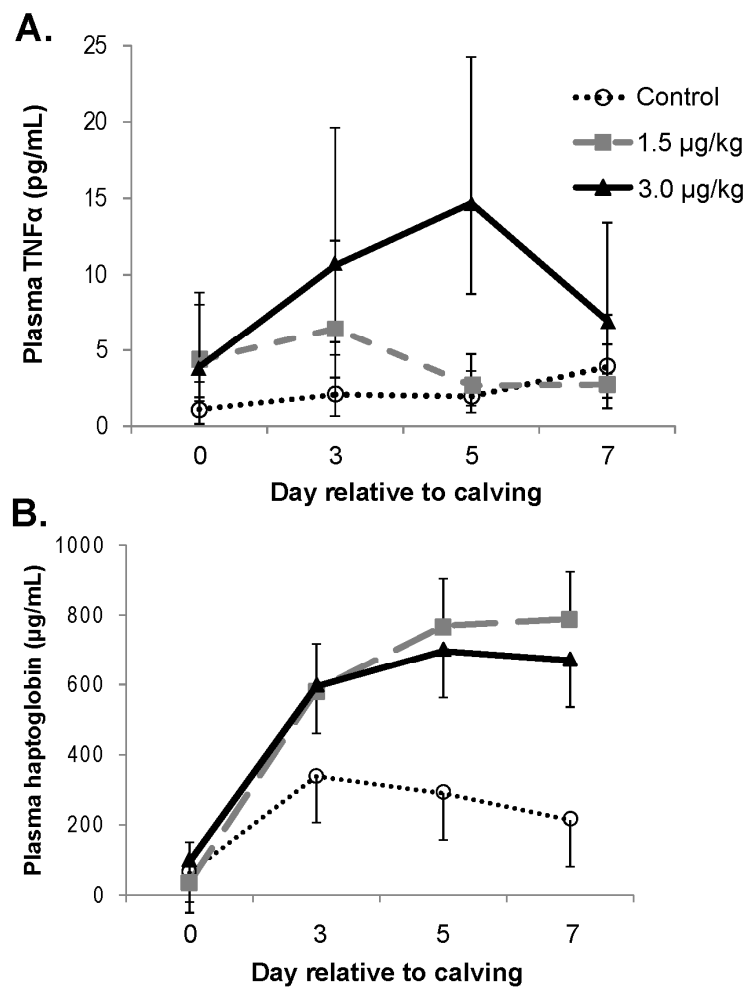


Figure 2-2 Composite plasma concentration of anti-inflammatory eicosanoids during the first 5 days of rbTNF α or Control administration. The total anti-inflammatory eicosanoid concentration represents the sum of resolvin D₁ and D₂, protectin, lipoxin A₄, 7-maresin 1, 9-oxoODE, and 13-oxoODE concentrations (Sordillo et al., 2009). The 3.0 $\mu\text{g/kg}$ dose tended to differ from the 1.5 $\mu\text{g/kg}$ rbTNF α dose ($P = 0.08$), but no overall rbTNF α was detected ($P = 0.22$). A tendency for a dose by time interaction was also observed ($P = 0.06$), with a significant dose contrast on day 5 ($P < 0.01$). Values are least squares means \pm SEM, $n = 10-11$.

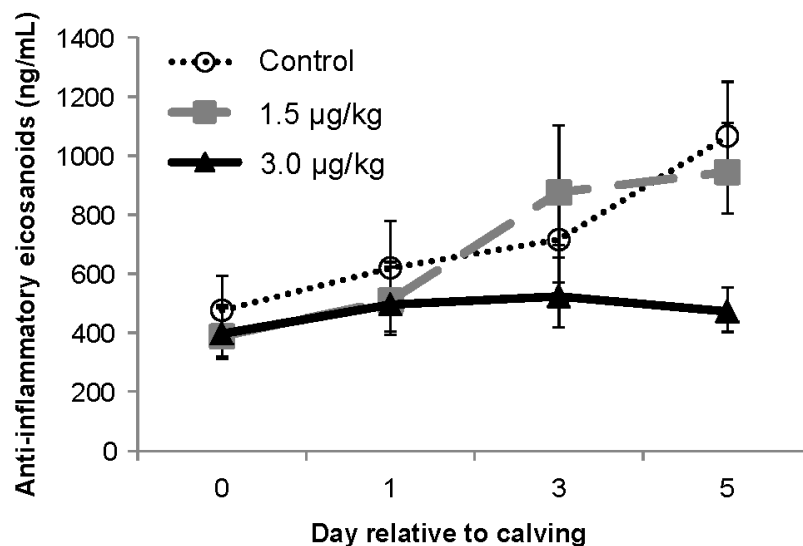
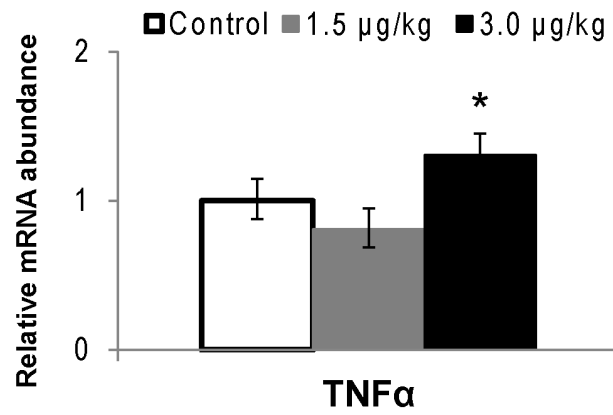
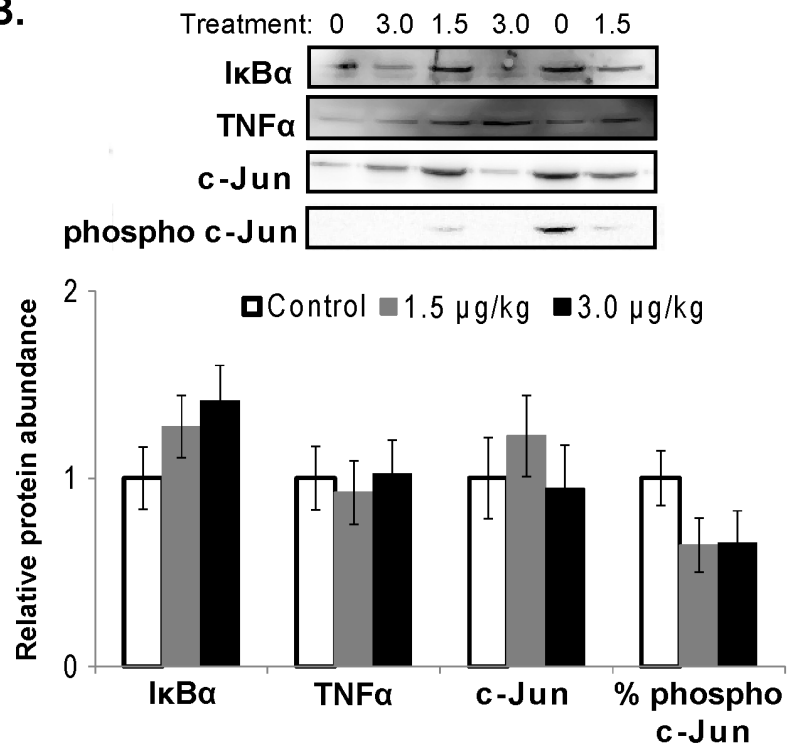


Figure 2-3 Hepatic mRNA abundance of *TNFα* and protein abundance of key mediators involved in inflammatory pathways. Liver samples were collected after 7 days of rbTNFα or Control administration. (A) Hepatic *TNFα* transcript abundance was increased by 3.0 vs. 1.5 μg/kg rbTNFα treatments ($P = 0.02$), but did not differ between rbTNFα treatments and Control ($P = 0.73$). (B) Western blot images are shown for 6 cows along with densitometry data from analysis of all samples for hepatic IκBα (37 kDa), TNFα (17 kDa), and total and phosphorylated c-Jun (39 kDa). There was a tendency for treatment by parity interaction ($P = 0.07$) for IκBα, reflecting increased ($P = 0.04$) IκBα abundance by rbTNFα treatment in primiparous cows (data not shown). No treatment effects ($P > 0.10$) were observed for hepatic TNFα, c-Jun, or relative c-Jun phosphorylation. (C): Parity significantly affected hepatic IκBα ($P = 0.02$), TNFα ($P = 0.04$), and relative c-Jun phosphorylation ($P = 0.04$). Values are means ± SEM, n = 10-11 (A and B) or 9-24 (C).

A.



B.



C.

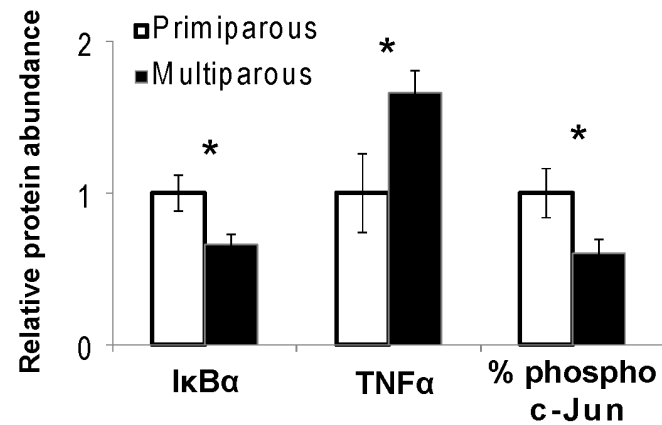
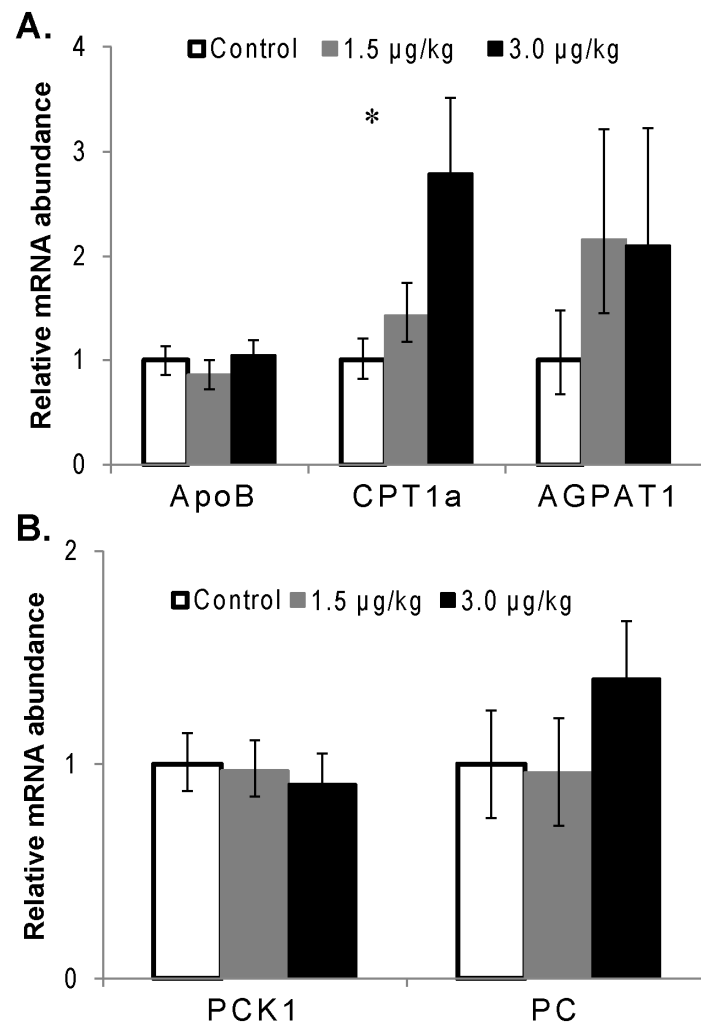


Figure 2-4 Hepatic abundance of transcripts involved in lipid metabolism (A) and gluconeogenesis (B). Liver samples were collected after 7 days of rbTNF α or Control administration. Differences were observed between rbTNF α treatments and Control ($P < 0.01$), and between 1.5 and 3.0 $\mu\text{g/kg}$ rbTNF α treatments ($P = 0.04$) for *CPT1a*. No treatment effects ($P > 0.10$) were detected for *ApoB*, *AGPAT1*, *PCK1*, or *PC*. *AGPAT1*: 1-acylglycerol-3-phosphate O-acyltransferase 1; *ApoB*: apolipoprotein B; *CPT1a*: mitochondrial carnitine palmitoyltransferase 1A; *PC*: pyruvate carboxylase; *PCK1*: phosphoenolpyruvate carboxykinase

1. Values are means \pm SEM, n = 10-11.



Chapter 3 - Effects of supplemental amino acids and chromium propionate on nutrient metabolism, neutrophil activation, and adipocyte size in dairy cows during peak lactation

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Abstract

The objective of this study was to evaluate effects of chromium propionate (CrPr), rumen-protected lysine and methionine (RPLM), or both on metabolism, neutrophil function, and adipocyte size in lactating dairy cows (38 ± 15 days in milk). Forty-eight individually fed Holstein cows (21 primiparous, 27 multiparous) were stratified by calving date in 12 blocks and randomly assigned to 1 of 4 treatments within block. Treatments were control, CrPr (8 mg/d Cr, KemTRACE brand Chromium Propionate 0.04%, Kemin Industries, Inc.), RPLM (10 g/d lysine and 5 g/d methionine intestinally available, from LysiPEARL and MetiPEARL, Kemin Industries, Inc.), or CrPr plus RPLM. Treatments were fed for 35 d; blood plasma samples were collected on d 21 and 35 of treatment, and blood neutrophils were isolated from 24 cows for analysis of tumor necrosis factor α ($TNF\alpha$) and interleukin 1β ($IL-1\beta$) transcript abundance in the basal state and after 12 h of lipopolysaccharide (LPS) activation. Tailhead subcutaneous adipose tissue samples were collected on d 35 for measurement of adipocyte size. Plasma glucose, NEFA, and glucagon concentrations were unaffected by treatments, whereas plasma insulin concentration was increased ($P = 0.03$) by RPLM. Basal $TNF\alpha$ transcript abundance in neutrophils was not affected ($P > 0.10$) by treatment, but basal $IL-1\beta$ transcript abundance was decreased ($P = 0.05$) by RPLM and tended to be increased by CrPr ($P = 0.08$). After LPS activation, CrPr increased ($P = 0.02$) neutrophil $TNF\alpha$ transcript abundance. In addition, RPLM \times parity interactions ($P < 0.01$) were detected for both $TNF\alpha$ and $IL-1\beta$ abundance after LPS activation, reflecting enhanced responses in primiparous cows and attenuated responses in multiparous cows supplemented with RPLM. Adipocyte size was not affected by treatment. Supplemental CrPr and RPLM had minimal effects on metabolism when fed for 35 d near peak lactation, but may modulate innate immune function in lactating dairy cows.

Key Words: metabolism, neutrophil, adipocyte, lysine, methionine, chromium

Introduction

Chromium is a nutrient that can influence animal metabolism. Supplementation of Cr in cattle increased milk production (Hayirli et al., 2001; McNamara and Valdez, 2005; Smith et al., 2005), improved glucose clearance and insulin sensitivity (Bunting et al., 1994; Spears et al., 2012; Sumner et al., 2007), reduced lipolysis (Besong, 1996; Bryan et al., 2004), altered adipose tissue metabolism (McNamara and Valdez, 2005), and modulated immune response (Burton et al., 1993; Burton, 1995; Burton et al., 1996). Although considerable research has been conducted with Cr in cattle, only recently has Cr supplementation in cattle diets been permitted by the U.S. Food and Drug Administration. Currently, Cr-propionate (CrPr) is the only source of Cr allowed for supplementation to cattle, at inclusion rates up to 0.5 mg Cr/kg of diet. By feeding CrPr to dairy cows from 21 d prepartum to 35 d postpartum, McNamara and Valdez (2005) increased DMI and milk yield in early lactation. Interestingly, they also observed that CrPr dramatically increased adipose tissue lipogenesis compared to that of controls in the postpartum period. Collectively, these results indicate that CrPr may improve production by modulating metabolism of lactating cows.

To improve the performance and productive efficiency of lactating cows, first limiting AA such as rumen-protected lysine and methionine (RPLM) have been commonly supplemented (Berthiaume et al., 2006; Chilliard and Doreau, 1997; Leonardi et al., 2003). A recent meta-analysis (Patton, 2010) indicated that feeding rumen-protected methionine increased milk protein content and yield and slightly increased milk yield across 35 studies in the literature. However, little research has been conducted to determine effects of RPLM on glucose or lipid metabolism or immune function in dairy cows. Enhanced metabolic and immune function may contribute to improved milk production.

Currently, there are no reports regarding the interaction between CrPr and RPLM supplementation in dairy cows. We hypothesized that supplementation of both may generate effects superior to supplementing either one alone, in part because enhanced milk production in response to CrPr would increase essential AA requirements. Therefore, the objective of this study was to determine if CrPr and RPLM supplementation affects neutrophil function, adipocyte size, or intermediary nutrient metabolism in dairy cows near peak lactation.

Materials and Methods

The Kansas State University Institutional Animal Care and Use Committee approved all experimental procedures. Production responses to these treatments are reported in a companion paper (Vargas-Rodriguez et al., 2014).

Design and Treatments

Forty-eight individually fed Holstein cows (21 primiparous, 27 multiparous, 38 ± 15 DIM, mean \pm SD) were stratified by calving date in 12 blocks and randomly assigned to 1 of 4 treatments within block. Treatments were control, CrPr (8 mg/d Cr in the form of 20 g/d KemTRACE Chromium Propionate 0.04%, Kemin Industries, Inc., Des Moines, IA), RPLM (10 g/d lysine and 5 g/d methionine, intestinally available), or both (CrPr+RPLM). The RPLM supplement was composed of 48.8 g/d of LysiPEARL and 15.3 g/d of MetiPEARL (Kemin Industries, Inc.). Treatments were premixed with ground corn and top-dressed at 200 g/d for 35 d. All cows were fed once daily (1600 h) for ad libitum intake of a diet formulated to meet NRC (2001) nutrient requirements (Table 1). Analysis by the Cornell Net Carbohydrate and Protein

System version 6.1 (NDS version 3, Ruminant Management & Nutrition, Reggio Emilia, Italy) estimated metabolizable Met supply at 47 g/d (2.03% of MP) and metabolizable Lys supply at 148 g/d (6.38% of MP) with 22 kg/d DMI in the control diet. The RPLM supplement was predicted to result in Lys and Met supplies of 6.77% and 2.23% of MP, respectively.

Plasma Samples

On d 21 and 35 (1430 h), approximately 14 mL of blood was collected from all cows from the coccygeal vessels into 2 evacuated tubes, one containing K₃-EDTA (for analyses of NEFA, glucagon, insulin, leptin, and adiponectin) and the other containing potassium oxalate with sodium fluoride (for analysis of glucose) as a glycolytic inhibitor (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). Samples were centrifuged at $2,000 \times g$ for 15 min immediately after sample collection, and plasma was harvested and frozen at -20°C until analysis. Plasma aliquots for glucagon analysis were added to tubes containing benzamidine (50 mM final concentration; Sigma-Aldrich Chemical Co., St Louis, MO) as a protease inhibitor. Plasma was analyzed for NEFA using an enzymatic colorimetric procedure (NEFA-HR, Wako Chemicals USA, Richmond, VA), glucose by a colorimetric kit (Autokit Glucose; Wako Chemicals USA), glucagon by a radioimmunoassay kit (#GL-32K Millipore Corp., Billerica, MA), and insulin by a bovine-specific sandwich ELISA (#10-1201-01, Mercodia AB, Uppsala, Sweden).

Plasma samples collected on d 35 were analyzed for leptin and adiponectin protein abundance by Western blot. Plasma samples (1 μL) were diluted with 19 μL Laemmli sample buffer (Bio-Rad, Richmond, CA). The homogenate was heated at 90°C for 5 min, cooled and loaded onto a 4-20% Tris-HCl gel for electrophoresis. Samples were separated by SDS-PAGE and dry-transferred onto nitrocellulose membranes (iBlot, Invitrogen, Carlsbad, CA).

Membranes were blocked for 2 h in blocking buffer (5% dry milk in Tris-HCl buffer, pH 7.5, with 0.05% Tween 20). After incubation with blocking buffer, the membranes were washed 3 times for 5 min each with washing buffer (phosphate-buffered saline, pH 7.5, containing 0.05% Tween 20). Membranes were then incubated with primary antibodies (1:500) against leptin (sc-8325, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and adiponectin (A6354, Sigma-Aldrich Co, St. Louis, MO) overnight at 4°C. After washing, a secondary antibody (sc-2313, Santa Cruz Biotechnology) diluted 1:10,000 was incubated for 1 h at room temperature. Immunodetection was performed by chemiluminescence (West-Dura; Thermo Scientific, Waltham, MA) and bands quantified by scanning densitometry (ChemiDoc-It Imaging System; UVP Inc., Upland, CA).

Neutrophil Isolation

On d 21 and 35 (0700 h), 50 mL of blood was obtained by jugular venipuncture from 6 blocks of the cows for isolation of neutrophils. Blood was collected in 50-mL tubes (Becton Dickinson) with 0.75 mL of heparin (1,000 units/mL, Acros Organics Inc., Fair Lawn, NJ). Blood was placed on ice for transport to the lab (< 60 min) and then 20 mL of whole blood was combined with 20 mL of Ficoll-Paque PLUS (GE Healthcare, Pittsburgh, PA) and centrifuged at $800 \times g$ for 50 min at 4°C. Centrifugation separated the blood into 6 distinct bands: plasma, monocytes, isolation media, neutrophils, more isolation media, and the red blood cell pellet. The top three layers (plasma, monocytes, and isolation media) were removed, and neutrophils were collected and washed with $\text{Ca}^{2+}/\text{Mg}^{2+}$ free Hank's balanced salt solution (Sigma-Aldrich Chemical Co.) 3 times. Isolated neutrophils from each cow were split into 2 samples; one sample was immediately frozen for subsequent analysis of basal neutrophil status. The remaining neutrophils were suspended in Dulbecco's modified Eagle's medium with 10% fetal bovine

serum and 1% penicillin/streptomycin (Sigma-Aldrich Chemical Co.) and incubated with 1 µg/mL lipopolysaccharide (LPS, from *E. coli* O55:B5, Sigma-Aldrich Chemical Co.) for 12 h. Cells were frozen at –80°C until analysis of transcript abundance of tumor necrosis factor α (*TNF α*) and interleukin 1 β (*IL-1 β*) in the basal state or after 12 h of LPS activation.

Transcript Abundance

Total RNA was extracted from neutrophils using a commercial kit (RNeasy Lipid Tissue Mini Kit, Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. Two micrograms of total RNA was used as template for the reverse transcriptase reaction using random primers (High-Capacity cDNA RT Kit, Applied Biosystems, Foster City, CA). Quantitative real-time PCR (qPCR) was performed in duplicate on 96-well plates with 1/20 of the cDNA product in the presence of 200 nmol/L gene-specific forward and reverse primers with real-time SYBR green fluorescent detection using SYBR Green Premix reagent (7500 Fast Real-Time PCR System, Applied Biosystems). Primers were designed (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>) using the following GenBank accessions to generate these primer sequences (all 5'–3'): *TNF α* (NM_173966.3), forward AAGTAACAAGCCGGTAGCCCA, reverse CTTCCAGCTTCACACCGTTG; *IL-1 β* (NM_174093.1), forward CACGTGGGCTGAATAACCCCGA, reverse AGGCAGTCGGGCATGGATCAGA; ribosomal protein subunit 9 (*RPS9*) (NM_001101152.2), forward GAACAAACGTGAGGTCTGGAGG, reverse TTACCTTCGAACAGACGCCG. All sample values were normalized against *RPS9* (Mamedova et al., 2010), and relative gene expression was quantified by using the $2^{-\Delta Ct}$ method.

Adipose Tissue Biopsy

On d 35, tailhead subcutaneous adipose tissue samples were collected from all cows. At the time of sampling, the site was shaved and aseptically prepared. The tailhead was then anesthetized with 2 mL of 2% lidocaine hydrochloride. An incision of 2 to 3 cm in length was made between the tailhead and the tuber ischii, and adipose tissue was collected as described previously (Smith and McNamara, 1990). Incisions were closed by a skin stapler (Oasis Disposable Skin Stapler, Med-Vet International, Mettawa, IL). Tissue samples were trimmed to eliminate contaminating tissue and were cut into small pieces (approximately 1 cm³). One sample from each cow was embedded in Optimal Cutting Temperature compound (OCT, Tissue-Tek, The Netherlands) and was frozen at -80°C for subsequent analysis of adipocyte size.

Adipocyte Morphology

Adipose tissue cryosections (14 µm) were prepared on a cryostat (Microm HM550, Thermo Fisher Scientific Inc., Kalamazoo, MI) and mounted on SuperFrost slides (Thermo Fisher Scientific Inc.). Tissue sections were fixed in 4% paraformaldehyde for 10 min, rinsed twice with phosphate buffered saline, and coverslipped. Representative images were captured using a Nikon Eclipse TI-U inverted microscope with 10× working distance magnification (Nikon Instruments Inc., Melville, NY). Five representative photomicrographs per animal were captured using a Nikon DS-QiMc digital camera (Nikon Instruments Inc.). All the adipocytes in each photomicrograph and at least 150 adipocytes per sample were analyzed for adipocyte cross-sectional area using NIS-Elements Imaging Software (Basic Research, 3.3; Nikon Instruments Inc.).

Calculations and Statistical Analysis

An insulin sensitivity measure, revised quantitative insulin sensitivity check index (RQUICKI) was calculated as (Holtenius and Holtenius, 2007): $RQUICKI = 1/[\log(G_b) + \log(I_b) + \log(NEFA_b)]$, where G_b = glucose (mg/dL), I_b = insulin (μ U/mL), and $NEFA_b$ = NEFA (mmol/L), such that a lower RQUICKI indicates greater insulin resistance. Insulin, determined in ng/mL units, was converted to μ U/mL units by assuming an activity of 25.1 μ U/ng (JAMA, 2010). The distribution of adipocyte area values within each cow (from 5 randomly selected cows) was plotted to assess the normality of the population. In contrast to some previous reports demonstrating bimodal adipocyte populations in relatively obese animals (Cruz et al., 2012), adipocyte populations within animal were unimodal in this study. However, distributions were right-skewed because of small numbers of very large adipocytes. Therefore, median adipocyte area from each cow was used instead of the mean value for further analysis.

One cow receiving CrPr+RPLM developed severe mastitis on d 20 of treatment and was subsequently removed from the study; no data were collected or analyzed for this cow. Data were analyzed using ProcMIXED of SAS (version 9.2; SAS Institute Inc., Cary, NC) to assess the fixed effects of day, CrPr, RPLM, parity, and all interactions, and the random effect of block. With the exception of CrPr \times RPLM, interactions were removed from models when $P > 0.30$. Data for *TNF α* and *IL-1 β* transcript abundance in neutrophils were log-transformed for analysis and reported values are back-transformed. Repeated measures over time within cow were modeled with an autoregressive (AR[1]) covariance structure. Denominator degrees of freedom were estimated using the Kenward-Rogers method. Values were deemed outliers and omitted from analysis when Studentized residuals were > 3.5 or < -3.5 . Significance was declared at $P \leq$

0.05 and trends at $0.05 < P \leq 0.10$. Treatment means were separated with pair-wise *t*-tests when interactions were significant.

Results

Plasma Data

Plasma metabolite and hormone concentrations are shown in Table 2. Glucose and NEFA concentrations were not affected ($P > 0.10$) by RPLM or CrPr. Compared with d 21, glucose tended to be decreased (50.5 vs. 53.1 ± 1.5 mg/dL, $P = 0.10$) and NEFA were decreased (223 vs. 342 ± 22 μ M, $P < 0.001$) on d 35. Insulin was unaffected ($P > 0.10$) by CrPr, but was increased ($P = 0.03$) by RPLM, and was greater on d 35 compared with d 21 (311 vs. 259 ± 23 pg/mL, $P = 0.04$). Although glucagon was not affected ($P > 0.10$) by CrPr or RPLM, there was an interaction of RPLM \times day ($P = 0.04$), reflecting increased ($P = 0.05$) glucagon concentration (173 v. 159 ± 5.6 pg/mL) by RPLM on d 35; no difference ($P = 0.53$) was detected on d 21. The RQUICKI measure of insulin sensitivity did not show treatment effects ($P > 0.10$), but tended to be increased on d 35 compared with d 21 (0.569 vs. 0.536 ± 0.014 , $P = 0.08$). Primiparous cows tended to have lesser RQUICKI values than multiparous cows (0.536 vs. 0.570 ± 0.014 , $P = 0.07$).

Plasma leptin and adiponectin protein abundance on d 35 is shown in Figure 1. Plasma leptin protein abundance (Figure 1A) tended to be increased ($P = 0.09$) by RPLM, but was unaffected ($P > 0.10$) by CrPr. In addition, there was a tendency for a CrPr \times parity interaction ($P = 0.08$), reflecting decreased ($P = 0.04$) leptin abundance by CrPr in multiparous cows, but

not ($P = 0.53$) in primiparous cows (Figure 1B). Plasma adiponectin protein abundance (Figure 1C) was not altered ($P > 0.10$) by treatments.

Neutrophil Transcript Abundance

Basal *TNF α* transcript abundance (Figure 2A) in neutrophils was not affected ($P > 0.10$) by treatment or day. After LPS activation, *TNF α* transcript abundance increased compared with non-stimulated controls, and tended ($P = 0.07$) to be greater in cells collected on d 21 compared with d 35. Dietary CrPr increased ($P = 0.02$) *TNF α* abundance in LPS-activated neutrophils (Figure 2B). There was a RPLM \times parity interaction ($P < 0.01$), reflecting increased *TNF α* transcript abundance in LPS-stimulated neutrophils from primiparous cows ($P < 0.01$) and a tendency for decreased *TNF α* in multiparous cows ($P = 0.07$) in response to RPLM (Figure 2C).

Basal *IL-1 β* transcript abundance in neutrophils was decreased ($P = 0.05$) by RPLM, and tended to be increased by CrPr ($P = 0.08$, Figure 3A). Basal *IL-1 β* transcript abundance was increased between d 21 and d 35 ($P = 0.01$), and was greater ($P = 0.04$) in primiparous cows than multiparous cows (12.2 vs. 2.7 ± 7.77 arbitrary units). After LPS activation, *IL-1 β* transcript abundance increased dramatically, but no treatment or day effects ($P > 0.10$) were observed (Figure 3B). However, there was a RPLM \times parity interaction ($P < 0.01$), reflecting increased *IL-1 β* transcript abundance in primiparous cows ($P = 0.03$) and decreased *IL-1 β* in multiparous cows ($P = 0.01$) in response to RPLM (Figure 3C).

Adipocyte Morphology

Figure 4A depicts a representative photomicrograph of an adipose tissue cross-section. Tailhead subcutaneous adipocyte cross-sectional area was not affected ($P > 0.10$) by RPLM or CrPr (Figure 4B), but was greater ($P = 0.02$) in primiparous compared with multiparous cows ($4,942$ vs. $3,577 \pm 427 \mu\text{m}^2$). Median adipocyte cross-sectional area was positively correlated with d 35 BCS ($P < 0.001$, Figure 5), but not BW ($P > 0.10$).

Discussion

In this study, CrPr supplementation did not affect plasma glucose, NEFA, insulin, or glucagon concentrations in lactating cows. Most studies evaluating the effects of Cr supplementation on dairy cattle metabolism have been conducted in the periparturient period, and effects on circulating hormones and metabolites in lactating animals have been limited. Hayirli et al. (2001) reported that supplementing periparturient cows with 0, 0.03, 0.06, and 0.12 mg/kg BW^{0.75} of Cr as chromium-L-methionine (Cr-Met) decreased plasma insulin but did not affect plasma glucose, glucagon, or NEFA concentrations in early lactation. Smith et al. (2008) reported that supplementation of 0.03 or 0.06 mg/kg BW^{0.75} of Cr as Cr-Met had no effects on these plasma concentrations in early lactation despite some alterations in late gestation. Interestingly, we found that RPLM supplementation increased plasma insulin concentration. Essential AA have long been considered to be physiologic stimuli for insulin secretion in humans (Floyd et al., 1966), but few studies have focused on the influence of dietary AA supplementation on insulin concentrations in dairy cows. By feeding a rumen-protected methionine product, Blum et al. (1999) observed increased plasma insulin concentrations in lactating cows within 5 d of supplementation. In the present study, although insulin was

significantly increased by RPLM, this increase was not likely of great consequence for the metabolism and performance of animals because other metabolites were unaffected.

One important characteristic of Cr is its modulatory effect on insulin sensitivity. It has been established that Cr supplementation alleviates insulin resistance, enhances glucose tolerance, and improves metabolism in obese or type 2 diabetic subjects (Cefalu et al., 2010; Kim et al., 2011; Wang and Cefalu, 2010). Although the mode of action is not precisely known, Cr has been shown to enhance insulin binding, insulin receptor abundance, and pancreatic β -cell sensitivity (Wang and Cefalu, 2010). A recent paper also proposed that Cr improves insulin action by activating glucose transporter 4 trafficking and enhancing insulin-stimulated glucose transport in adipocytes (Chen et al., 2006). In the bovine, Sumner et al. (2007) reported that CrPr supplementation improved glucose tolerance in growing Holstein heifers. There has also been interest in effects of Cr supplementation on insulin sensitivity in the periparturient period (Sano et al., 1993), when insulin resistance typically occurs (Bell and Bauman, 1997). Subiyatno et al. (1996) found that supplementation of 0.5 mg Cr/kg DM beginning 6 wk prepartum increased insulin sensitivity in primiparous but not multiparous dairy cows at 2 wk before parturition. Hayirli et al. (2001) reported that Cr-Met supplementation from 21 d prepartum to 28 d postpartum improved glucose tolerance and insulin responsiveness measured at 28 d postpartum in multiparous dairy cows. Therefore, by modulating insulin action, Cr may have significant implications for health and performance of lactating dairy cows. In the current study, we used RQUICKI to estimate insulin sensitivity (Holtenius and Holtenius, 2007). We found that RQUICKI tended to be lesser on d 21 compared with d 35, which is consistent with insulin resistance in early lactation. Furthermore, RQUICKI tended to be lesser in primiparous compared with multiparous cows, indicating that in this study first-lactation heifers were

relatively more insulin resistant. However, RQUICKI was not affected by treatments, suggesting that CrPr and RPLM did not alter insulin sensitivity.

Neutrophils are the first line of immune defense in dairy cows. Upon maturation, neutrophils are released into circulation and play key roles for immune surveillance and defense against pathogens (Burton and Erskine, 2003). Impaired neutrophil trafficking, phagocytosis, or killing directly contribute to immunosuppression and increased disease incidence in early-lactation cows (Kehrli et al., 1989). Among other functions, neutrophils serve as a significant source of pro-inflammatory cytokines, such as $TNF\alpha$ and $IL-1\beta$ (Sohn et al., 2007). These cytokines are critical in activating and coordinating immune responses. As a cell wall component of Gram-negative bacteria, LPS frequently has been used to stimulate immune cells; the capacity of these activated cells to produce pro-inflammatory cytokines can be used to assess the competence of these immune cells (Mani et al., 2012).

In this study, we evaluated the impact of CrPr and RPLM supplementation on neutrophil function by determining $TNF\alpha$ and $IL-1\beta$ transcript abundance in neutrophils in the basal state and after 12 h of LPS activation. We found that CrPr did not affect basal $TNF\alpha$ transcript abundance, but significantly increased its abundance after LPS activation. These results suggest that CrPr supplementation may improve innate immune response upon activation. Several studies have highlighted the role of Cr in bovine immunity. Burton et al. (1993) reported that Cr supplementation at 0.5 mg Cr/kg DM from 6 wk prepartum through 16 wk postpartum increased antibody responses to ovalbumin administration and mitogen-stimulated blastogenesis of blood monocytes in lactating cows. In addition, $TNF\alpha$ and $IL-2$ production from mitogen-stimulated monocytes was significantly decreased for cows supplemented with Cr compared with controls, particularly around peak lactation (Burton et al., 1996).

Interestingly, RPLM increased LPS responsiveness of neutrophils from primiparous cows but decreased responsiveness of neutrophils from multiparous cows, based on both *TNF α* and *IL-1 β* results. The reasons underlying the discrepant responses to RPLM supplementation in primiparous and multiparous cows are unclear. We also observed that basal *IL-1 β* transcript abundance in neutrophils was decreased by RPLM and tended to be increased by CrPr. Recent research has suggested that AA play an important role in immune modulation by regulating immune cell activation, proliferation, antibody production, and cytokine production, whereas dietary AA deficiency directly impairs immune function of animals (Li et al., 2007). For example, lysine deficiency limits cytokine synthesis and lymphocyte proliferation, and impairs immune responses in chickens (Konashi et al., 2000). Methionine supplementation markedly enhanced several key aspects of innate and humoral immune responses in chickens infected with virus (Li et al., 2007). Although little research has directly assessed the effects of these AA on bovine inflammation and immune function, the interaction between these nutrients and immunity warrants further research.

Several studies have been conducted in an attempt to modulate lipolysis in lactating dairy cows by dietary manipulations (Baumgard et al., 2002; McNamara and Valdez, 2005; Yuan et al., 2012). For example, conjugated linoleic acid (CLA, *trans*-10,*cis*-12 isomer) decreased milk fat synthesis in lactating cows and reduced body fat accretion in growing animals by decreasing de novo lipogenesis (Bauman et al., 2000). Recently, Akter et al. (2011) reported that supplementation of dairy cows with 100 g of CLA/d (about 6% each of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers) for the first 100 d of lactation considerably decreased adipocyte sizes in different fat depots. This finding supports the concept that adipocyte size is sensitive to altered rates of lipogenesis or lipolysis in dairy cows (McNamara, 1991). By feeding 10 mg/d Cr as CrPr

to dairy cows from 21 d prepartum to 35 d postpartum, McNamara and Valdez (2005) dramatically increased adipose tissue lipogenesis postpartum, but did not affect rates of lipolysis. In the present study, however, we did not detect effects of CrPr or RPLM on subcutaneous adipocyte size. Interestingly, adipocyte size was positively correlated with BCS, supporting the observation that fatter cows typically have greater adipocyte size (McNamara, 1991). In addition, we found that adipocyte size was greater in primiparous than multiparous cows ($4,942$ vs. $3,577 \pm 427 \mu\text{m}^2$, $P = 0.02$), which is consistent with the observation that first-lactation heifers tended to have greater BCS than multiparous cows (2.94 vs. 2.70 ± 0.085 , $P = 0.07$).

We also measured the protein abundance of several key hormones secreted from adipocytes to assess adipose tissue endocrine status. Leptin is primarily secreted by adipocytes and plays a central role in the regulation of feed intake, energy metabolism, and body condition (Block et al., 2003; Ingvarsen and Boisclair, 2001). In dairy cows, plasma leptin concentrations have been shown to be positively related to body fatness (Kokkonen et al., 2005; Reist et al., 2003). In this study, however, plasma leptin protein abundance was not correlated with BCS ($P = 0.53$, $r^2 = 0.009$, data not shown). Leptin abundance tended to be increased by RPLM, and was decreased by CrPr in multiparous cows. Adiponectin, another adipokine secreted by adipose tissue, plays a key role in glucose and lipid metabolism (Kadowaki and Yamauchi, 2005). Paradoxically, despite being produced in adipose tissue, adiponectin was negatively correlated to body fatness in humans (Ahima, 2006; Kadowaki and Yamauchi, 2005). In dairy cows, however, a previous study did not detect a relationship between plasma adiponectin concentrations and BCS (Raddatz et al., 2008). Interestingly, we found a negative correlation ($P = 0.02$, $r^2 = 0.16$, data not shown) between plasma adiponectin protein abundance and BCS, which agrees with the

human data mentioned above. Here, we did not find effects of CrPr or RPLM on adiponectin protein abundance.

Taken together, our results indicate that supplemental CrPr (8 mg/d Cr), RPLM (10 g/d Lys and 5 g/d Met), or both provided for 35 d had minimal effects on metabolism and adipocyte size, but may modulate neutrophil function in lactating dairy cows. Neutrophil inflammatory responses to pathogen-associated molecules may be enhanced by CrPr supplementation and RPLM can also influence these responses in a parity-dependent manner.

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Table 3-1 Ingredient and nutritional composition of the basal diet

Ingredient	% of DM
Corn silage	31.5
Alfalfa hay	23.4
Wet corn gluten feed ¹	6.8
Ground corn	23.1
Whole cottonseed	4.6
Mechanically extracted soybean meal ²	2.1
Solvent extracted soybean meal	5.1
Ca salts of long-chain fatty acids ³	0.8
Micronutrient premix ⁴	2.6
Nutrient, % as-fed	
DM	57.9
OM	91.3
CP	16.7
NDF	31.7
ADF	20.1
fNDF ⁵	22.1
NFC	39.8
Ether extract	3.1
GE, Mcal/kg	4.11
DE ⁶ , Mcal/kg	3.34

ME ⁷ , Mcal/kg	2.92
NE _L ⁸ , Mcal/kg	1.87
Model-predicted ME ⁹ , Mcal/kg	2.50

¹SweetBran, Cargill Inc., Blair, NE

²Soy Best, Grain States Soya, West Point, NE

³Megalac-R, Church & Dwight Co, Princeton, NJ

⁴Premix consist of 45.1% limestone, 32.2% of sodium bicarbonate, 6.4% magnesium oxide, 5.2% sodium chloride, 5.2% vitamin E premix (44 IU/g), 0.45% vitamin A premix (30 IU/g), 0.19% vitamin D premix (30 IU/g), 2.1% 4-Plex (Zinpro Corp., Eden Prairie, MN; contains 2.58% Zn, 1.48% Mn, 0.90% Cu, 0.18% Co, 8.21% Met, and 3.80% Lys), 0.96% selenium premix (600 ppm Se), 0.45% Zinpro 100 (Zinpro Corp.; contains 10% Zn and 20% Met), 0.03% ethylenediamine dihydriodide premix (3.65% I), 0.88% Kallsil (Kemin Industries), and 0.88% Myco CURB (Kemin Industries).

⁵forage NDF

⁶DE = (Gross energy intake – gross energy in feces)/DMI.

⁷ME = [1.01 × (DE, Mcal/kg) - 0.045] + 0.0046 × (EE, % -3).

⁸NE_L = 0.703 × ME (Mcal/kg) – 0.19 + [(0.097 × ME, Mcal/kg + 0.19)/97] × [EE, % -3].

⁹ME predicted by CNCPS 6.1 (NDS version 3, Ruminant Management & Nutrition, Reggio Emilia, Italy).

Table 3-2 Effects of chromium propionate (CrPr) and rumen-protected lysine and methionine (PRLM) on plasma metabolites and hormones in lactating dairy cows on d 21 and 35 of dietary treatment. Values are least squares means across d 21 and 35.

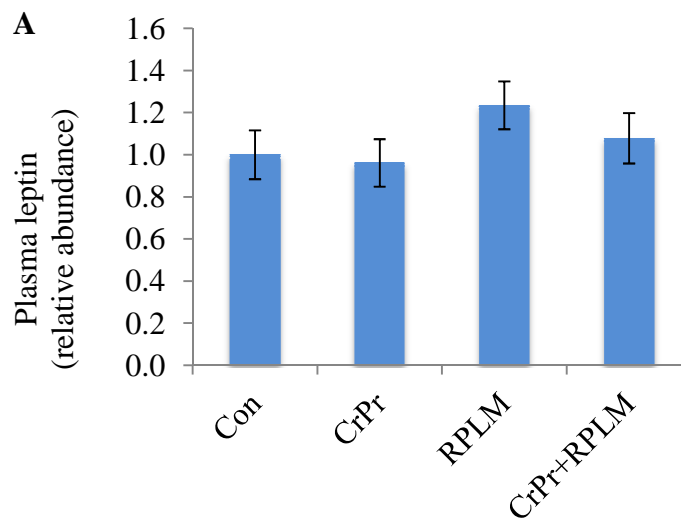
Item	Treatment				SEM	<i>P</i> value ¹		
	Control	CrPr	RPLM	CrPr+RPLM		CrPr	RPLM	Day
Glucose, mg/dL	51.4	51.9	52.2	51.8	2.0	0.96	0.83	0.10
Insulin, ng/mL	0.259	0.247	0.273	0.357	0.032	0.20	0.03	0.04
NEFA, μ M	314	292	287	238	38	0.34	0.28	<0.001
Glucagon ² , ng/mL	0.163	0.173	0.169	0.173	0.006	0.30	0.38	0.27
RQUICKI ³	0.522	0.533	0.534	0.533	0.015	0.68	0.64	0.08

¹ No interactions were significant except as noted for glucagon.

² RPLM \times day interaction ($P = 0.04$). Glucagon concentration was increased by RPLM on d 35 (0.173 vs. 0.159 ± 0.006 ng/mL, $P = 0.05$), but not on d 21 (0.173 vs. 0.169 ± 0.006 ng/mL, $P = 0.53$).

³ Revised quantitative insulin sensitivity check index (Holtenius and Holtenius, 2007).

Figure 3-1 Plasma leptin and adiponectin protein abundance in lactating dairy cows supplemented with control, chromium propionate (CrPr), rumen-protected lysine and methionine (RPLM), or both (CrPr+RPLM). Plasma samples were collected on d 35 of dietary treatment, and protein abundance was measured by Western blot. **(A):** There were a tendency for increased leptin abundance in response to RPLM ($P = 0.09$), but no effects of CrPr ($P = 0.33$) or RPLM \times CrPr ($P = 0.56$). **(B):** A tendency for a CrPr \times parity interaction was detected ($P = 0.08$), reflecting decreased ($P = 0.04$) leptin abundance by CrPr in multiparous cows, but not in primiparous cows ($P = 0.53$). **(C):** Relative abundance of adiponectin was not affected by RPLM ($P = 0.18$), CrPr ($P = 0.66$), or RPLM \times CrPr ($P = 0.19$). Error bars are SEM, $n = 12$.



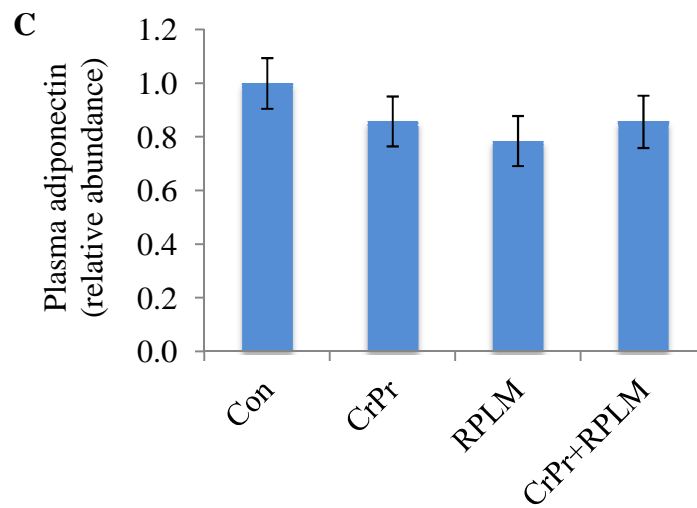
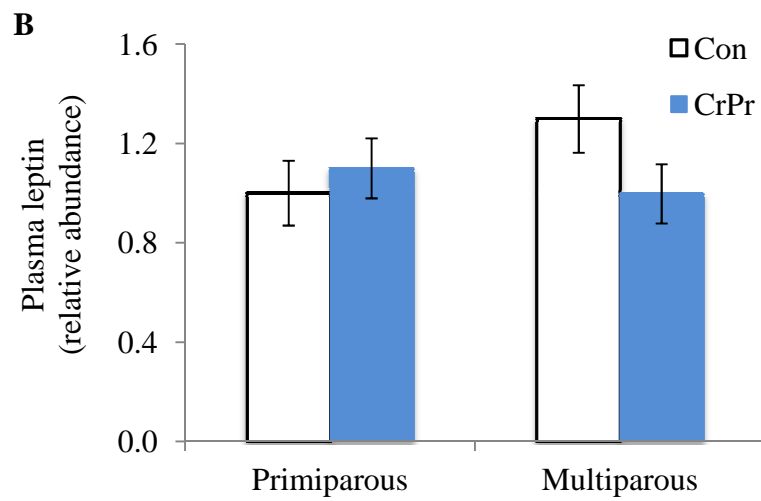


Figure 3-2 Neutrophil transcript abundance of tumor necrosis factor α ($TNF\alpha$) in the basal state or after 12 h of lipopolysaccharide (LPS, 1 $\mu\text{g/mL}$) activation. Cows were supplemented with control, chromium propionate (CrPr), rumen-protected lysine and methionine (RPLM), or both (CrPr+RPLM). Neutrophils were isolated from blood samples collected on d 21 and 35 of dietary treatments. **(A):** Basal $TNF\alpha$ abundance was not affected by RPLM ($P = 0.59$), CrPr ($P = 0.82$), or RPLM \times CrPr ($P = 0.53$). There was a tendency for a CrPr \times day interaction ($P = 0.09$), but CrPr did not affect ($P > 0.10$) $TNF\alpha$ transcript abundance on either d 21 or 35 (data not shown). **(B):** LPS-activated $TNF\alpha$ transcript abundance was increased ($P = 0.02$) by CrPr supplementation but there were no effects for RPLM ($P = 0.14$) or RPLM \times CrPr ($P = 0.76$); $TNF\alpha$ transcript abundance tended to be greater ($P = 0.07$) on d 21 compared with d 35 (data not shown). **(C):** LPS-activated $TNF\alpha$ transcript abundance was affected by an RPLM \times parity interaction ($P < 0.01$), reflecting increased $TNF\alpha$ transcript abundance in primiparous cows ($P < 0.01$) and a tendency for decreased $TNF\alpha$ transcript abundance in multiparous cows ($P = 0.07$) in response to RPLM. Error bars are SEM, $n = 6$.

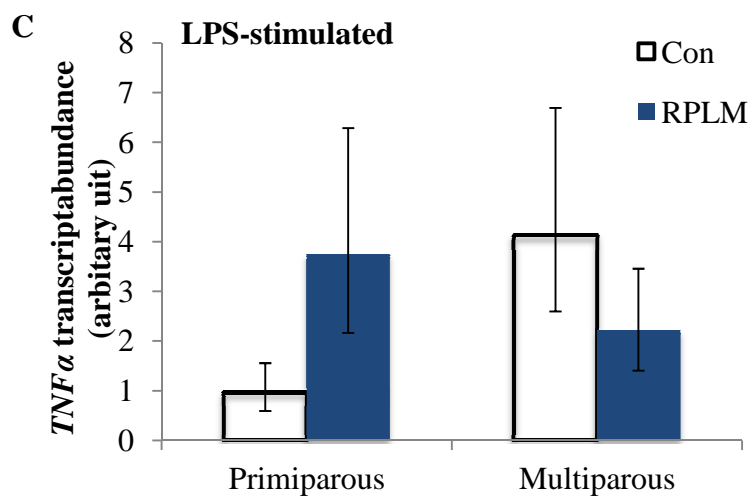
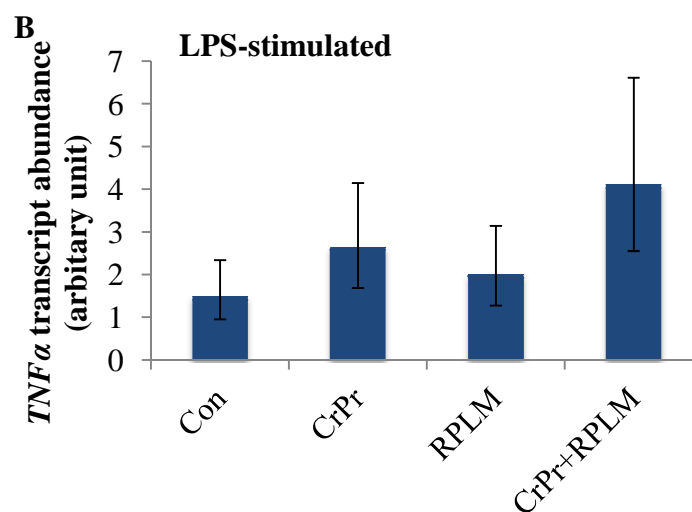
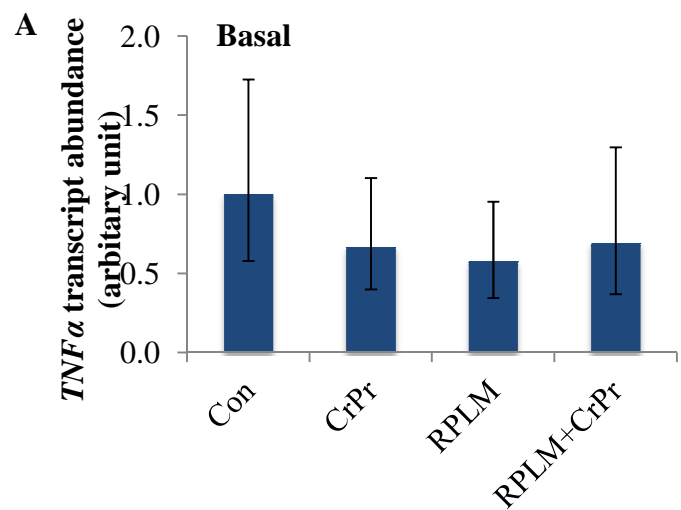
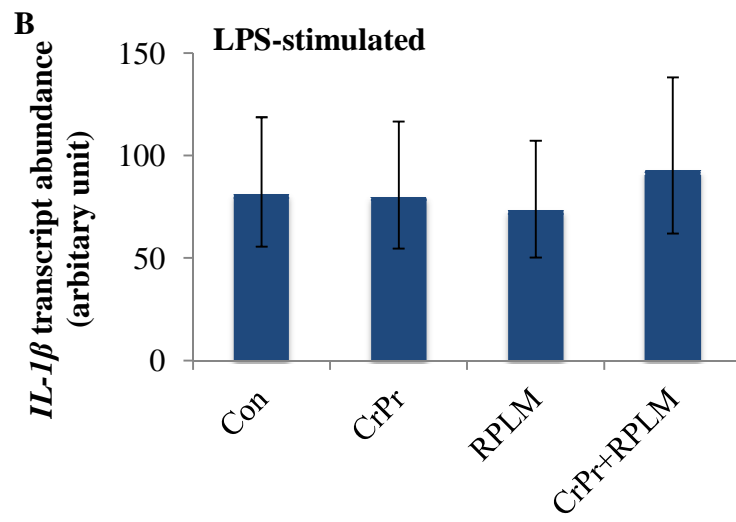
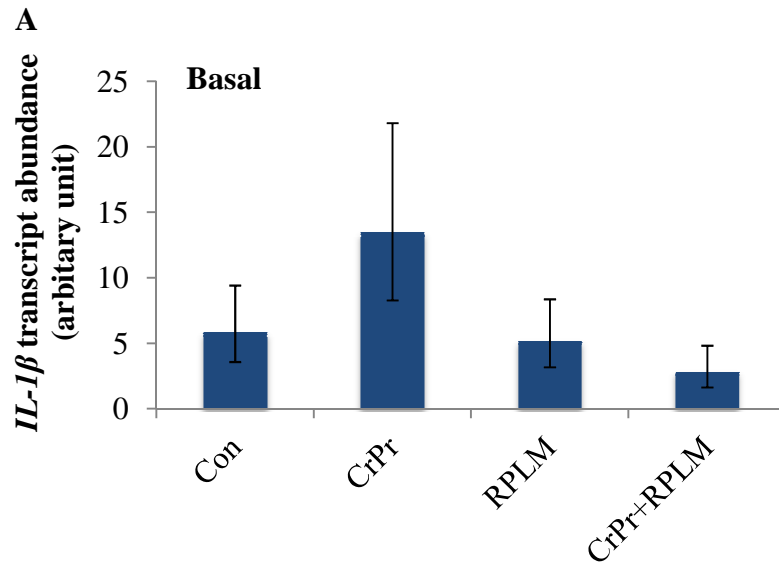


Figure 3-3 Neutrophil transcript abundance of interleukin 1 β (*IL-1 β*) in the basal state or after 12 h of lipopolysaccharide (LPS, 1 μ g/mL) activation. Cows were supplemented with control, chromium propionate (CrPr), rumen-protected lysine and methionine (RPLM), or both (CrPr+RPLM). Neutrophils were isolated from blood samples collected on d 21 and 35 of dietary treatments. (A): Basal *IL-1 β* transcript abundance was decreased ($P = 0.05$) by RPLM, and tended to be increased ($P = 0.08$) by CrPr, but there was no RPLM \times CrPr interaction ($P = 0.13$). (B): LPS-activated *IL-1 β* transcript abundance was not affected by RPLM ($P = 0.91$), CrPr ($P = 0.60$), or RPLM \times CrPr ($P = 0.54$). (C): LPS-activated *IL-1 β* transcript abundance was affected by a RPLM \times parity interaction ($P < 0.01$), reflecting increased *IL-1 β* transcript abundance in primiparous cows ($P = 0.03$) and decreased *IL-1 β* transcript abundance in multiparous cows ($P = 0.01$) in response to RPLM. Error bars are SEM, $n = 6$.



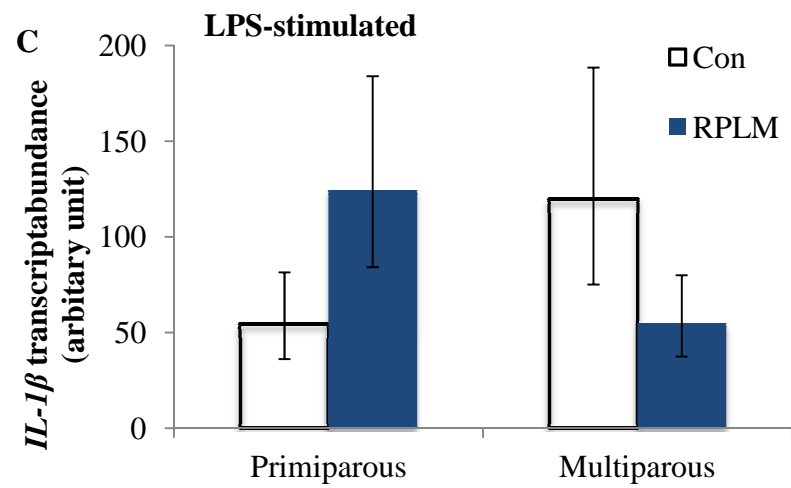


Figure 3-4 Subcutaneous adipocyte size in lactating dairy cows. Cows were supplemented with control, chromium propionate (CrPr), rumen-protected lysine and methionine (RPLM), or both (CrPr+RPLM). Adipose tissue samples were collected from the tailhead on d 35 of dietary treatment for assessment of median (≥ 150 cells/cow) cross-sectional area of adipocytes. **(A):** there were no effects of RPLM ($P = 0.14$), CrPr ($P = 0.58$), or RPLM \times CrPr ($P = 0.46$) on adipocyte size. **(B):** a representative photomicrograph (original magnification $10\times$) of adipose tissue section. Scale bar, $100\ \mu\text{m}$.

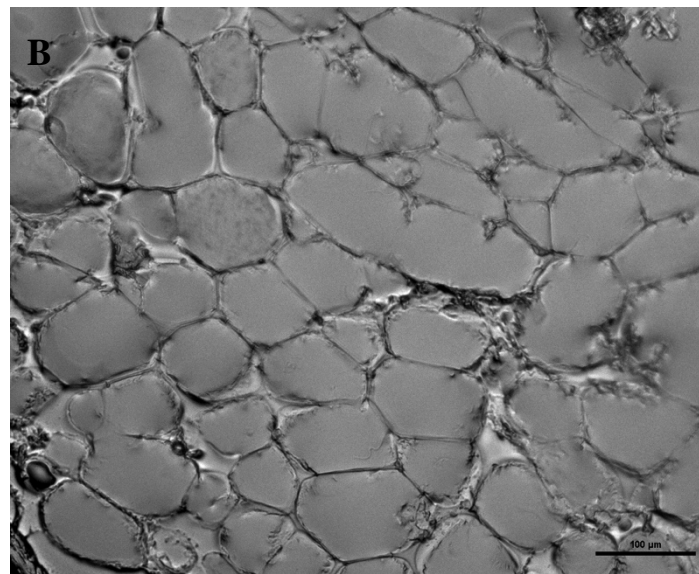
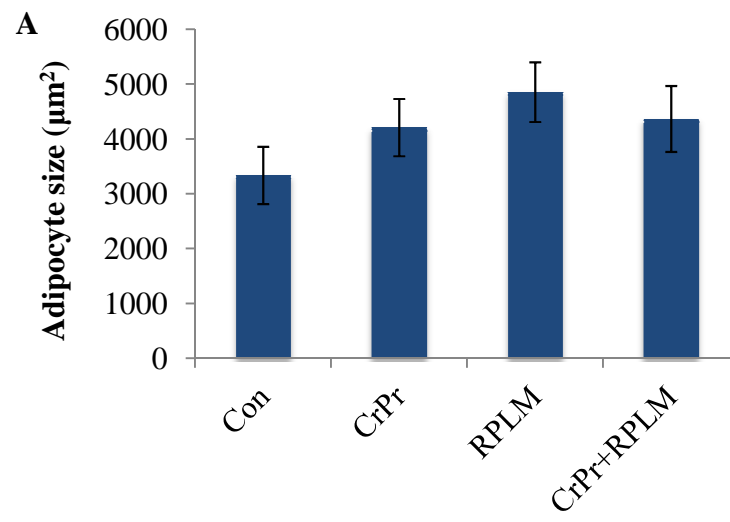
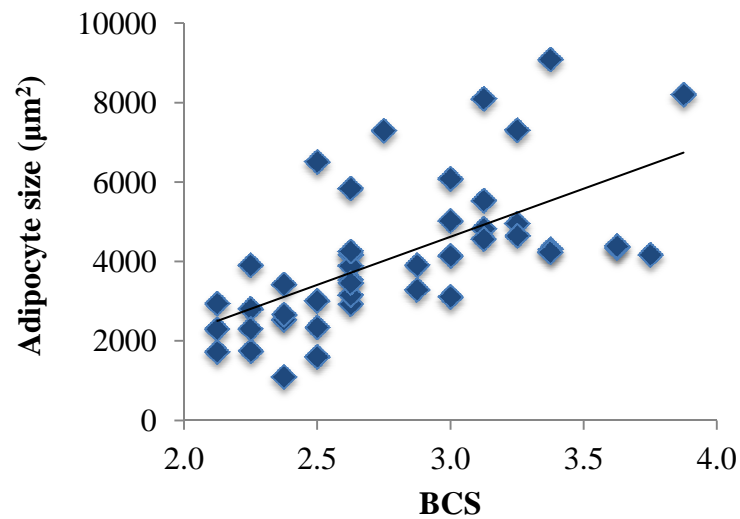


Figure 3-5 Correlation between median cross-sectional area of subcutaneous adipocytes and body condition score (BCS, on d 35) in lactating dairy cows. Adipose tissue samples were collected on d 35 of dietary treatment. Adipocyte sizes were positively correlated with BCS ($P < 0.001$, $r^2 = 0.38$).



Chapter 4 - Yeast product supplementation modulated feeding behavior and metabolism in transition dairy cows

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Abstract

The objective of this study was to assess the effects of supplementing a yeast product derived from *Saccharomyces cerevisiae* on production, feeding behavior, and metabolism in transition cows. Forty multiparous Holstein cows were blocked by expected calving date and randomly assigned within block to 1 of 4 treatments ($n = 10$) from 21 d before expected calving to 42 d postpartum. Rations were top-dressed with a yeast product (yeast culture plus enzymatically hydrolyzed yeast; Celmanax, Vi-COR, Mason City, IA) at the rate of 0, 30, 60, or 90 g/d throughout the experiment. Dry matter and water intake, feeding behavior, and milk production were monitored. Plasma collected on -21, -7, 1, 4, 7, 14, 21, and 35 d relative to calving were analyzed for glucose, β -hydroxybutyrate (BHBA), and nonesterified fatty acids (NEFA). Data were analyzed using mixed models with repeated measures over time. Pre- or postpartum dry matter intake (DMI) and water intake did not differ ($P > 0.10$) among treatments. There were quadratic dose effects ($P < 0.05$) for prepartum feeding behavior, reflecting decreased meal size, meal length, and intermeal interval, and increased meal frequency for cows received 30 and 60 g/d of yeast products. Postpartum feeding behavior was unaffected ($P > 0.10$) by treatments. Milk yields were not affected ($P > 0.10$; 45.3, 42.6, 47.8, and 46.7 kg/d for 0, 30, 60, and 90 g/d, respectively) by treatments. Tendencies for increased ($0.05 < P \leq 0.10$) percentages of milk fat, protein, and lactose were detected for cows receiving yeast. Yeast product increased ($P < 0.01$) plasma BHBA and tended to decrease ($P = 0.06$, quadratic dose effect) glucose, but did not affect NEFA. Yeast product supplementation during the transition period did not affect milk production and DMI, but modulated feeding behavior and metabolism.

Key words: feeding behavior, metabolism, transition cow, yeast

Introduction

The transition from late gestation to early lactation in dairy cows is characterized by dramatic energy requirements for milk synthesis and secretion, inadequate feed intake, and substantial metabolic stress (Grummer, 1995). Therefore, the transition period directly determines the lactational performance, health, and profitability of dairy cows. Yeast products derived from *Saccharomyces cerevisiae* have been added to diets in an attempt to improve ruminal fermentation, feed intake, and milk yield. Dann et al. (2000) reported that yeast culture supplementation at 60 g/d from approximately 21 d prepartum to 140 d postpartum increased DMI during both the last 7 d prepartum ($P = 0.01$) and the first 42 d of lactation ($P = 0.05$) in Jersey cows. A recent transition cow study (Ramsing et al., 2009) reported that yeast culture supplementation at 57 g/d from approximately 21 d prepartum to 21 d postpartum improved prepartum DMI ($P < 0.01$) and postpartum milk production ($P < 0.01$). Desnoyers et al. (2009) conducted a quantitative meta-analysis using 157 experiments to assess the responses to yeast supplementation, and found that yeast products increased rumen pH, DMI, milk yield, and tended to increase milk fat content. These data indicate that yeast products may modulate the metabolism and improve the production of dairy cows. The objectives of this study were to assess whether a yeast product alters milk production, feeding behavior, and biomarkers of lipid and glucose metabolism in transition dairy cows.

Materials and Methods

The Kansas State University Institutional Animal Care and Use Committee approved all the experimental procedures.

Design and Treatments

Forty multiparous Holstein transition cows from the Kansas State University Dairy Cattle Teaching and Research Facility were used in a randomized complete block design. Cows were blocked by expected calving date (10 blocks), and randomly assigned within block to 1 of 4 treatments 21 d before their expected calving date. Cows remained on their respective treatments through 42 d postpartum. Cows received a yeast product (yeast culture plus enzymatically hydrolyzed yeast; Celmanax, Vi-COR, Mason City, IA) at a rate of 0, 30, 60, or 90 g/d. Yeast product was administered daily to each cow in the treatment groups by top-dressing and manually mixing the premix into the upper part of each TMR. Diets were formulated to meet or exceed NRC (2001) requirements (Table 1). Samples of corn silage were collected weekly; all other dietary ingredients were collected biweekly and stored at -20°C. Upon study completion, feed ingredients were composited monthly for analysis by NIR or wet chemistry by Dairy One Forage Laboratory (Ithaca, NY).

Management of Cows, Data Collection, and Sample Analysis

Dry cows were moved into the maternity barn approximately 1 wk before entering the study. Cows were allowed ad libitum access to the designated treatment rations by an electronic gating system (Roughage Intake System, Insentec B.V., Marknesse, The Netherlands). After parturition, cows were moved into a tie-stall facility where they remained through 42 d postpartum. Individual feed bunks in the tie-stall facility were suspended from individual load cells and bunk weight was monitored continuously by computer. Feed weights and times were

stored prior to and immediately after any deviation in bunk weight. Dry cows were fed twice daily (1100 and 1800 h), and lactating cows were fed twice daily (1200 and 1900 h). All cows were fed for ad libitum intake. All feeding activity, including meal length and size, were recorded electronically. As-fed feed intake of each cow was recorded on a daily basis. As-fed ration consumption was adjusted for DM content for determination of meal and daily DMI. Dry matter percentage was determined for each monthly composited feed ingredient; these values were used to determine ration DM for each month. Water was offered ad libitum and individual water consumption was measured daily throughout the study.

Cows were milked 3 times daily in a milking parlor. Milk yields were recorded at each milking. Milk samples were collected at each milking on 3 consecutive days each week, and were analyzed for concentrations of fat, true protein, lactose (B-2000 Infrared Analyzer; Bentley Instruments, Chaska, MN), and urea nitrogen (MUN spectrophotometer, Bentley Instruments). Energy-corrected milk yield was calculated as: $(0.327 \times \text{milk yield}) + (12.86 \times \text{fat yield}) + (7.65 \times \text{protein yield})$ (Dairy Records Management Systems, 2010). Solids-corrected milk production was calculated as: $(12.3 \times \% \text{fat}) + (6.56 \times \% \text{solids-non-fat}) - (0.0752 \times \text{milk yield})$ (Tyrrell and Reid, 1965). Body condition score was evaluated weekly.

Blood samples were collected on d -21, -14, -7, 1, 4, 7, 21, and 35 relative to calving from the coccygeal vessels 1 h prior to feeding. Approximately 20 mL of blood was collected into 4 tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA): one 7-mL tube containing potassium EDTA, one 3-mL tube containing potassium EDTA, one 5-mL tube containing heparin, and another 7-mL tube containing potassium oxalate with sodium fluoride as a glycolytic inhibitor. Blood samples from the 7-mL potassium EDTA tube and the 7-mL tube containing potassium oxalate with sodium fluoride were centrifuged at $2000 \times g$ for 10 min

immediately after sample collection, and plasma was harvested and frozen at -20°C until subsequent analysis of glucose, BHBA, and NEFA. Plasma samples were analyzed for NEFA using an enzymatic colorimetric procedure (NEFA-HR, Wako Chemicals USA, Richmond, VA), glucose by a colorimetric kit (kit #439-90901, Wako Chemicals USA), and BHBA using an enzymatic reaction (kit #H7587-58, Pointe Scientific, Inc.). Absorbance was read on a spectrophotometer (Powerwave XS, Biotek Instruments, Winooski, VT, USA) and calculations were conducted using Gen5 software (Biotek Instruments). Blood collected from the 3-mL potassium EDTA tube and the 5-mL tube containing heparin were analyzed for hematology and whole blood bactericidal capacity, respectively (reported in Yuan et al., 2014).

Data and Statistical Analysis

One cow in the 0 g/d treatment group was removed from the study on d 30 postpartum due to difficulty standing up in the tie-stall. Data obtained from this cow prior to removal were included in all analyses. Feeding behavior variables were calculated from logged data that included the start and end weights as well as start and end times of meals. Meals were combined if intermeal interval was less than 12 min, a criteria used in our previous study (Mullins et al., 2012) that used the same feeding systems and housing facilities. Data were analyzed using mixed models with repeated measures over time. Models included the fixed effects of treatment, time, and their interaction, and the random effect of cow. Contrast statements were used to assess the overall effect of yeast product (control vs. all yeast product treatments), as well as the linear and quadratic effects of dose. If treatment by time effects were significant, slices were used to assess effects on specific days. Values were deemed outliers and omitted from analysis when Studentized residuals were greater than $> |3.0|$. After initial outlier removal, the model was

repeated and Studentized residuals greater than $> |3.5|$ were deemed outliers. Prepartum and postpartum measures were analyzed separately for DMI, water intake, and feeding behavior variables. Significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$.

Results

There were no significant differences among groups in prepartum dietary treatment length (18.1, 18.8, 17.8, and 17.2 ± 1.32 d for 0, 30, 60, and 90 g/d, respectively; $P = 0.86$). Actual calving dates ranged from 13 d before expected calving to 5 d after expected calving.

Feed Intake, Water Intake, and Feeding Behavior

Results of feed intake, water intake, and feeding behavior are shown in Table 2 and Figure 1. As expected, across all treatments, DMI decreased ($P < 0.01$) before calving, and increased ($P < 0.01$) after parturition. Water intake tended to be decreased ($P = 0.09$) before calving, and increased dramatically ($P < 0.01$) after parturition. Pre- or postpartum DMI and water intake did not differ ($P > 0.10$) among treatments. There were quadratic dose effects ($P < 0.05$) for prepartum meal size, meal length, meal frequency, and intermeal interval, reflecting decreased meal size, meal length, and intermeal interval, and increased meal frequency for cows receiving 30 and 60 g/d doses. Postpartum feeding behavior was not affected ($P > 0.10$) by treatments.

Milk Production and Body Condition

As shown in Table 3 and Figure 1A, milk yield was not affected ($P > 0.10$) by treatments. There was a tendency for increased ($P = 0.09$, quadratic dose effect) milk fat percentage for cows receiving 30 and 60 g/d doses. Tendencies for increased percentages of milk protein ($P = 0.08$, linear dose effect) and lactose ($P = 0.10$, linear dose effect) were detected with increasing dose. Milk composition yields, SCM, and ECM (Figure 1B) were not affected ($P > 0.10$) by treatments. Body condition score (Figure 1C) decreased dramatically ($P < 0.01$) after calving, but was unaffected ($P = 0.90$) by treatments.

Plasma Metabolites

As shown in Figure 3, significant day effects ($P < 0.01$) were observed for plasma glucose, BHBA, and NEFA, reflecting dramatic metabolic and endocrine changes during the transition period. There was a tendency for a quadratic dose effect ($P = 0.06$) for glucose, indicating that 30 and 60 g/d doses tended to decrease plasma glucose (69.6, 66.1, 67.0, and 68.7 \pm 1.33 mg/dL for 0, 30, 60, and 90 g/d, respectively, throughout the experimental period), but no yeast product vs. control ($P = 0.14$), linear dose ($P = 0.75$), or treatment \times day ($P = 0.99$) effects were detected. Interestingly, compared with control, yeast product increased plasma BHBA ($P < 0.01$; 661.0, 882.0, 759.8, and 736.4 \pm 41.3 μ M for 0, 30, 60, and 90 g/d, respectively, throughout the experimental period). A significant quadratic dose effect ($P < 0.01$) was also detected. Although plasma NEFA was numerically increased by 30 and 60 g/d doses ($P = 0.12$, quadratic dose effect; 496.8, 565.7, 566.3, and 480.9 \pm 48.7 μ M for 0, 30, 60, and 90 g/d, respectively, throughout the experimental period), no yeast product vs. control ($P = 0.47$) or treatment \times day ($P = 0.73$) effects were detected.

Discussion

The transition period imposes tremendous stress on dairy cows and may result in inadequate nutrient and energy intake to support the onset of lactation. In this study, we attempted to evaluate if yeast product supplementation would modulate DMI, feeding behavior, and milk production of transition cows. We found that DM and water intake decreased before parturition and increased dramatically after calving, but were not affected by treatments either pre- or postpartum. Dann et al. (2000) reported that yeast culture increased DMI both pre- and postpartum in Jersey cows. Ramsing et al. (2009) recently reported that cows supplemented with yeast culture at 57 g/d throughout the transition period had an increased prepartum DMI by 1.5 kg/d ($P < 0.05$), but postpartum DMI was not affected by treatments. Other studies (Robinson, 1997; Arambel and Kent, 1990; Swartz et al., 1994), however, reported no effects of yeast products on DMI.

Changes in DMI ultimately result from changes in feeding behavior, quantified as number of meals consumed daily, the length of each meal, and the rate of eating that occurs during meals (Grant and Albright, 1995). Similar to the changes in DMI, we observed that most of the feeding behavior variables changed over time either pre- or postpartum. Consistent with the finding (Ramsing et al., 2009) that yeast culture increased ($P = 0.02$) the number of meals per day in prepartum cows, we observed increased meal frequency for cows received 30 and 60 g/d treatments. Ramsing et al. (2009) reported no difference in prepartum meal size, but here we detected a quadratic decrease in treated cows. The reasons for the discrepancies between the 2 studies are not clear, but differences in feeding systems, meal criteria and calculation methods, and treatment products may have contributed. In our study, the increase in meals consumed per

day and decreased meal size may result in more consistent prepartum intake patterns throughout the day. Although not measured directly, this modulated feeding behavior may be associated with improved rumen fermentation characteristics in response to yeast product supplementation (Marden et al., 2008; Piva et al., 1993; Yoon and Stern, 1996). Although no significant treatment effects were observed for postpartum feeding behavior, the numerically increased meals consumed per day ($P = 0.19$) and decreased meal size ($P = 0.14$) postpartum did seem to be similar to the patterns observed prepartum. If these changes would attenuate fluctuations of rumen pH, then yeast product would especially benefit these early-lactation cows by allowing them to better adapt to the sudden dietary shift. It should be noted that cows were housed in free-stall prepartum and in tie-stall facilities postpartum, and the changes in feeding patterns from pre- to postpartum likely reflect differences in feeding behavior of cows in pen vs. tie-stall housing (Grant and Albright, 1995) in addition to a true stage of production effect. Regulation of feeding behavior and feed intake is complex, and is controlled by gut fill and chemostatic mechanisms. Factors such as feeding management, environment, health, and social interactions could affect feeding behavior and feed intake as well (Grant and Albright, 1995).

Milk and milk component yields were not affected by yeast product supplementation, although there were tendencies for a quadratic increase in milk fat percentage and linear increases in milk protein and lactose percentages with increasing dose. Many studies (Arambel and Kent, 1990; Swartz et al., 1994; Robinson, 1997) reported no significant effects of yeast products on milk yield, and a few experiments reported positive milk responses (Nocek et al., 2011; Ramsing et al., 2009). These data suggest that milk production responses to yeast product supplementation are rather variable; differences in parities, stages of lactation, product derivation, and environmental conditions could all contribute to the discrepancies. Given the

large variability in lactational performance in early lactation, more animals are needed to achieve adequate power to detect differences in milk responses.

The dramatic changes in plasma metabolites throughout the transition period are similar to the patterns observed in previous studies (Morey et al., 2011; Mullins et al., 2012). Supplementation of 30 and 60 g/d tended to decrease plasma glucose. Although not significant, 30 and 60 g/d treatments numerically increased plasma NEFA. Interestingly, yeast product increased plasma BHBA, and a significant quadratic dose effect was also detected, reflecting that the 30 g/d dose had the greatest effect in elevating BHBA. These changes point to an enhanced lipid mobilization in treated cows, which is consistent with the finding that 30 and 60 g/d doses tended to increase milk fat percentage. The reasons underlying this observation are not clear, but it is unlikely that yeast product had a direct effect on lipid metabolism. In fact, cows receiving 30 g/d had a numerical decrease in postpartum DMI of 2.1 kg/d, and decreased feed intake in the transition period is a key risk factor for elevated BHBA and ketosis (Grummer, 1995).

Together, these data suggest that yeast product supplementation did not affect milk production or DMI, but modulated feeding behavior and metabolism in transition dairy cows. Given the numerical differences in milk responses and DMI, together with changes of lipid metabolism markers, the 30 g/d dose did not benefit these cows; rather, it seems that 60 g/d dose provided the most benefits from the production and metabolic health standpoint.

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Table 4-1 Ingredient and nutrient composition of diets.

Item	Prepartum	Postpartum
Ingredient, % of DM		
Corn silage	29.5	15.9
WCGF ¹	21.3	34.3
Alfalfa hay	-	14.2
Wheat straw	10.9	3.3
Prairie hay	16.8	-
Cottonseed	-	5.0
Ground corn	3.4	11.2
Dry rolled sorghum grain	3.4	6.4
Mechanically extracted soybean meal ²	12.3	4.8
Molasses	1.2	1.2
Ca salts of long-chain fatty acids ³	-	0.8
Micronutrient premix ⁴	1.3	2.9
Nutrient, % of DM		
DM, % as-fed	45.4	51.1
CP	13.0	17.7
Starch	21.1	20.2

ADF	24.4	16.9
NDF	42.5	31.0
NFC	33.8	41.1
Ether extract	3.3	4.2
Ash	6.0	8.3

¹ SweetBran, Cargill Inc., Blair, NE

² Soy Best, Grain States Soya, West Point, NE

³ Megalac-R, Church & Dwight Co, Princeton, NJ

⁴ Prepartum premix consist of 53.0% vitamin E premix (44 IU/g), 11.6% selenium premix (600 ppm Se), 10.5% trace mineral salt, 9.6% vitamin A premix (30 IU/g), 6.4% 4-Plex (Zinpro Corp., Eden Prairie, MN; contains 2.58% Zn, 1.48% Mn, 0.90% Cu, 0.18% Co, 8.21% Met, and 3.80% Lys), 4.3% vitamin D premix (30 IU/g), 4.3% Rumensin 90, and 0.48% ethylenediamine dihydriodide premix (3.65% I).

Postpartum premix consist of 47.5% limestone, 27.9% of sodium bicarbonate, 10.1% trace mineral salt, 6.4% magnesium oxide, 4.5% vitamin E premix (44 IU/g), 1.79% 4-Plex (Zinpro Corp., Eden Prairie, MN; contains 2.58% Zn, 1.48% Mn, 0.90% Cu, 0.18% Co, 8.21% Met, and 3.80% Lys), 1.1% selenium premix (600 mg/kg Se), 0.56% vitamin A premix (30 IU/g), 0.01% vitamin D premix (30 IU/g), and 0.03% ethylenediamine dihydriodide premix (3.65% I).

Table 4-2 Feed intake and feeding behavior during the experimental period. Values are least squares means \pm SEM, n = 9-10.

Item	Treatment				SEM	<i>P</i> value		
	0g	30g	60g	90g		Yeast vs. Control	Linear	Quadratic
Prepartum DMI, kg/d	12.1	11.9	12.6	12.1	0.51	0.86	0.77	0.76
Prepartum water intake, L/d	49.0	48.3	49.0	51.3	2.38	0.84	0.48	0.53
Meal frequency, d ⁻¹	10.1	11.4	11.2	10.0	0.45	0.12	0.80	0.01
Intermeal interval, h	2.08	1.87	1.94	2.08	0.08	0.18	0.86	0.02
Meal size, kg DM	1.23	1.06	1.16	1.24	0.06	0.27	0.68	0.04
Meal length, min	20.6	17.7	17.3	21.1	1.58	0.31	0.87	0.04
Postpartum DMI, kg/d	21.8	19.7	21.5	22.8	1.13	0.73	0.34	0.14
Postpartum water intake, L/d	105.6	96.1	105.6	105.2	5.15	0.59	0.72	0.40
Meal frequency, d ⁻¹	12.8	13.1	13.5	12.2	0.62	0.85	0.62	0.19
Intermeal interval, h	1.50	1.50	1.42	1.60	0.08	0.90	0.53	0.29
Meal size, kg DM	1.81	1.69	1.76	1.93	0.10	0.87	0.34	0.14
Meal length, min	26.4	25.0	25.3	26.7	1.11	0.56	0.80	0.19

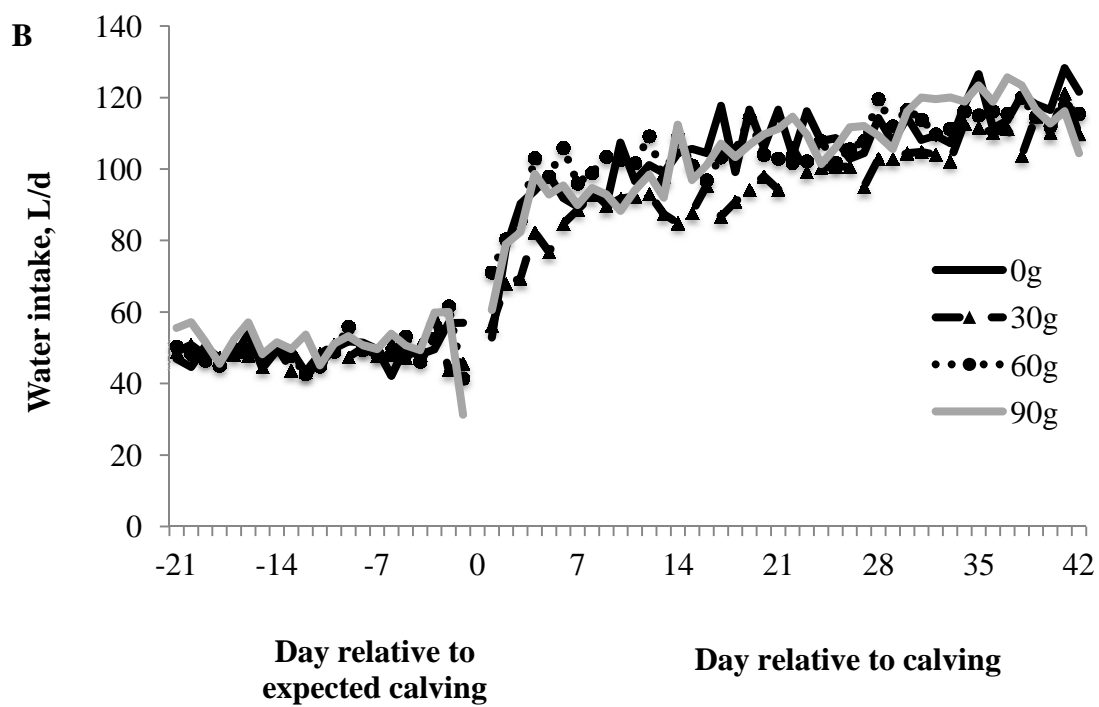
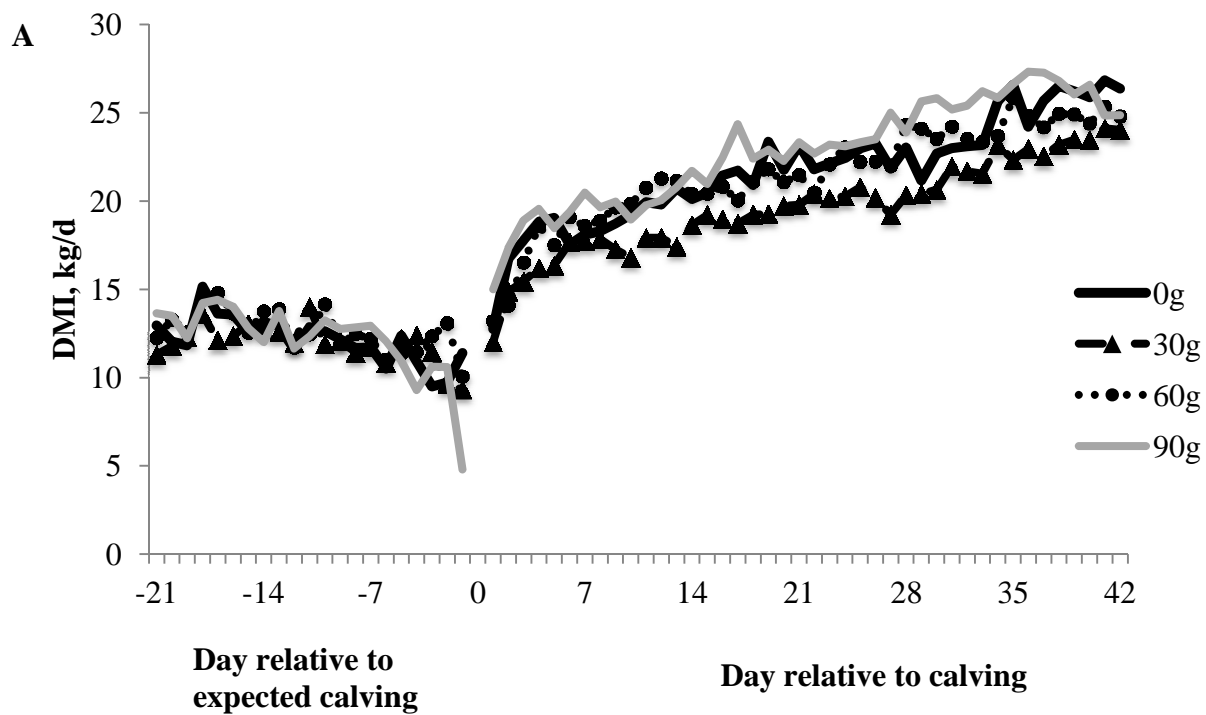
Table 4-3 Milk production and composition during the experimental period. Values are least squares means \pm SEM, n = 9-10.

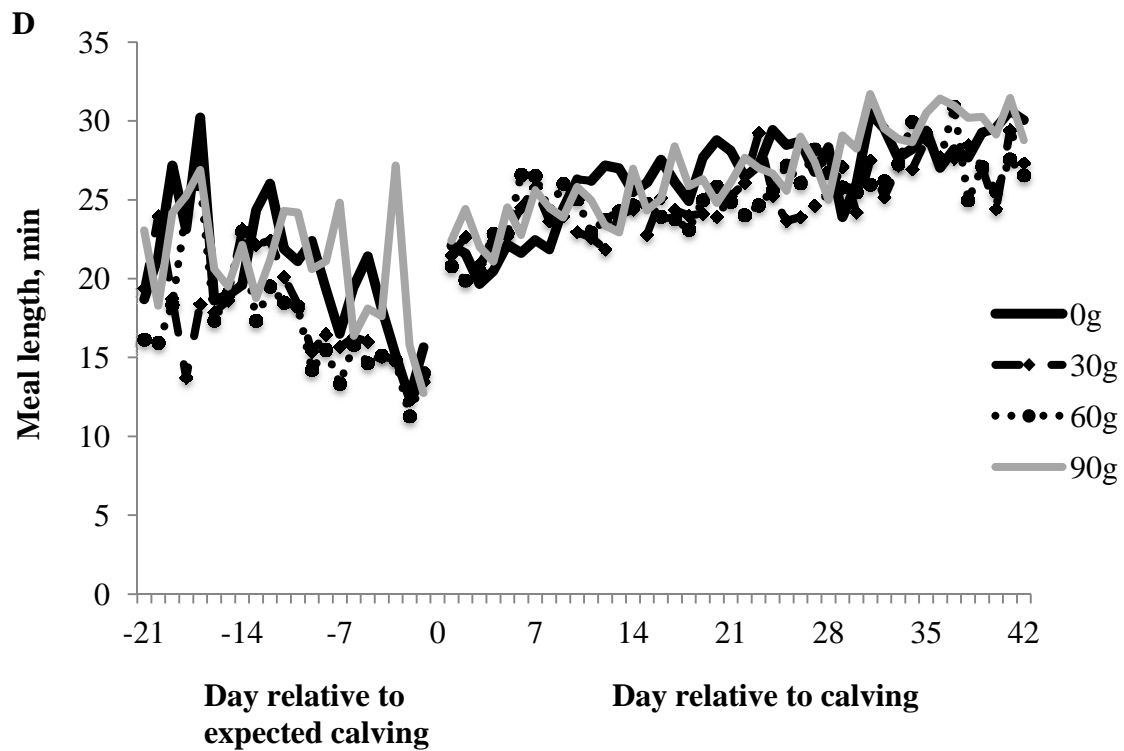
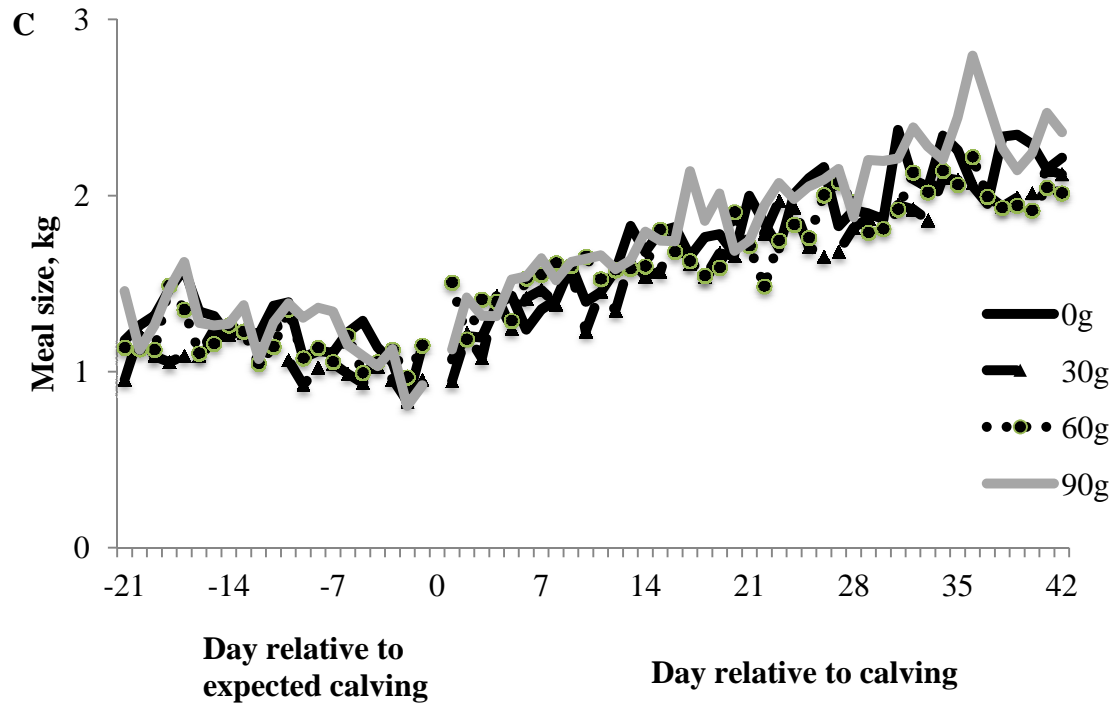
Item	Treatment				SEM	<i>P</i> value		
	0g	30g	60g	90g		Yeast vs. Control	Linear	Quadratic
Milk yield, kg/d	45.3	42.6	47.8	46.7	2.53	0.90	0.39	0.72
Milk fat, %	4.11	4.38	4.33	4.17	0.13	0.20	0.80	0.09
Milk protein, %	2.99	2.89	3.04	3.12	0.07	0.76	0.08	0.17
Milk lactose, %	4.77	4.74	4.84	4.85	0.05	0.45	0.10	0.67
MUN, mg/dl	13.3	14.1	13.4	13.9	0.63	0.44	0.65	0.81
Fat yield, kg/d	1.81	1.82	2.02	1.90	0.09	0.32	0.24	0.46
Protein yield, kg/d	1.34	1.21	1.42	1.42	0.08	0.86	0.17	0.42
Lactose yield, kg/d	2.16	2.03	2.32	2.26	0.13	0.78	0.30	0.79
SCM ¹ , kg/d	44.5	42.7	48.5	46.5	2.32	0.58	0.24	0.96
ECM ² , kg/d	48.5	46.5	52.4	50.5	2.45	0.63	0.26	0.99

¹ SCM = $(12.3 \times \% \text{fat}) + (6.56 \times \% \text{ solids-non-fat}) - (0.0752 \times \text{milk yield})$; (Tyrell and Reid, 1965)

² ECM = $(0.327 \times \text{milk yield}) + (12.86 \times \% \text{fat}) + (7.65 \times \% \text{protein})$; (Dairy Records Management Systems, 2010)

Figure 4-1 Dry matter intake (A), water intake (B), meal size (C), meal length (D), number of meals (E), and intermeal interval (F) during the experimental period. Yeast product was supplemented at 0, 30, 60, or 90 g/d to transition dairy cows from 21 d before expected calving to 42 d after calving. No treatment ($P > 0.10$) or treatment \times day ($P > 0.10$) effects were observed for these variables. (A): Day effects ($P < 0.01$) both pre- and postpartum were observed; prepartum SEM = 0.86, postpartum SEM = 1.44. (B): A tendency for day effect prepartum ($P = 0.09$) and a day effect postpartum ($P < 0.01$) were observed; prepartum SEM = 4.63, postpartum SEM = 7.02. (C): Day effects ($P < 0.01$) both pre- and postpartum were observed; prepartum SEM = 0.11, postpartum SEM = 0.17. (D): Day effects ($P < 0.01$) both pre- and postpartum were observed; prepartum SEM = 2.65, postpartum SEM = 1.81. (E): A tendency for day effect prepartum ($P = 0.07$) and a day effect postpartum ($P = 0.03$) were observed; prepartum SEM = 0.93, postpartum SEM = 1.00. (F): A day effect was observed prepartum ($P < 0.01$), but not postpartum ($P = 0.18$); prepartum SEM = 0.17, postpartum SEM = 0.14. Values are least squares means \pm SEM, $n = 9-10$ for all.





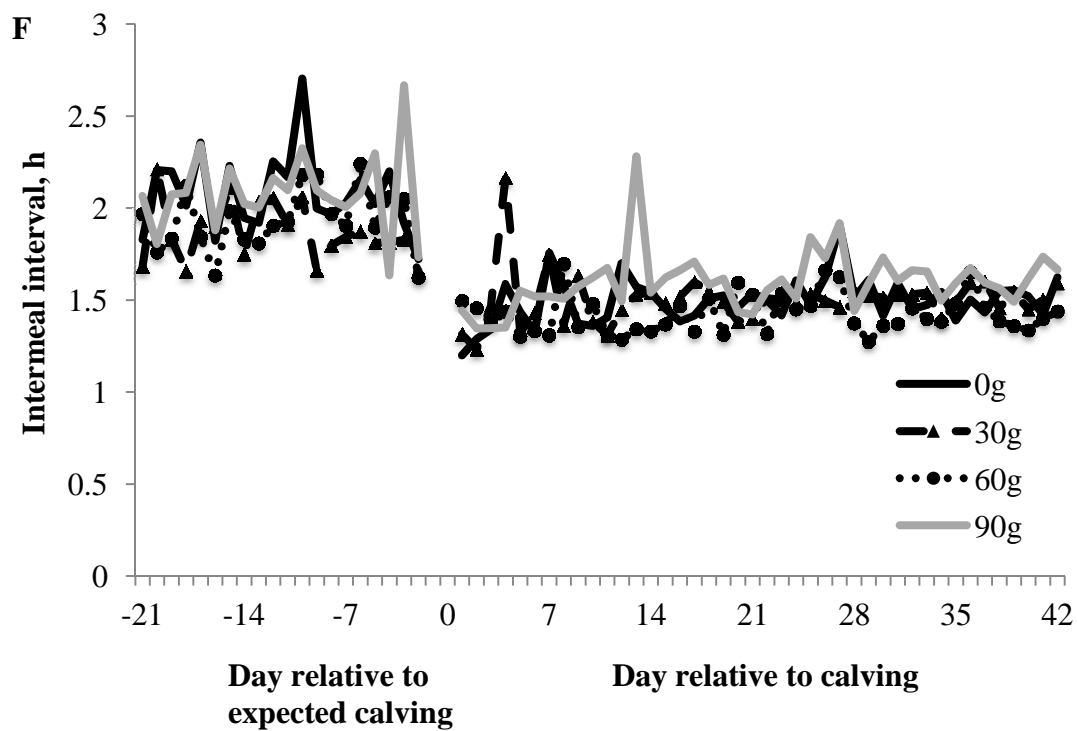
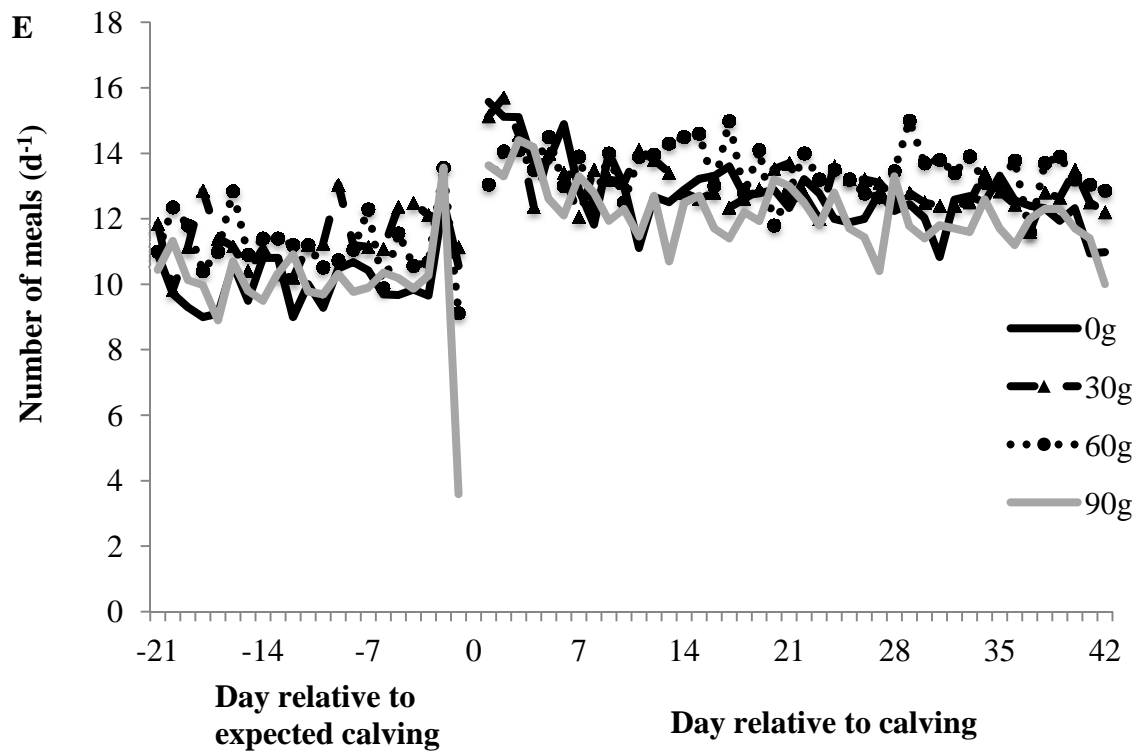
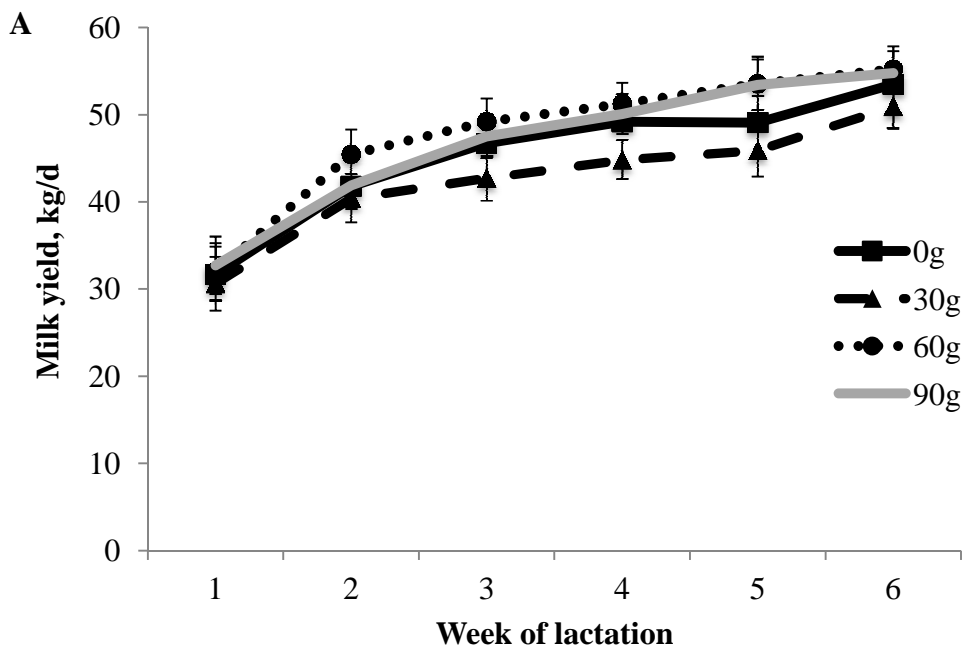


Figure 4-2 Milk yield (A), ECM (B), and BCS (C) during the experimental period. Yeast product was supplemented at 0, 30, 60, or 90 g/d to transition dairy cows from 21 d before expected calving to 42 d after calving. No treatment ($P > 0.10$) or treatment \times day ($P > 0.10$) effects were observed for these variables. **(A)** A week effect ($P < 0.01$) was observed. **(B)** A week effect ($P < 0.01$) was observed. **(C)** A day effect ($P < 0.01$) was observed. Values are least squares means \pm SEM, $n = 9-10$ for all.



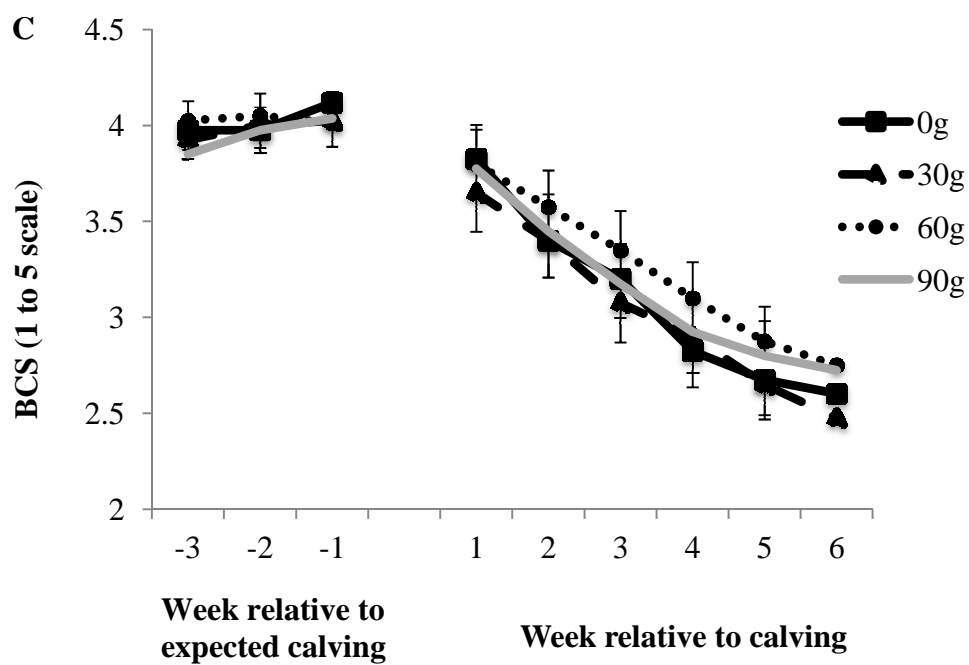
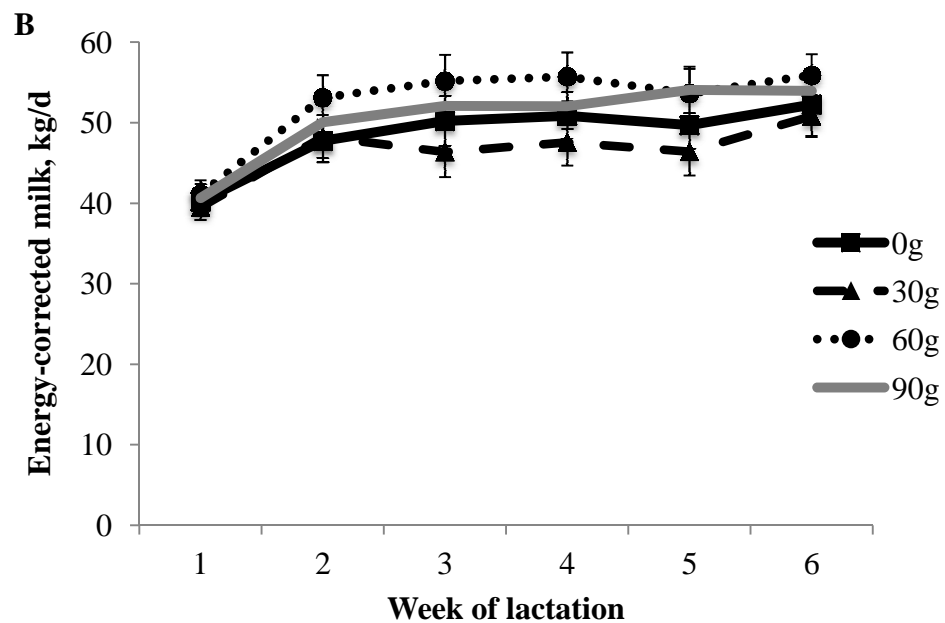
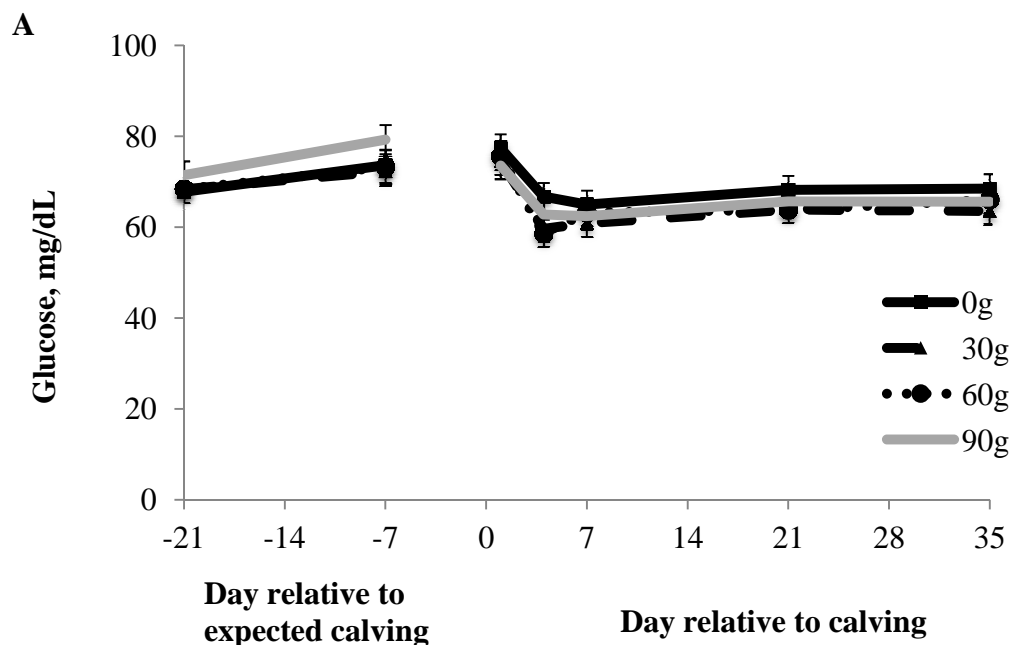
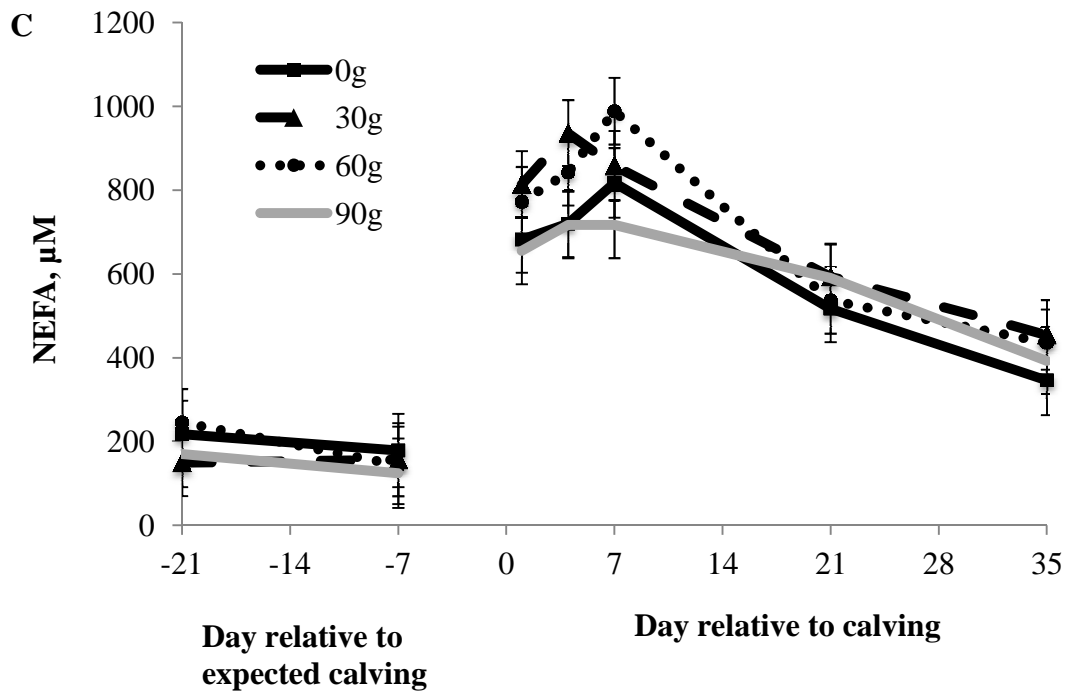
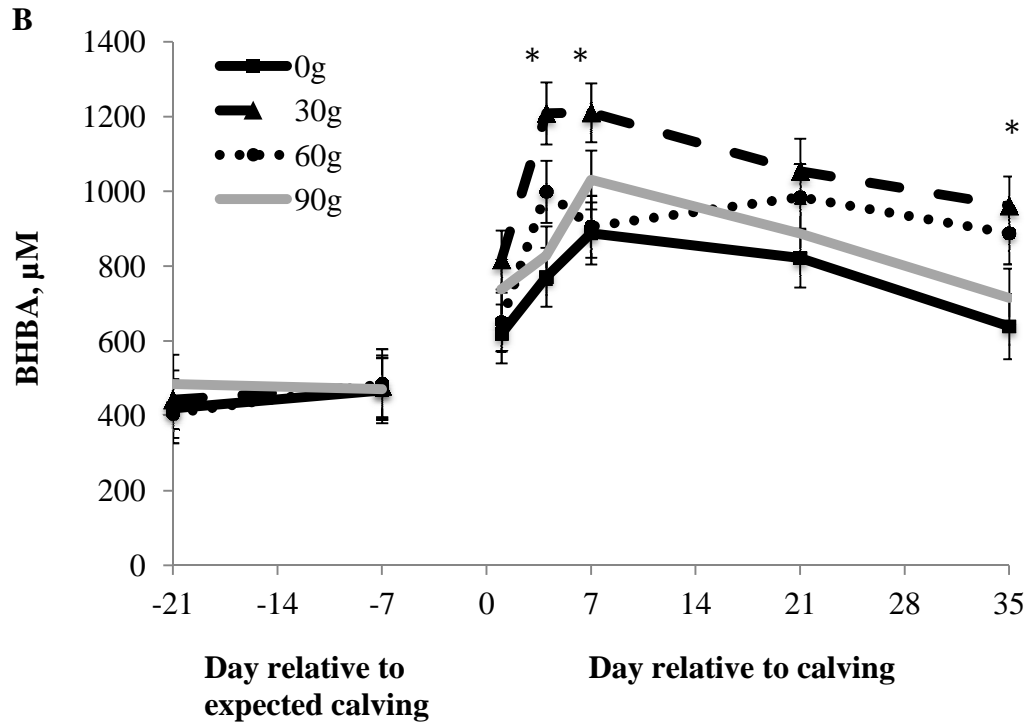


Figure 4-3 Concentrations of plasma metabolites during the experimental period. Yeast product was supplemented at 0, 30, 60, or 90 g/d to transition dairy cows from 21 d before expected calving to 42 d after calving. **(A)** NEFA: There was a day effect ($P < 0.01$), and a tendency for quadratic dose effect ($P = 0.06$), but no yeast product vs. control ($P = 0.14$), linear dose ($P = 0.75$), or treatment \times day ($P = 0.99$) effects. **(B)** BHBA: There were day ($P < 0.01$), yeast product vs. control ($P < 0.01$), and quadratic dose ($P < 0.01$) effects, but no linear dose ($P = 0.57$) or treatment \times day ($P = 0.20$) effects. * indicates significant differences ($P < 0.05$). **(C)** NEFA: There was a day effect ($P < 0.01$), but no yeast product vs. control ($P = 0.47$), linear dose ($P = 0.83$), quadratic dose ($P = 0.12$), or treatment \times day ($P = 0.73$) effects. Values are least squares means \pm SEM, $n = 9-10$ for all.





Chapter 5 - Yeast product supplementation modulated humoral and mucosal immunity and uterine inflammatory signals in transition dairy cows

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Abstract

The transition from late gestation to early lactation is characterized by substantial metabolic stress and suppressed immune function. The objective of this study was to assess the effects of supplementing a yeast product derived from *Saccharomyces cerevisiae* on immunity and uterine inflammation in transition cows. Forty multiparous Holstein cows were blocked by expected calving date and randomly assigned within block to 1 of 4 treatments ($n = 10$) from 21 d before expected calving to 42 d postpartum. Rations were top-dressed with a yeast product (yeast culture plus enzymatically hydrolyzed yeast; Celmanax, Vi-COR, Mason City, IA) at the rate of 0, 30, 60, or 90 g/d throughout the experiment. Colostrum samples were collected immediately after calving for analysis of immunoglobulin G (IgG) concentrations. Milk somatic cell count (SCC) was analyzed and converted to somatic cell linear score (SCS). Blood samples collected on -21, -7, 1, 4, 7, 14, 21, and 35 d relative to calving were analyzed for hematology and leukocyte differential and plasma haptoglobin; whole blood samples were incubated with *E. coli* (# 51813) to assess the ability of blood to kill bacteria. Cows were challenged on d -21, -7, and 14 with subcutaneous administration of ovalbumin with adjuvant; plasma samples collected on d -21, -14, and 21 were analyzed for anti-ovalbumin IgG content to assess humoral immune response. Fecal samples were collected on d 7 and 21 for analysis of IgA concentration. Uterine samples were collected on d 7 and 42 postpartum by cytobrush technique to determine neutrophil populations and relative abundance of transcripts involved in inflammation. Data were analyzed using mixed models with repeated measures over time. Concentrations of colostrum IgG were unaffected ($P > 0.10$) by treatments. A treatment \times wk interaction ($P < 0.01$) was observed for SCS, reflecting a tendency for a quadratic dose effect on wk 1 (2.34, 2.85, 1.47, and 4.06 ± 0.59 for 0, 30, 60, and 90 g/d, respectively; $P = 0.08$), and a quadratic dose effect on wk 5 (1.36, -0.15,

-1.07, and 0.35 ± 0.64 for 0, 30, 60, and 90 g/d, respectively; $P = 0.02$). Platelet count was increased by yeast product ($P = 0.04$). Plasma haptoglobin concentrations were not affected ($P > 0.10$) by treatments. Percentages of *E. coli* killed by whole blood were not affected ($P > 0.10$) by treatments. Yeast product linearly increased ($P < 0.01$) plasma anti-ovalbumin IgG levels on d 21, suggesting that treatments enhanced humoral immunity. Yeast product quadratically increased fecal IgA concentrations ($P = 0.03$), suggesting that 30 and 60 g/d doses enhanced mucosal immunity. Uterine neutrophil populations were much greater in samples collected on d 7 compared with those on d 42 (32.0 vs. 7.6 ± 3.7 % of cells, $P < 0.01$), reflecting neutrophil infiltration immediately after calving, but no treatment effect ($P = 0.53$) was detected. There were significant day effects ($P \leq 0.01$) for *IL-6*, *IL-8*, *neutrophil myeloperoxidase*, and *neutrophil elastase* mRNA in uterine sample, reflecting greater abundance of these transcripts collected on d 7 compared with d 42. Interestingly, there was a quadratic dose effect ($P = 0.02$) for *IL-6*, indicating that 30 and 60 g/d decreased uterine *IL-6* mRNA. The mRNA abundance of *neutrophil myeloperoxidase* and *elastase* was increased ($P < 0.05$) by yeast product. Yeast product supplementation enhanced humoral and mucosal immunity, and modulated uterine inflammatory signals and mammary gland health in transition dairy cows.

Key words: immunity, transition cow, uterine inflammation, yeast

Introduction

The transition from late gestation to early lactation is characterized by substantial metabolic stress and suppressed immune function, which may contribute to the markedly elevated incidence of health disorders in early-lactation dairy cows (Goff and Horst, 1997). Therefore, it is critical to develop effective nutritional strategies that could modulate the immunity of these animals. Yeast products derived from *Saccharomyces cerevisiae* have been shown to improve feed intake and milk yield in transition cows ((Dann et al., 2000; Ramsing et al., 2009). Recent findings suggest yeast products may also enhance immune function. Kim et al. (2011) reported that neonatal calves fed hydrolyzed yeast showed enhanced acute phase response, antibody production, and health scores when challenged with live vaccines, compared with controls. Such responses could be attributed to improved energy status via effects on digestive function, or on activation of the immune system through cell sensing of yeast components in the gut and subsequent cross-talk between immune cells.

One overlooked aspect of the response to yeast is the potential to influence mucosal immunity. Mucosal surfaces of the intestinal and reproductive tracts are among the most important routes of entry of microbial pathogens into the host. Epithelial cells that line mucosal surfaces are important mechanical barriers to prevent most microbes found in the environment, including commensal microbes, from entering epithelial cells (Kagnoff and Eckmann, 1997). A recent study (Zanello et al., 2011) reported that yeast modulated the expression of genes involved in inflammation and recruitment and activation of immune cells in porcine intestinal epithelial cells. Specifically, yeast decreased both the transcript and protein abundance of pro-inflammatory genes, including interleukine-6 (IL-6) and IL-8, in response to *E. coli* exposure.

Together, interest in yeast products for dairy cows has expanded beyond a focus on improving production. There are intriguing data suggesting that yeast products can improve immune function, particularly during physiological challenges. The objectives of this study were to assess whether yeast product alters: 1) immunity, assessed as milk SCS, colostrum immunoglobulin G (IgG) concentrations, hematology and leukocyte differential, the ability of blood immune components to kill *E. coli*, IgG antibody production after vaccination, and IgA secretion from the gut mucosa; 2) uterine inflammation, as determined by neutrophil populations and transcript abundance of inflammatory genes in uterine tissues.

Materials and Methods

The Kansas State University Institutional Animal Care and Use Committee approved all the experimental procedures.

Design and Treatments

Forty multiparous Holstein transition cows from the Kansas State University Dairy Cattle Teaching and Research Facility were used in a randomized complete block design. Cows were blocked by expected calving date (10 blocks), and randomly assigned within block to 1 of 4 treatments 21 d before their expected calving date. Cows remained on their respective treatments through 42 d postpartum. Cows received a yeast product (yeast culture plus enzymatically hydrolyzed yeast; Celmanax, Vi-COR, Mason City, IA) at a rate of 0, 30, 60, or 90 g/d. Yeast product was administered daily to each cow in the treatment groups by top-dressing and manually mixing the premix into the upper part of each TMR. Diets were formulated to meet or

exceed NRC (2001) requirements; ingredient and nutrient composition of the diet were reported in a companion paper (Yuan et al., 2014).

Management of Cows and Data Collection

Dry cows were moved into the maternity barn approximately 1 wk before entering the study. Cows were allowed ad libitum access to the designated treatment rations by an electronic gating system (Roughage Intake System, Insentec B.V., Marknesse, The Netherlands). After parturition, cows were moved into a tie-stall facility where they remained through 42 d postpartum. Dry cows were fed ad libitum twice daily (1100 and 1800 h), and lactating cows were fed twice daily (1200 and 1900 h). Lactating cows were milked 3 times daily in a milking parlor. Feed intake, feeding behavior, and milk yields were recorded as described in Yuan et al. (2014). Milk samples were collected 3 consecutive days each week, and were analyzed for somatic cells (SCC 500, Bentley Instruments; Heart of America DHIA, Manhattan, KS). Colostrum samples were collected immediately after calving and were kept frozen at -20°C until analysis of IgG concentration (Cornell Diagnostic Lab, Ithaca, NY).

Cows were challenged (subcutaneous injection) with 1 mg of chicken egg ovalbumin (Sigma-Aldrich, St. Louis, MO) diluted in vaccine adjuvant (VET-SAP, Desert King International, San Diego, CA; 0.5 mg of adjuvant dissolved in 1 mL of saline) on d -21, -7, and 14. Blood samples were collected on d -21, -14, -7, 1, 4, 7, 21, and 35 relative to calving from the coccygeal vessels 1 h prior to feeding. Approximately 20 mL of blood was collected into 4 tubes: one 7-mL tube containing potassium EDTA, one 3-mL tube containing potassium EDTA, one 5-mL tube containing heparin, and another 7-mL tube containing potassium oxalate with sodium fluoride as a glycolytic inhibitor (Vacutainer, Becton Dickinson, Franklin Lakes, NJ,

USA). Fecal samples were collected on d 7 and 21, and were frozen at -20°C until analysis of IgA concentration.

Endometrial samples were collected approximately on d 7 (7.2 ± 1.0 d across treatments; 7.2, 6.8, 7.3, and 7.3 ± 0.35 d for 0, 30, 60, and 90 g/d, respectively; $P = 0.68$) and 42 (40.9 ± 1.6 d across treatments; 40.9, 41.4, 40.2, and 41.0 ± 0.49 d for 0, 30, 60, and 90 g/d, respectively; $P = 0.40$) postpartum by cytobrush. Prior to the procedure, the vulva was cleaned with paper towels. Then, a cytological sample of the endometrium from the dorsal aspect of the uterine body was collected using a cytobrush (Cytobrush Plus; CooperSurgical Inc., Trumbull, CT) as reported before (Mendonça et al., 2013). Briefly, the cytobrush was placed into the stainless steel tube of an insemination pipette. The device was placed into a sanitary plastic chemise (Agtech, Inc., Manhattan, KS). Endometrial sampling was performed by inserting the instrument through the vagina into the uterine body. Once the device was pushed forward into the uterine body, the sanitary chemise was punctured and the plastic sheath retracted to expose the cytobrush. Once the brush had been rolled onto the uterine wall, it was pulled back into the plastic sheath and removed from the vagina. Slides for cytological examination were prepared by rolling the cytobrush on a clean glass microscope slide.

Cows were monitored daily for health status. Ketosis was recorded when urine acetoacetate exceeded 80 mg/dL on any day or 40 mg/dL for 2 consecutive days (Ketostix; Bayer Corp. Diagnostics Division, Elkhart, IN). Other disorders or diseases were diagnosed according to the guidelines by Kelton et al. (1998).

Sample Analysis

Blood samples from the 7 mL potassium EDTA tube and the 7 mL tube containing potassium oxalate with sodium fluoride were centrifuged at $2,000 \times g$ for 10 min immediately after sample collection, and plasma was harvested and frozen at -20°C until subsequent analysis of glucose (reported in Yuan et al., 2014), BHBA (reported in Yuan et al., 2014), NEFA (reported in Yuan et al., 2014), and haptoglobin concentrations. Haptoglobin was analyzed by a colorimetric method based on peroxidase activity (Cooke and Arthington, 2013). The standard curve was prepared using plasma samples from another research project (Yuan et al., 2013) with known haptoglobin concentrations, which were analyzed by ELISA (ELISA kit #2410-7; Life Diagnostics, West Chester, PA). Absorbance was read on a spectrophotometer (Powerwave XS, Biotek Instruments, Winooski, VT, USA) and calculations were conducted using Gen5 software (Biotek Instruments).

Blood collected from the 3-mL potassium EDTA tube was analyzed for hematology using a ProCyte hematology analyzer (IDEXX Laboratories, Westbrook, ME). Whole blood collected from the 5-mL tube containing heparin was used to test its bactericidal capacities against live cultures of environmental *E. coli* (ATCC# 51813) according to methods previously described (Ballou, 2012). Briefly, an overnight broth culture of the *E. coli* was diluted in nonpyrogenic $1\times$ PBS to an approximate concentration of $25\text{ cfu}/\mu\text{L}$ and kept in an ice bath. Whole blood was diluted 1:1 with RPMI-1640 to a final volume of $200\text{ }\mu\text{L}$. All tubes were placed in an ice bath for 5 min. Then, $50\text{ }\mu\text{L}$ of the working *E. coli* culture was added to each tube of diluted whole blood, vortexed, and incubated in a water bath at 37.5°C for 5 min. Following incubation, the cultures were vortexed, $50\text{ }\mu\text{L}$ of each culture was pipetted and spread plated onto tryptic soy agar plates in duplicate, and the plates were incubated overnight at 36.5°C .

before determination of the number of cfu. Data are expressed as the percentage of reduction in cfu for blood samples vs. controls.

Plasma samples collected on d -21, -14, and 21 were analyzed for anti-ovalbumin IgG using an ELISA modified from a method described by Mallard et al. (1997). All cows were analyzed for d -21 and -14 samples; only cows that received 3 doses of chicken egg ovalbumin injection (on d -21, -7, and 14) were retained for the analysis of d 21 samples. Briefly, 96-well plates (Corning Costar high binding 96 well plate, Thermo Fisher Scientific Inc.) were coated with 100 μ L of coating solution: 16.8 chicken egg ovalbumin (Sigma-Aldrich) diluted in 12 mL 0.05 M carbonate-bicarbonate buffer (pH 9.4). Plates were incubated overnight at room temperature and washed 5 times with wash buffer (PBS and 0.05% Tween solution, pH 7.4). Plates were then blocked with 300 μ L of blocking buffer (4% bovine serum albumin, 5% sucrose in PBS, 0.2 μ m filtered) and incubated overnight at 4°C. Plates were washed 5 times, and blank (wash buffer), diluted plasma (1:200), and pooled samples from d -21 (negative control, 1:200) and 21 (positive control, 1:200) were added in duplicates. Plates were incubated for 1 h at room temperature and washed 5 times. Subsequently, anti-bovine IgG (Anti-Bovine IgG -peroxidase antibody produced in rabbit, Sigma-Aldrich) was diluted with 10 mM Tris buffer solution at 1:30,000, added to the plates and incubated for 1 h at room temperature. Plates were then washed 5 times, and 100 μ L of 3,3',5,5'-tetramethylbenzidine substrate solution (TMB Solution; Thermo Fisher Scientific Inc.) was added to each well and allowed to incubate for 3 min at room temperature, followed by the addition of 100 μ L of stop solution (0.18 M sulfuric acid). Optical density (OD, blank-adjusted) was measured at 450 nm using a plate reader (PowerWaveXS, BioTek, Winooski, VT). The final OD was corrected across different plates to achieve a positive control optical density = 0.5. For each sample, final OD = $0.5 \times \text{original OD} \div \text{positive control OD of that plate}$.

For fecal IgA analysis, the frozen fecal samples were thawed and placed immediately in ultra purified water at a concentration of 10% w/v by weighing 1 g of feces and adding 9 mL of water. Samples were then vortexed vigorously and incubated overnight before being spun at $2,000 \times g$ for 15 min. The supernatants were collected and analyzed for IgA by ELISA according to the manufacturer's instructions (Bovine IgA ELISA Quantitation Set, Bethyl Laboratories, Montgomery, TX).

For morphological assessment of uterine samples, slides were stained with modified Wright Giemsa stain (Protocol-Hema3, Bio-chemical Sciences, Swedesboro, NJ). Each slide was examined at $400 \times$ magnification to perform a differential cell count of at least 200 cells (neutrophils + endometrial cells) by a single observer. The result of this examination was reported as neutrophils as a percentage of all cells.

Total RNA was extracted from uterine tissue using a commercial kit (RNeasy Lipid Tissue Mini Kit; Qiagen Inc., Valencia, CA) according to the manufacturer's instructions, and spectroscopy was used to quantify RNA (Nanodrop-1000, Nanodrop Technologies Inc., Wilmington, DE). Quality of RNA (integrity number = 6.78 ± 1.09 for a random subset of 16 samples) was verified with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Then, RNA was reverse-transcribed to cDNA and mRNA abundance of *IL-6*, *IL-8*, *neutrophil myeloperoxidase*, and *neutrophil elastase* was determined by qPCR as described (Yuan et al., 2013). Briefly, 2 mg of total RNA was used as the template for the reverse transcriptase reaction using random primers (High-Capacity cDNA RT Kit; Life Technologies, Carlsbad, CA). Quantitative real-time PCR (RT-PCR) was performed in duplicate on 96-well plates with 5% of the cDNA product in the presence of 200 nM gene-specific forward and reverse primers with real-time SYBR green fluorescent detection using SYBR Green Premix reagent (7500 Fast Real-

Time PCR System, Life Technologies). Primers were designed (www.ncbi.nlm.nih.gov/tools/primer-blast/) using GenBank sequences (Table 1). Data were recorded and analyzed with Sequence Detector software (Life Technologies). All sample values were normalized against the geometric mean of 3 control genes, *ribosomal protein subunit 9* (*RPS9*), *RPS15*, and β -*actin*, and relative transcript abundance was quantified by using the $2^{-\Delta Ct}$ method.

Data and Statistical Analysis

One cow in the 0 g/d group was removed from the study on d 30 postpartum due to difficulty standing up in the tie-stall. Data obtained from this cow prior to removal were included in all analyses. Data were analyzed using mixed models with repeated measures over time. Models included the fixed effects of treatment, time, and their interaction, and the random effect of cow. Contrast statements were used to assess the overall effect of yeast product (control vs. all yeast product treatments), as well as the linear and quadratic effects of dose. If treatment by time effects were significant, slices were used to assess effects on specific days. Values were deemed outliers and omitted from analysis when Studentized residuals were greater than $> |3.0|$. After initial outlier removal, the model was repeated and Studentized residuals greater than $> |3.5|$ were deemed outliers. When necessary, data (including plasma haptoglobin, fecal IgA, uterine transcript abundance of *IL-6*, *IL-8*, *neutrophil myeloperoxidase*, and *neutrophil elastase*) were log-transformed prior to analysis to achieve normal residual distributions, and reported means and standard errors are back-transformed. Milk SCC was converted to SCS (Shook et al., 1993). Regression analyses were used to explore relationships between different measures. For each regression analysis, the distribution of Cook's D statistic was visually checked and outliers were

removed from the analysis. Significance was declared at $P \leq 0.05$ and tendency at $0.05 < P \leq 0.10$.

Results

There were no differences among groups in prepartum dietary treatment length (18.1, 18.8, 17.8, and 17.2 ± 1.32 d for 0, 30, 60, and 90 g/d, respectively; $P = 0.86$). Actual calving dates ranged from 13 d before expected calving to 5 d after expected calving. Results for feed intake and milk production were reported in Yuan et al. (2014), and they were not affected ($P > 0.10$) by yeast product treatments.

Milk Constituents

As shown in Figure 1, there was a treatment \times wk interaction ($P < 0.01$) for milk SCS, reflecting a tendency for a quadratic dose effect on wk 1 (2.34, 2.85, 1.47, and 4.06 ± 0.59 for 0, 30, 60, and 90 g/d, respectively; $P = 0.08$), and a quadratic dose effect on wk 5 (1.36, -0.15, -1.07, and 0.35 ± 0.64 for 0, 30, 60, and 90 g/d, respectively; $P = 0.02$). As shown in Figure 2, concentrations of IgG in colostrum collected immediately after calving were not affected by treatments ($P = 0.96$).

Hematology and Leukocyte Differential

Results of hematology and leukocyte differential analysis are shown in Table 2. There were no treatment effects ($P > 0.10$) for erythrocyte count, hemoglobin, or leukocyte count. Hematocrit was linearly decreased ($P = 0.04$) by treatment. Platelet count was increased by yeast

product compared with control ($P = 0.04$). Concentrations and fractions of lymphocytes, monocytes, eosinophils, and basophils were not affected ($P > 0.10$) by treatments; neutrophil concentrations, but not fraction, were decreased ($P < 0.05$) by yeast supplementation. As shown in Table 3, there were day effects ($P < 0.01$) for erythrocyte count, hemoglobin, hematocrit, and leukocyte count. Specifically, hemoglobin, hematocrit, and erythrocyte count were decreased on d 21 and 35 compared with other dates; leukocyte count was increased on d 1 after calving; platelet count was decreased at the week around parturition compared with d -21, and increased on d 21 and 35. Both concentrations and fraction of neutrophils were elevated at calving; those of lymphocytes were greater at d -21 compared with other dates, monocytes were increased postpartum compared with prepartum, and eosinophils were decreased postpartum compared with prepartum.

Plasma Haptoglobin

As shown in Figure 3, haptoglobin was not altered by yeast product ($P = 0.15$, yeast product vs. control). No linear dose ($P = 0.28$), quadratic dose ($P = 0.35$) or treatment \times day ($P = 0.35$) effects were detected.

Whole Blood Killing of Bacteria

As shown in Figure 4, the proportion of bacteria killed by heparinized whole blood was not affected by treatments ($P = 0.28$). Although the values decreased numerically at the time around parturition compared with 3 wk before or after calving, this change did not reach a significant day effect ($P = 0.19$). No treatment \times day effect ($P = 0.99$) was detected.

Antibody Production

As shown in Figure 5, anti-ovalbumin IgG levels increased dramatically ($P < 0.01$) after 3 ovalbumin challenges (d 21) compared with pre-immunization (d -21). Throughout the experimental period, there was a tendency for increased anti-ovalbumin IgG in cows receiving yeast product ($P = 0.06$; linear dose effect). A treatment \times day effect ($P < 0.01$) was also detected, reflecting that yeast product linearly increased ($P < 0.01$) anti-ovalbumin IgG on d 21. As shown in Figure 6, fecal IgA concentrations were not different ($P = 0.61$) between d 7 and 21, but yeast product quadratically increased ($P = 0.03$) fecal IgA concentrations.

Uterine Inflammation

As shown in Figure 7, uterine neutrophil populations were much greater in samples collected on d 7 compared with d 42 (32.0 vs. 7.6 ± 3.7 % of cells, $P < 0.01$). There were no treatment ($P = 0.53$) or treatment \times day effects ($P = 0.75$). No correlation ($P = 0.64$, $R^2 = 0.01$) between d 7 and 42 uterine neutrophil populations was detected. No treatment effect ($P = 0.39$) was detected for incidence of subclinical endometritis, as defined by the presence of $>10\%$ neutrophil in uterine samples collected at d 42 (Kasimanickam et al., 2004). As shown in Table 1, the median Ct values of RT-PCR for *IL-6*, *IL-8*, *neutrophil myeloperoxidase*, and *neutrophil elastase* were 26.6, 20.0, 36.3, and 34.0 ± 3.5 , respectively, indicating that the abundance of *neutrophil myeloperoxidase* and *elastase* is much less compared with *IL-6* and *IL-8*. As shown in Figure 8, there were significant ($P \leq 0.01$) day effects for *IL-6*, *IL-8*, *neutrophil myeloperoxidase*, and *neutrophil elastase*, reflecting greater abundance of these transcripts in uterine tissues collected on d 7 compared with d 42. Specifically, compared with d 42, the

abundance of uterine *IL-6* and *IL-8* was over 25-fold greater on d 7, and that of *neutrophil myeloperoxidase* and *elastase* was over 2-fold greater. Interestingly, there was a quadratic dose effect ($P = 0.02$) for *IL-6*, indicating that 30 and 60 g/d doses decreased uterine *IL-6* mRNA abundance. No treatment effects ($P > 0.10$) were detected for *IL-8*. The abundance of *neutrophil myeloperoxidase* ($P = 0.05$, linear dose effect) and *elastase* ($P = 0.03$, linear dose effect) was increased by yeast product. The abundance of *neutrophil elastase* was positively correlated with neutrophil populations on d 42 ($P = 0.02$, $R^2 = 0.29$), but not on d 7 ($P = 0.65$, $R^2 = 0.08$). Abundance of other transcripts was not correlated with neutrophil populations ($P > 0.10$). To explore the possible link between systemic and uterine tissue inflammation, we examined the correlation between d 7 plasma haptoglobin concentrations and d 7 uterine neutrophil populations, and a significant positive association was detected ($P < 0.01$, $R^2 = 0.23$, Figure 9).

Health Disorders

Incidence of health disorders was not affected by treatments ($P > 0.10$, Table 4). To determine if retained placenta was linked to uterine inflammation, we compared neutrophil populations in cows with and without retained placenta. As shown in Figure 10, cows with retained placenta had greater ($P < 0.01$) neutrophil populations. In addition, cows that developed retained placenta also had much greater concentrations of plasma haptoglobin (4781 vs. 739 ± 301 $\mu\text{g/mL}$, $P < 0.01$, $R^2 = 0.83$) and BHBA (1295 vs. 991 ± 150 μM , $P = 0.04$, $R^2 = 0.19$) on d 7.

Discussion

Increasing evidence suggests that yeast product may improve immune function during physiological challenges. We found a tendency for a quadratic dose effect for SCS on wk 1 and a quadratic dose effect on wk 5 of lactation, reflecting decreased SCS by 30 and 60 g/d doses. These results suggest that yeast product modulated mammary gland health in early lactation cows. Similarly, Nocek et al. (2011) reported that supplementation of yeast product throughout the first 14 wk of lactation decreased SCC and cases of clinical mastitis. Interestingly, the finding that 90 g/d increased SCS on the first week of lactation suggests that this dose might have exceeded the optimal level of supplementation, somehow promoting an adverse inflammatory response in the mammary gland.

Colostrum is a rich source of immune components. As the major immunoglobulin class present in bovine colostrum, IgG plays a key role in passive immunization of the offspring and protection of the mammary gland itself (Stelwagen et al., 2009). In this study, colostrum IgG concentrations were not affected by treatments, indicating that yeast product did not affect the quality of main colostrum immune component. However, because colostrum volume was not recorded, we were unable to assess treatment effects on the amounts of IgG produced.

Both concentrations and relative proportions of neutrophils in blood were elevated at calving, which is consistent with the fact that cows typically experience a surge of blood neutrophils at parturition (Burton and Erskine, 2003). Total leukocyte count was also increased on d 1 after calving. Despite increased immune cell numbers, their function is often impaired, resulting in immune deficiency in the transition period (Burton and Erskine, 2003). Interestingly, yeast product supplementation decreased concentrations of neutrophils, indicating a modulatory effect on immune cells. Although there were no treatment effects, hemoglobin, hematocrit, and

erythrocyte count were decreased on d 21 and 35 compared with other dates; it is possible that these changes resulted from increased plasma volume associated with increased milk output as lactation proceeds (Burton et al., 1992). Platelet count was decreased in the week around parturition compared with d -21, and increased on d 21 and 35. Because of their abundance and their ability to rapidly release cytokines and other immune mediators, platelets play a central role in modulating immune function (Jenne et al., 2013; Li et al., 2012). Recent evidence suggests that platelets can directly modulate neutrophil function and clear pathogens (Jenne et al., 2013). The temporal of changes in platelet counts in our study seem to be consistent with the changes of neutrophil function in the transition period (Kehrli et al., 1989). Given the importance of platelets in immune modulation, it is possible that platelets can be used as an indicator of immune function in transition cows. We also found that yeast product increased platelet count, which is consistent with the finding that hydrolyzed yeast supplementation dramatically increased platelet count in neonatal calves challenged with vaccine (Kim et al., 2011). Whether this increase reflects an enhanced immune function is not clear.

As an acute phase protein released primarily by the liver in inflammation, haptoglobin has consistently been shown to be increased at the time around parturition (Saremi et al., 2012; Mullins et al., 2012; Yuan et al., 2013). Recent work (Sabedra, 2012) using 161 transition cows found that serum haptoglobin was elevated around calving even in cows that were apparently healthy; cows experienced diseases had significantly greater concentrations compared with healthy animals. In this study, plasma haptoglobin means for yeast product treatments were ~50% of control means; however, this was not significant ($P = 0.15$), reflecting large variations in haptoglobin concentrations among cows. Although previous *in vitro* studies (Jensen et al.,

2008; Zanello et al., 2011) demonstrated that yeast and yeast culture possess anti-inflammatory effects, further *in vivo* studies are needed to verify these findings.

Several studies (Ballou, 2012; Ballou et al., 2009) have used whole blood killing of bacteria to assess immune function in cattle. It is known that transition cows experience a dramatic decrease in immune cell function (Kehrli et al., 1989), and this change was reflected in significant decrease in whole blood anti-microbial capacity at calving (Ballou et al., 2009). In this study, we observed a numerical decrease in blood killing ability at the time around parturition compared with 3 wk before or after calving, but this did not reach a significant day effect. It is possible that the assay we used is not sensitive enough to detect the previously-reported changes in immune function, as the killing capacity can be largely affected by types of bacteria used, dilution of blood, incubation time, and many other assay conditions (Liebl and Martin, 2009; Millet et al., 2007). Under our experimental conditions, the percentage of bacteria killed by whole blood was not affected by yeast product treatments.

To assess humoral immune response, we injected chicken egg ovalbumin, an antigen that these animals naïve to, to cows on d -21, -7 and 14, and measured plasma anti-ovalbumin IgG levels on d -21 (pre-immunization), -14, and 21. By using a similar vaccination approach, Burton et al. (1993) reported that Cr supplementation enhanced humoral immune response. Mallard et al. (1997) partitioned transition cows into 3 groups based on antibody response (i.e. high, low, and no responders) to ovalbumin challenge, and found that cows with the highest antibody response also tended ($P \leq 0.10$) to have the highest response to *E. coli* challenge at parturition and had the lowest incidence of diseases, particularly mastitis. As expected, after 3 ovalbumin challenges, anti-ovalbumin IgG increased dramatically. Yeast product also linearly increased ($P < 0.01$) levels of anti-ovalbumin IgG on d 21, indicating that yeast product supplementation

enhanced humoral immunity. The positive effects of yeast products on humoral immunity have been observed across different species. Franklin et al. (2005) reported that supplementation of yeast cell wall components, mannanoligosaccharides, to cows during the last 3 wk of the dry period enhanced their humoral immune response to rotavirus and tended to enhance the subsequent transfer of rotavirus antibodies to calves. Muthusamy et al. (2011) reported that feeding hydrolyzed yeast and yeast cell wall components increased blood antibody level against Newcastle disease virus vaccination in broilers. Another key constituent of yeast cell wall, β -glucan, has also been shown to enhance the defense against infections in rodent models (Samuelsen et al., 2014).

Mucosal immune responses are the first line of defense because most pathogenic agents enter the host via mucosal surfaces, and IgA secreted by gut plays crucial roles in this mucosal defense by entrapping microorganisms and preventing the adherence of pathogens to mucosal surface (Neutra and Kozlowski, 2006). Recent research also indicates that secretions of IgA are critical in the homeostasis of gut microbiota (Suzuki et al., 2004). Several studies have used fecal IgA concentration as an indicator of mucosal immunity. Gurzell et al. (2013) observed that fish oil supplementation in mice improved mucosal immune function by increasing secretory fecal IgA, and the numbers and function of B cells in gut-associated lymphoid tissues. Scholtens et al. (2008) reported that prebiotic oligosaccharide supplementation in milk formula increased fecal IgA concentrations in infants; together with the altered composition of intestinal microbiota in response to treatment, the authors concluded that the increased IgA suggests a positive effect of prebiotic on mucosal immunity. Viljanen et al. (2005) found probiotic supplementation increased fecal IgA and decreased tumor necrosis factor- α (TNF α) concentrations in infants with food allergic atopic eczema, and proposed that this increased IgA indicates an attempt of the host to

protect the gut from allergic food antigens, whereas decreased TNF α suggests that probiotics decreased inflammation in the gut. In this study, yeast product quadratically increased fecal IgA concentrations, suggesting that 30 and 60 g/d doses enhanced mucosal immunity. Previous research has demonstrated several possible mechanisms of the immunostimulatory effects of yeast product components. For example, mannanoligosaccharides can act as anti-adhesive agents and prevent colonization of pathogens in the intestinal tract by providing alternative adhesion sites to intestinal bacteria (Ganner and Schatzmayr, 2012). Furthermore, yeast components have been reported to have structural benefits to the intestine by increasing villus height and number of goblet cells (Muthusamy et al., 2011; Solis De Los Santos et al., 2007), which are responsible for the secretion of intestinal mucus. Increased mucus would in turn help to lubricate intestinal surfaces, trap and neutralize bacteria, and protect epithelial cells (Johansson et al., 2013), all of which could contribute to intestinal immune health.

After the transition period, one major hurdle for the dairy cow is the return to estrus and breeding. We proposed to explore the impact of yeast product on neutrophil populations and expression of inflammatory mediators in the uterus at 2 key time points: d 7 and 42 postpartum. It is common that at least some bacteria are present in the uterus in the first week after calving (LeBlanc et al., 2011), and that the presence of neutrophils in the uterus is needed to clear pathogens and facilitate the involution of reproductive tissues (Challis et al., 2009). By day 42 postpartum, cows that mounted a sufficient immune response will have cleared the bacteria, and neutrophil populations and expression of inflammatory mediators should be minimal, but other cows will still be fighting persistent infections (LeBlanc et al., 2011). It is widely believed that such persistent infections greatly impair fertility. Therefore, these measures investigated a potential link between dietary yeast product and reproductive success. We did not find treatment

effects for uterine neutrophil populations, but across treatments, there were much greater numbers of neutrophils in samples collected on d 7 compared with d 42. Most studies (LeBlanc, 2008; Deguillaume et al., 2012; Dubuc et al., 2010) have examined cytobrush samples in cows > 35 postpartum; in this study, we did not detect a correlation between d 7 and 42 neutrophil populations, indicating that greater presence of neutrophils in the uterus on d 7 does not predict incidence of subclinical endometritis on d 42. Neutrophil infiltration immediately after calving is not necessary detrimental but rather is a normal physiological response needed to clear pathogens and repair reproductive tissues (Challis et al., 2009).

We also evaluated transcript abundance of *IL-6* and *IL-8* as indicators of uterine inflammation. As important pro-inflammatory cytokines, *IL-6* and *IL-8* have been shown to be expressed in the uterine tissues of postpartum cows, and the abundance of these transcripts was highly correlated with subclinical endometritis (Ghasemi et al., 2012). In order to directly assess neutrophil inflammatory status, we also evaluated the mRNA abundance of 2 neutrophil-derived enzymes, *neutrophil myeloperoxidase* and *neutrophil elastase*. Myeloperoxidase is a lysosomal protein stored in neutrophil azurophilic granules and plays a key role in the microbicidal activity of neutrophils (Klebanoff, 2005). As a serine protease, neutrophil elastase is responsible for degradation of proteins during phagocytosis, as well as degradation of connective tissue during an inflammatory process (Helmig et al., 2002). Interestingly, we found that the abundance of *IL-6* and *IL-8* was over 25-fold greater in samples collected on d 7 compared with d 42, indicating much greater inflammation in uterus immediately after calving. Similarly, the abundance of *neutrophil myeloperoxidase* and *neutrophil elastase* was over 2-fold greater on d 7 compared with d 42, consistent with the finding that neutrophil populations in uterine samples were greater on d 7 compared with d 42. Osmers et al. (1995) reported a significant increase in *IL-8*

concentrations in human uterine tissue at the time of parturition, and this increase was strongly correlated with concentrations of multiple neutrophil collagenases, enzymes involved in the breakdown of extracellular matrix. Thomson et al. (1999) demonstrated that not only neutrophil populations, but also the expression of neutrophil adhesion molecules, such as E-selectin, were increased in endothelium during labor. These data suggest that inflammatory mediators play a crucial role in neutrophil influx to facilitate parturition.

In the present study, the abundance of *neutrophil myeloperoxidase* and *elastase* was much less compared with *IL-6* and *IL-8*. This is not surprising because *IL-6* and *IL-8* are expressed by multiple cell types, whereas *myeloperoxidase* and *elastase* are only present in neutrophils, the relative quantities of which are very small. Despite the low abundance of *myeloperoxidase* and *elastase*, they nevertheless can serve as sensitive indicators of uterine inflammatory status. Indeed, abundance of *neutrophil elastase* was the only transcript investigated which was positively associated with uterine neutrophil infiltration on d 42, suggesting that uterine *neutrophil elastase* abundance could serve as a diagnostic marker for subclinical endometritis. We also observed some treatment effects on the abundance of these transcripts. Supplementation of yeast product at 30 and 60 g/d, but not 90 g/d, decreased uterine *IL-6* mRNA abundance. The abundance of *neutrophil myeloperoxidase* and *elastase* was linearly increased by treatments. Although several lines of evidence (Kim et al., 2011; Jensen et al., 2008; Zanello et al., 2011) indicate yeast or yeast culture may modulate inflammation, no previous research evaluated the effects of dietary yeast products on the inflammatory profiles of reproductive tissues. Although yeast product exerted inconsistent effects on different uterine inflammatory signals and the implications are unclear, our findings nevertheless indicate that

certain nutritional strategies are effective in modulating inflammation, and perhaps reproductive function.

To explore the relationships among reproductive disorders, uterine inflammation, and plasma markers, we examined the correlations among the incidence of retained placenta, uterine neutrophil populations, and plasma haptoglobin and BHBA. We found a positive link between d 7 plasma haptoglobin and d 7 uterine neutrophil populations, and that cows with retained placenta had greater neutrophil infiltration on both d 7 and 42. In addition, cows that experienced retained placenta had much greater concentrations of plasma haptoglobin and BHBA on d 7. In this study, all of the cows that had retained placenta (a total of 6) also developed metritis, consistent with previous reports of a direct link between these 2 conditions (LeBlanc, 2008). Previous studies (Skinner et al., 1991; Huzzey et al., 2009) have demonstrated that blood haptoglobin is a sensitive marker of uterine diseases. These results indicate that inflammation in local tissues (i.e. the reproductive tract) may lead to systemic inflammation and possibly impaired health. Consistent with our findings, Galvão et al. (2010) reported that cows that developed metritis had much greater concentrations of plasma haptoglobin and BHBA. Hammon et al. (2006) found that cows that developed metritis or subclinical endometritis had greater plasma BHBA and impaired blood neutrophil function in the transition period. Elevated BHBA is known to directly impair a number of functions of immune cells, including neutrophil migration, phagocytosis, and pathogen killing (Suriyasathaporn et al., 2000; Sartorelli et al., 1999). Furthermore, as a marker of energy deficiency, greater concentrations of plasma BHBA likely indicate more severe negative energy balance, which is known to suppress immune function (Kimura et al., 1999; Goff and Horst, 1997). Together, the resulting immune deficiency would in turn promote retained placenta (Kimura et al., 2002).

Together, these data suggest that yeast product supplementation enhanced humoral and mucosal immunity, and modulated uterine inflammation and mammary gland health in transition dairy cows. Although the 30 g/d dose was sufficient to elicit most of these responses, given the numerical differences observed in the majority of immune function measures, together with the production and metabolism responses reported in the companion paper (Yuan et al., 2014), it seems that 60 g/d provided the most benefits to transition dairy cows.

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Table 5-1 Primers used for RT-PCR detection of genes in the uterine tissue.

Gene	Accession Number ¹	Forward primer	Median Ct	Efficiency
		Reverse primer		
<i>IL-6</i>	X57317	AGGACGGATGCTTCCAATCTG GAAGACCAGCAGTGGTTCTGAT	26.6	105%
<i>IL-8</i>	NM-173925	GCTCTCTTGGCAGCTTTCCT GGCATCGAAGTTCTGTACTCATTCT	20.0	95%
<i>Myeloperoxidase</i>	XM_005219889.1	GCCCTGGAAC TTCAGAGAGAT GGGCTGGAGCATGATCAGAA	36.3	117%
<i>Elastase</i>	NM_001105653.1	TTGTCTGAACGGCCTGAACT AAGACCCTCCGGACTCTGAA	34.0	92%
<i>RPS9</i>	NM_001101152.2	GAACAAACGTGAGGTCTGGAGG TTACCTTCGAACAGACGCCG	17.1	103%
<i>RPS15</i>	NM_001024541.2	GGCGGAAGTGGAACAGAAGA GTAGCTGGTCGAGGTCTACG	16.2	106%

<i>β-actin</i>	NM_173979.3	ACGACATGGAGAAGATCTGG	15.6	94%
		ATCTGGGTCATCTTCTCACG		

¹ From NCBI Entrez Nucleotide Database (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide>).

Table 5-2 Hematology and leukocyte differential during the experimental period. Values are least squares means \pm SEM, n = 9-10.

	Treatment					<i>P</i> value		
Item	0g	30g	60g	90g	SEM	Yeast vs. Control	Linear	Quadratic
Hematology								
Leukocyte count, 10 ⁹ cells/L	12.0	11.2	12.0	10.9	1.17	0.63	0.64	0.91
Erythrocyte count, 10 ¹² cells/L	5.94	5.76	5.82	5.82	0.12	0.33	0.59	0.47
Hemoglobin, g/dL	10.3	10.1	10.3	9.9	0.15	0.39	0.20	0.38
Hematocrit, %	30.7	30.0	30.4	29.0	0.46	0.10	0.03	0.43
Platelet count, 10 ⁹ cells/L	204	260	264	243	25	0.04	0.09	0.27
Leukocyte concentrations, 10 ³ cells /μL								
Neutrophils	4.26	3.66	3.65	3.43	0.28	0.05	0.06	0.51
Lymphocytes	5.54	5.36	6.22	5.46	0.96	0.90	0.89	0.76
Monocytes	1.74	1.73	1.84	1.66	0.22	0.99	0.90	0.70
Eosinophils	0.37	0.31	0.32	0.36	0.05	0.58	0.97	0.35
Basophils	0.006	0.005	0.006	0.005	0.001	0.47	0.44	0.80

Leukocyte fraction, % of total

Neutrophils	37.7	33.9	30.4	33.3	3.2	0.17	0.25	0.30
Lymphocytes	44.4	46.8	50.9	47.7	3.5	0.32	0.38	0.43
Monocytes	14.5	15.9	15.8	14.4	1.2	0.53	0.97	0.26
Eosinophils	3.43	2.97	2.91	3.68	0.52	0.69	0.76	0.24
Basophils	0.05	0.05	0.05	0.05	0.01	0.98	0.81	0.66

Table 5-3 Hematology and leukocyte differential with significant day effect ($P < 0.05$) during the experimental period. Values are least squares means \pm SEM, n = 39-40.

	Day ¹							
Item	-21	-7	1	4	7	21	35	SEM
Hematology								
Leukocyte count, 10 ⁹ cells/L	11.3 ^{ab}	11.6 ^b	12.8 ^c	10.4 ^a	11.1 ^{ab}	11.5 ^b	12.1 ^{bc}	0.69
Erythrocyte count, 10 ¹² cells/L	6.10 ^d	6.07 ^{cd}	6.16 ^d	5.92 ^c	5.76 ^b	5.47 ^a	5.37 ^a	0.08
Hemoglobin, g/dL	10.7 ^d	10.6 ^d	10.8 ^d	10.3 ^c	10.1 ^b	9.5 ^a	9.2 ^a	0.13
Hematocrit, %	31.6 ^d	31.8 ^{de}	32.7 ^c	31.3 ^d	29.9 ^c	27.1 ^b	26.0 ^a	0.41
Platelet count, 10 ⁹ cells/L	243 ^b	180 ^a	213 ^{ab}	184 ^a	207 ^{ab}	349 ^c	364 ^c	23.5
Leukocyte concentrations, 10 ³ cells /μL								
Neutrophils	3.37 ^{ab}	4.19 ^c	4.92 ^d	2.76 ^a	3.16 ^{ab}	3.67 ^{bc}	4.17 ^c	0.28
Monocytes	1.13 ^a	1.42 ^a	1.87 ^b	2.10 ^b	1.84 ^b	1.95 ^b	1.92 ^b	0.15
Lymphocytes	6.22 ^c	5.53 ^{ab}	5.45 ^{ab}	5.27 ^a	5.68 ^b	5.63 ^{ab}	5.72 ^{ab}	0.51
Eosinophils	0.62 ^d	0.60 ^d	0.32 ^c	0.24 ^b	0.15 ^a	0.20 ^{ab}	0.27 ^{bc}	0.04

Leukocyte fraction, % of total								
Neutrophils	32.9 ^{bc}	36.6 ^{cd}	38.8 ^d	27.3 ^a	32.0 ^b	33.6 ^{bc}	35.5 ^{bd}	2.04
Lymphocytes	51.1 ^d	45.5 ^{ab}	43.2 ^a	49.3 ^{cd}	49.5 ^{cd}	47.5 ^{bc}	46.0 ^{ab}	4.22
Monocytes	10.3 ^a	12.3 ^a	14.9 ^b	20.2 ^c	16.9 ^b	16.3 ^b	15.2 ^b	0.97
Eosinophils	5.66 ^d	5.56 ^d	3.00 ^c	2.51 ^{bc}	1.58 ^a	2.00 ^{ab}	2.43 ^{ac}	0.41

¹ Days that do not share a common superscript are significantly different ($P < 0.05$). Pairwise differences were evaluated by the PDIFF option when the overall effect of day was significant.

Table 5-4 Incidence of health disorders during the experimental period.

Disorder ²	Treatment ¹			
	0g	30g	60g	90g
Ketosis	1	3	1	1
Retained Placenta	1	1	3	1
Metritis	3	1	3	1
Subclinical endometritis	1	3	2	2
Clinical mastitis	2	1	0	1
Displaced abomasum	0	1	0	1
≥ 1 event	5	4	4	4

¹ There were no treatment effects ($P > 0.10$) on the incidence of any disorders. Yeast product was supplemented at 0, 30, 60, or 90 g/d to transition dairy cows from 21 d before expected calving to 42 d after calving.

² Ketosis was recorded when the urine ketone dipstick test (Ketostix; Bayer Corp. Diagnostics Division, Elkhart, IN) detected acetoacetate > 80 mg/dL on any day or > 40 mg/dL for 2 consecutive days. Other health disorders were diagnosed according to the guidelines by Kelton et al. (1998).

Figure 5-1 Milk SCS during the experimental period. Yeast product was supplemented at 0, 30, 60, or 90 g/d to transition dairy cows from 21 d before expected calving to 42 d after calving. There was a week effect ($P < 0.01$), but no yeast product vs. control ($P = 0.25$), linear dose ($P = 0.53$), or quadratic dose ($P = 0.11$) effects. There was a treatment \times wk ($P < 0.01$) effect, reflecting a tendency for quadratic dose effect on wk 1 ($P = 0.08$), and a quadratic dose effect on wk 5 ($P = 0.02$). * indicates significant differences ($P < 0.05$), † indicates tendencies ($0.05 < P \leq 0.10$). Values are least squares means \pm SEM, n = 9-10.

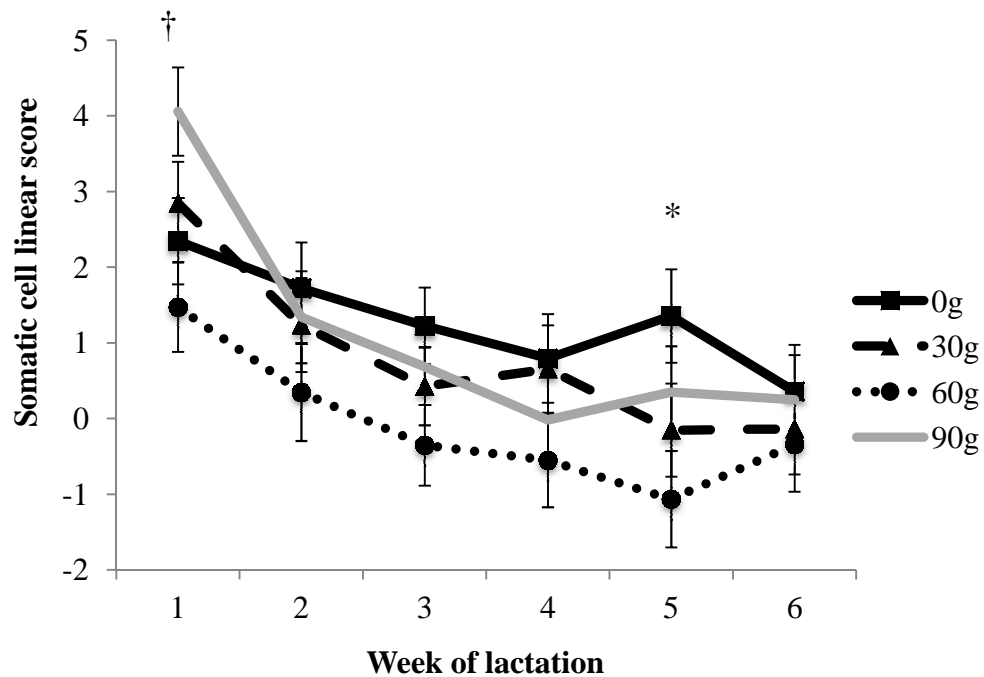


Figure 5-2 Concentrations of IgG in colostrum samples collected immediately after calving.

Yeast product was supplemented at 0, 30, 60, or 90 g/d to transition dairy cows from 21 d before expected calving to 42 d after calving. There were no yeast product vs. control ($P = 0.96$), linear dose ($P = 0.45$), or quadratic dose ($P = 0.39$) effects. Values are least squares means \pm SEM, $n = 8-10$.

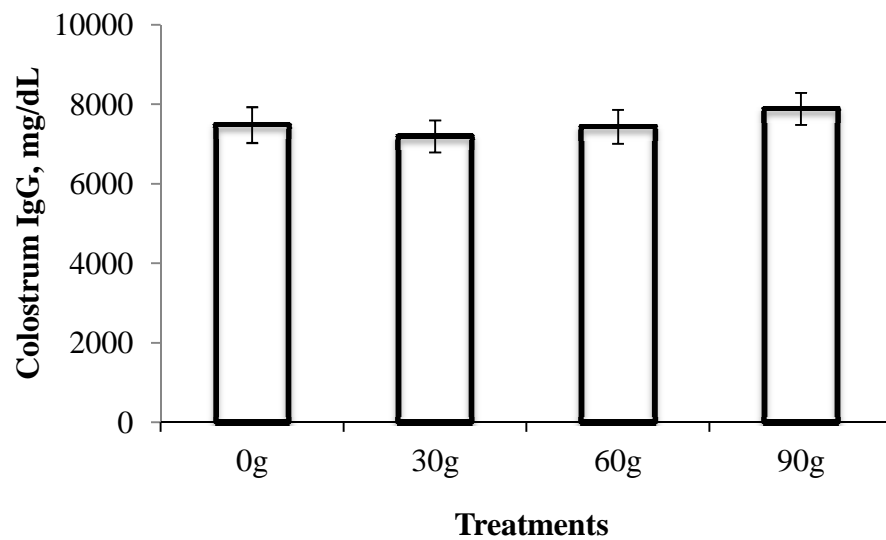


Figure 5-3 Concentrations of plasma haptoglobin during the experimental period. Yeast product was supplemented at 0, 30, 60, or 90 g/d to transition dairy cows from 21 d before expected calving to 42 d after calving. There was a day effect ($P < 0.01$), but no yeast product vs. control ($P = 0.15$), linear dose ($P = 0.28$), quadratic dose ($P = 0.35$), or treatment \times day ($P = 0.35$) effects. Values are least squares means \pm SEM, $n = 9-10$.

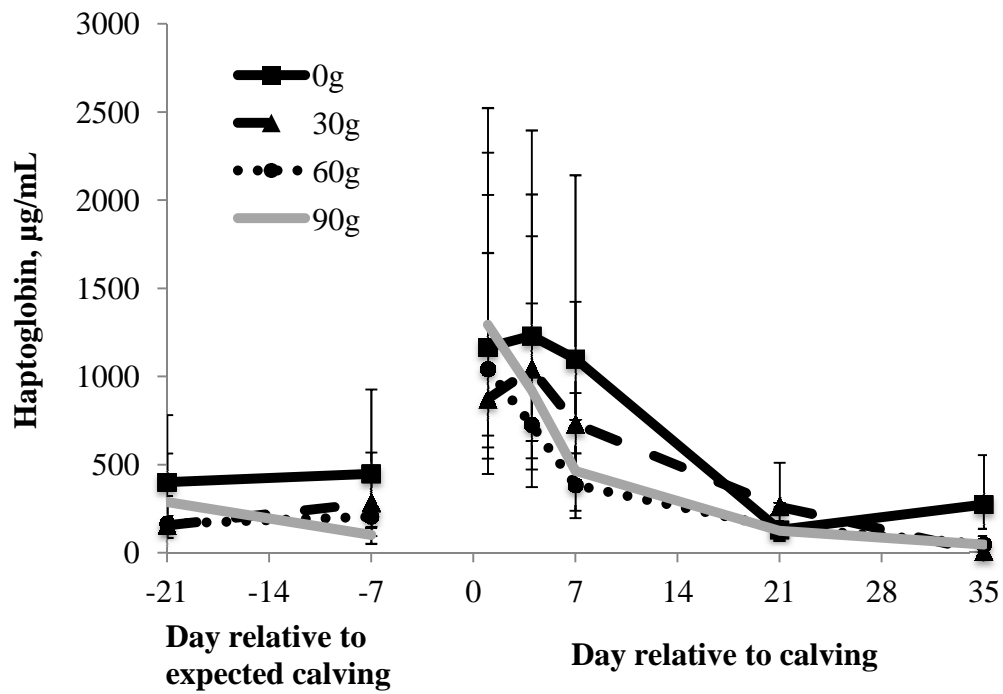


Figure 5-4 Killing capacity of heparinized whole blood when incubated for 5 min with an *E. coli* strain during the experimental period. Yeast product was supplemented at 0, 30, 60, or 90 g/d to transition dairy cows from 21 d before expected calving to 42 d after calving. There were no yeast product vs. control ($P = 0.28$), linear dose ($P = 0.30$), quadratic dose ($P = 0.40$), day ($P = 0.19$), or treatment \times day ($P = 0.99$) effects. Values are least squares means \pm SEM, $n = 9-10$.

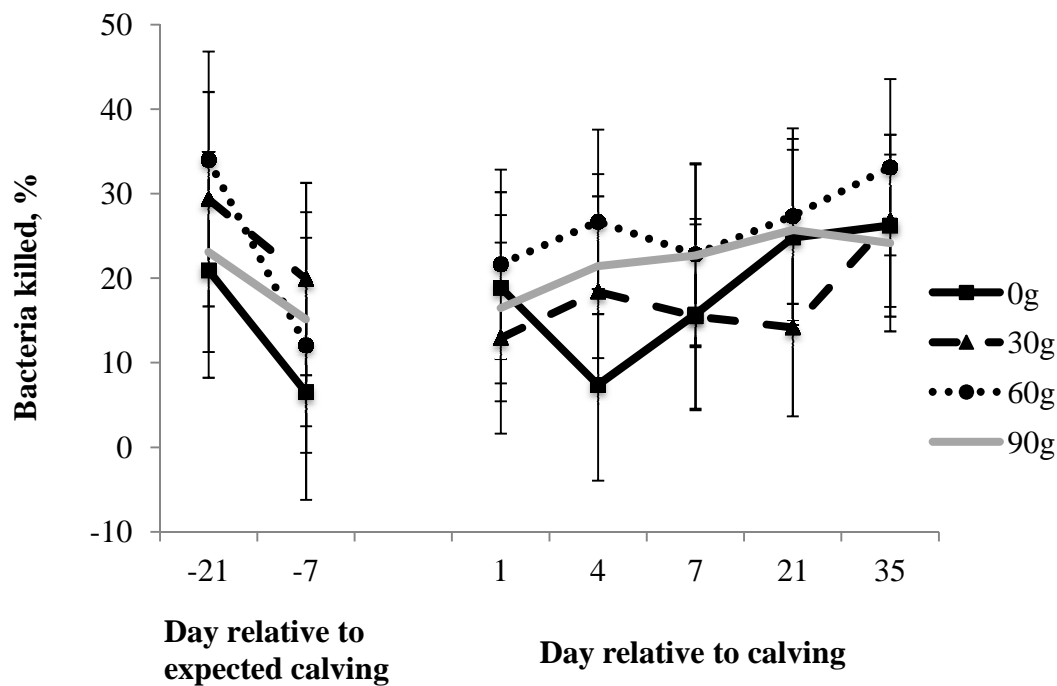


Figure 5-5 Plasma concentrations of anti-ovalbumin IgG collected on d -21, -14, and 21 relative to calving. Yeast product was supplemented at 0, 30, 60, or 90 g/d to transition dairy cows from 21 d before expected calving to 42 d after calving. Cows were challenged on d -21, -7, and 14 with an ovalbumin. There was a tendency for linear dose effect ($P = 0.06$) and a day effect ($P < 0.01$), but no yeast product vs. control ($P = 0.41$) or quadratic dose ($P = 0.50$) effects. There was a treatment \times day ($P < 0.01$) effect, reflecting that yeast product linearly increased ($P < 0.01$) anti-ovalbumin IgG on d 21. Values are least squares means \pm SEM, $n = 10$.

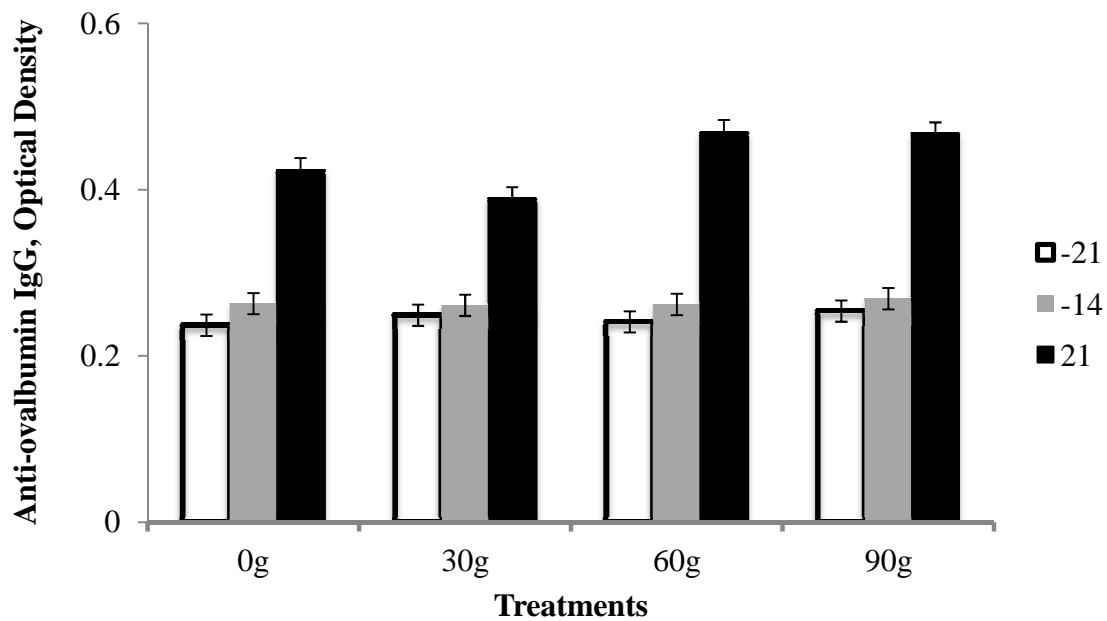


Figure 5-6 Concentrations of IgA in fecal samples collected on d 7 and 21 relative to calving. Yeast product was supplemented at 0, 30, 60, or 90 g/d to transition dairy cows from 21 d before expected calving to 42 d after calving. There was a significant quadratic dose effect ($P = 0.03$), but no yeast product vs. control ($P = 0.16$), linear dose ($P = 0.73$), day ($P = 0.61$), or treatment \times day ($P = 0.42$) effects. Values are least squares means \pm SEM, $n = 10$.

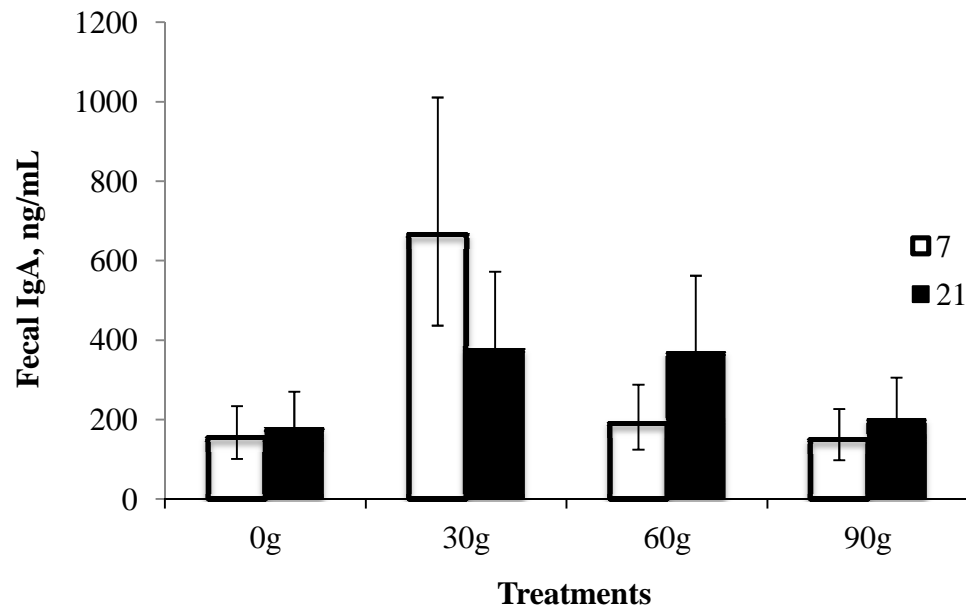


Figure 5-7 Neutrophil populations of uterine samples collected on d 7 and 42 relative to calving. Yeast product was supplemented at 0, 30, 60, or 90 g/d to transition dairy cows from 21 d before expected calving to 42 d after calving. (A) There were no yeast product vs. control ($P = 0.53$), linear dose ($P = 0.69$), quadratic dose ($P = 0.85$), or treatment \times day ($P = 0.75$) effects. (B) There was a day effect ($P < 0.01$). Values are least squares means \pm SEM, $n = 8-10$ (A) or 38 (B).

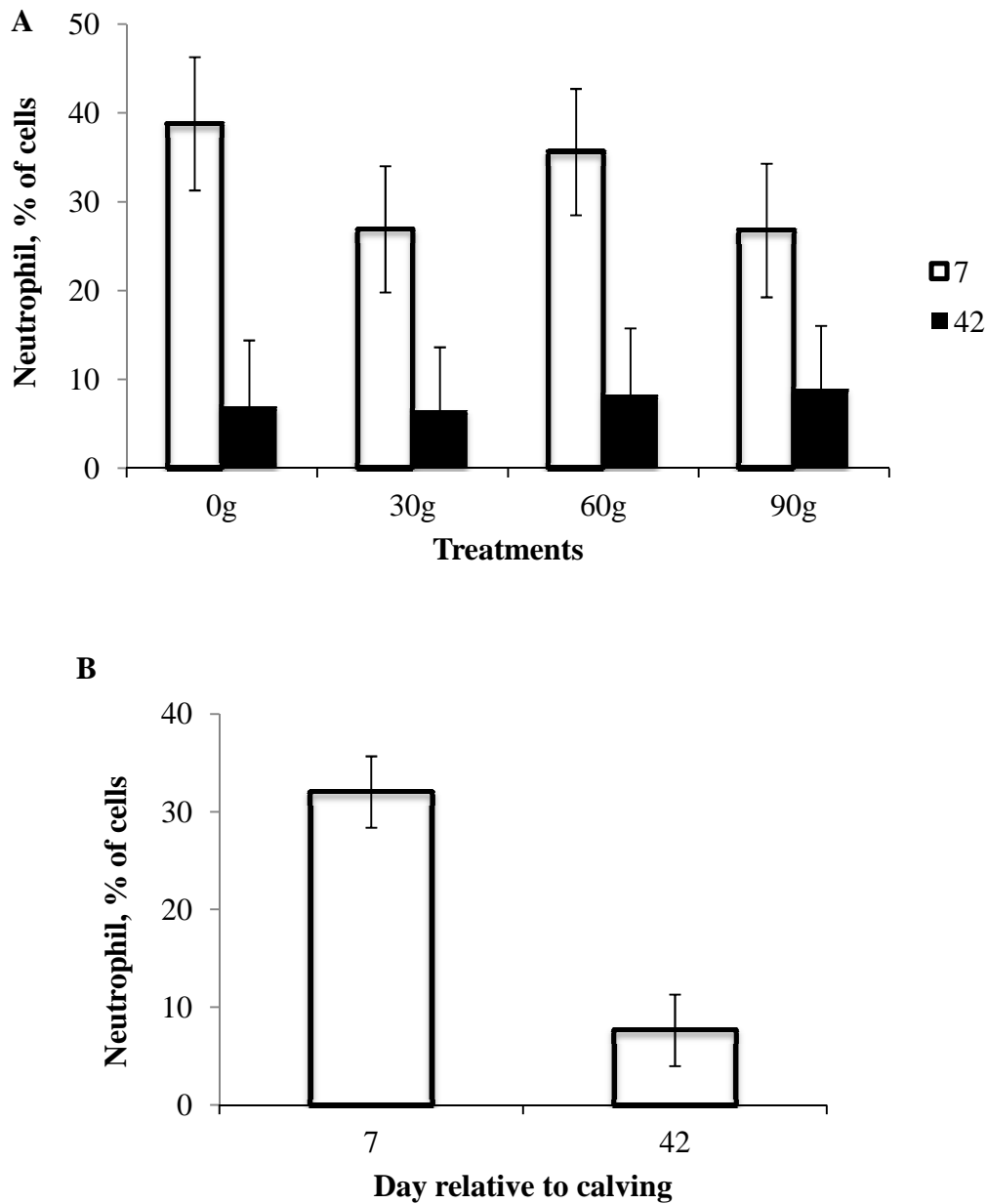
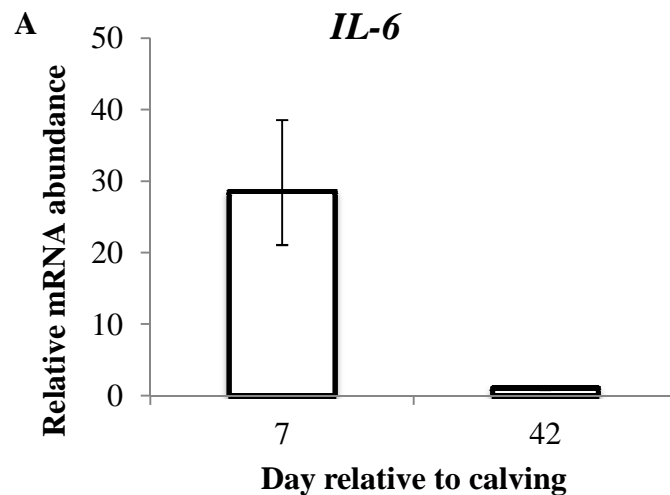
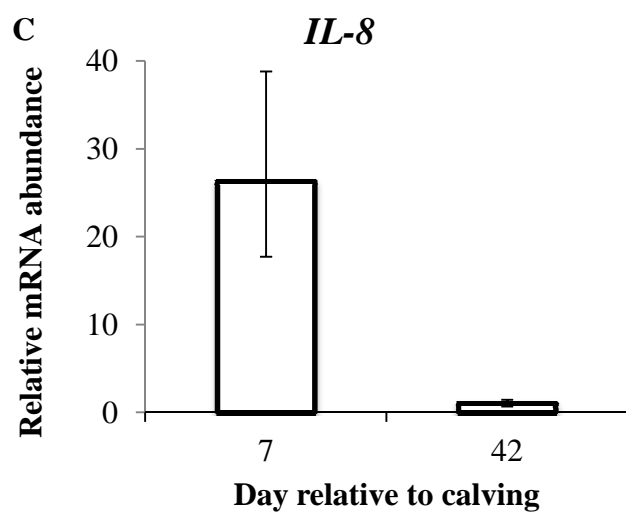
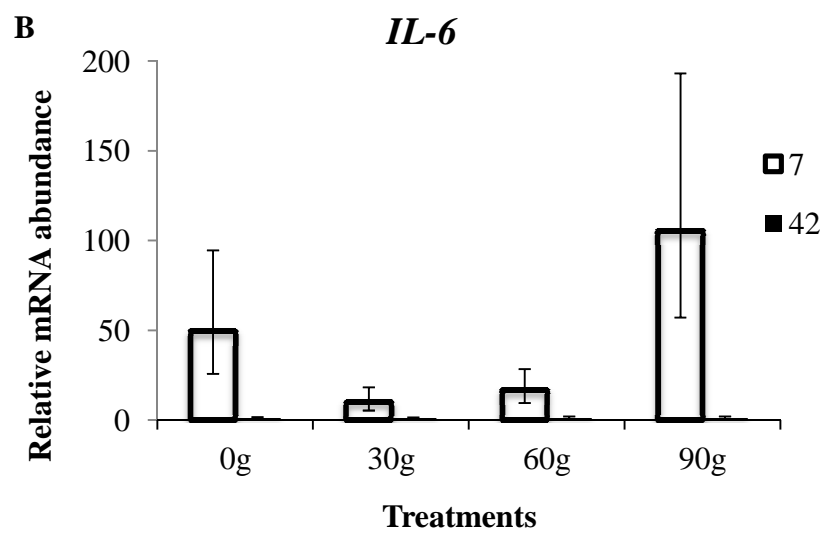
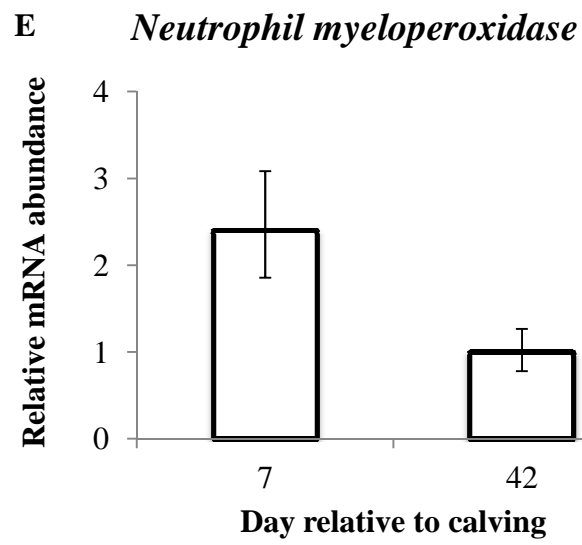
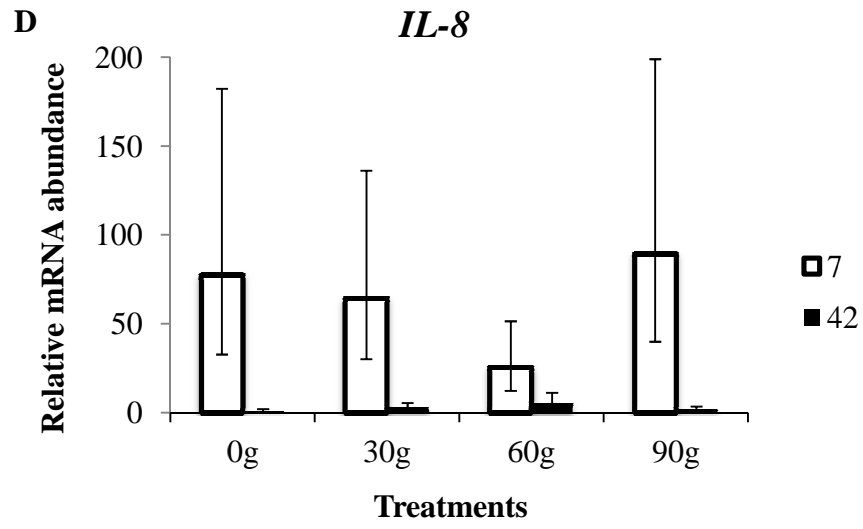
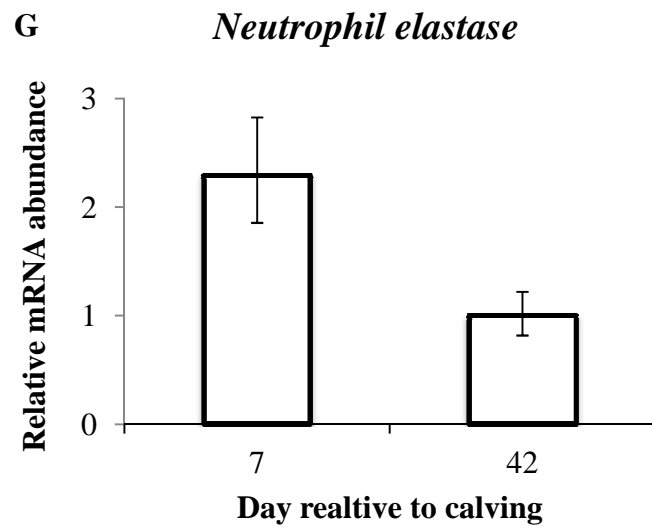
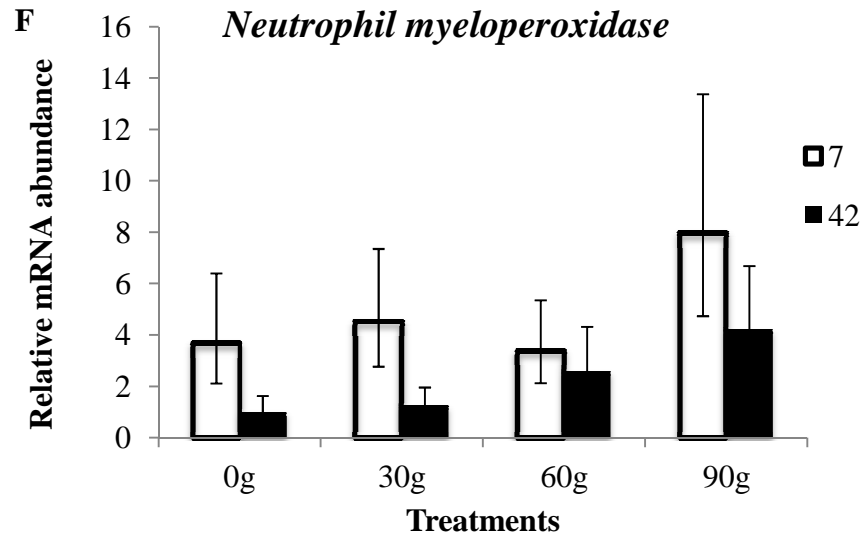


Figure 5-8 Relative mRNA abundance of inflammatory genes in uterine tissues collected on d 7 and 42 relative to calving. Yeast product was supplemented at 0, 30, 60, or 90 g/d to transition dairy cows from 21 d before expected calving to 42 d after calving. **(A-B)** *IL-6*: There were day ($P < 0.01$) and quadratic dose ($P = 0.02$) effects, but no yeast product vs. control ($P = 0.53$), linear dose ($P = 0.30$), or treatment \times day ($P = 0.32$) effects. **(C-D)** *IL-8*: There was a day effect ($P < 0.01$), but no yeast product vs. control ($P = 0.60$), linear dose ($P = 0.71$), quadratic dose ($P = 0.77$), or treatment \times day ($P = 0.35$) effects. **(E-F)** *Neutrophil myeloperoxidase*: There were day ($P = 0.01$) and linear dose ($P = 0.05$) effects, but no yeast product vs. control ($P = 0.20$), quadratic dose ($P = 0.54$), or treatment \times day ($P = 0.61$) effects. **(G-H)** *Neutrophil elastase*: There were day ($P = 0.01$), yeast product vs. control ($P = 0.01$), and linear dose ($P = 0.03$) effects, but no quadratic dose ($P = 0.25$) or treatment \times day ($P = 0.62$) effects. Values are least squares means \pm SEM, $n = 35-37$ (A, C, E, G) or 8-10 (B, D, F, H).









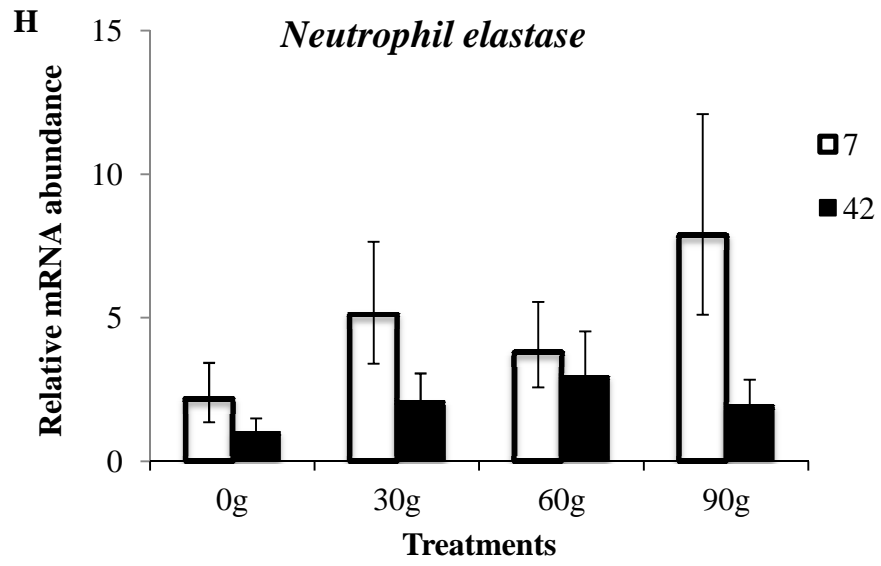


Figure 5-9 Correlation between plasma haptoglobin concentrations on d 7 and neutrophil populations of uterine samples collected on d 7. Yeast product was supplemented at 0, 30, 60, or 90 g/d to transition dairy cows from 21 d before expected calving to 42 d after calving. A significant correlation ($P < 0.01$, $R^2 = 0.23$) was detected. There were no treatment differences ($P > 0.10$) affecting this correlation.

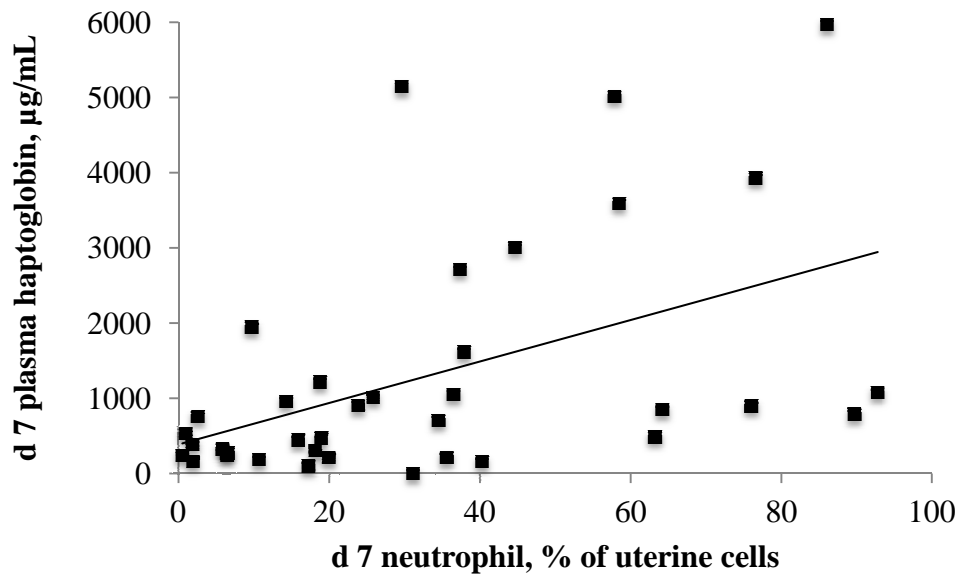
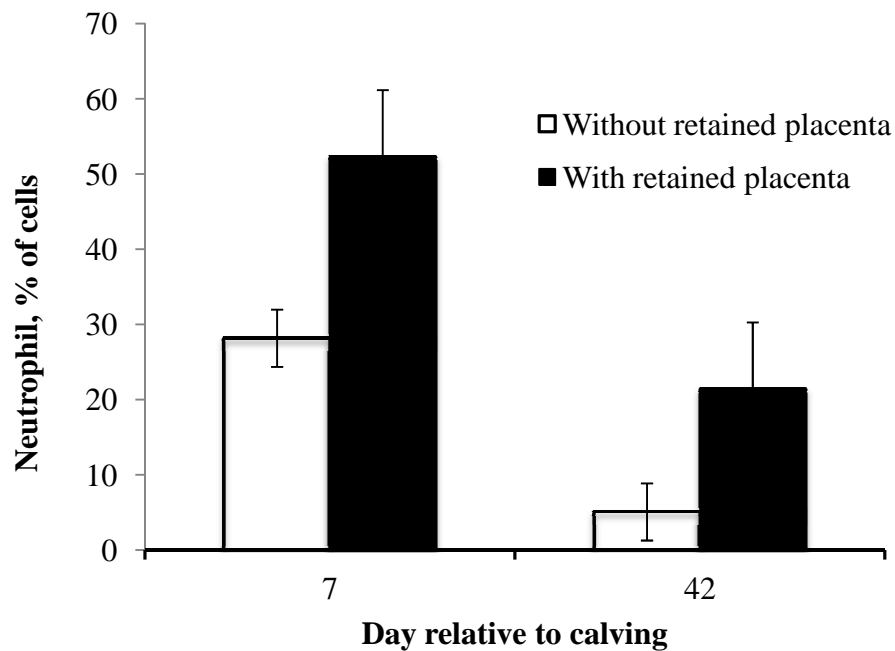


Figure 5-10 Comparison of neutrophil populations of uterine samples in cows with or without retained placenta. Yeast product was supplemented at 0, 30, 60, or 90 g/d to transition dairy cows from 21 d before expected calving to 42 d after calving. Across treatments, there were 6 out of 40 cows had retained placenta. Cows with retained placenta had greater ($P < 0.01$) neutrophil populations compared with cow without retained placenta. Values are least squares means \pm SEM, $n = 6$ (with retained placenta) or 32 (without retained placenta).



Chapter 6 - Overall Conclusions

The transition from late gestation to early lactation is often the most problematic period in the production cycle of dairy cows. Cows experience dramatic nutrient and energy requirements for milk production, tremendous metabolic stress, and suppressed immune function. Inadequate adaptation to these changes often contributes to markedly elevated incidence of metabolic disorders and infectious diseases during this time. Increasing evidence suggests that elevated inflammation is common during the transition period. Unlike the classical inflammation associated with acute infection or tissue injury, the postpartum inflammatory state is low-grade and intertwined with metabolic function. This metabolic inflammation plays a key role in numerous disorders. An improved understanding of inflammatory pathways in transition cows may improve our ability to predict and prevent disorders.

To mimic metabolic inflammation, we administered low amounts of a pro-inflammatory cytokine rbTNF α , to early lactation cows, and evaluated whether rbTNF α affects milk production, metabolism, and health (**Chapter 2**). We found that rbTNF α administration increased plasma concentrations of TNF α and haptoglobin, indicating increased systemic inflammation. Dry matter and water intake, milk yield, and milk fat and protein yields were all decreased by rbTNF α treatments by 15 to 18%. Administration of rbTNF α did not affect energy balance or markers of glucose and lipid metabolism, but increased the incidence of ketosis by 3-fold in the first week of lactation. These data suggest that low-grade inflammation may impair production independent of altering systemic metabolism. Conversely, modulating this inflammation has the potential to improve productivity and health of dairy cows.

To identify nutritional strategies that could modulate metabolism and immunity, we evaluated the efficacy of several feed supplements. In **Chapter 3**, we evaluated effects of

chromium propionate, rumen-protected lysine and methionine, or both on metabolism, immune cell function, and adipocyte size in lactating cows. Our results showed that feeding these supplements for 35 d had minimal effects on metabolism or adipocyte size, but modulated immune function in lactation cows. Neutrophil inflammatory responses to pathogen-associated molecules may be enhanced by chromium supplementation, and amino acids can also influence these responses in a parity-dependent manner.

Supplementation of yeast products has been shown to improve production and metabolism, and enhance immune function particularly during physiological challenges. In **Chapter 4**, we determined whether supplementation of a yeast product to transition cows could modulate production, feeding behavior, and metabolism. We found that yeast product supplementation did not affect milk production and DMI, but modulated feeding behavior and metabolism. Specifically, we detected quadratic dose effects for prepartum feeding behavior, reflecting decreased meal size, meal length, and intermeal interval, and increased meal frequency for cows received 30 and 60 g/d of yeast products. The increase in meals consumed per day and decreased meal size may result in more consistent feed intake and rumen fermentation patterns throughout the day. Although we found that treatments increased plasma BHBA, it is unlikely that yeast product had a direct effect on lipid metabolism. In **Chapter 5**, we found that yeast product supplementation modulated immunity and uterine inflammatory signals in transition dairy cows. As a humoral immunity indicator, plasma anti-ovalbumin IgG production in response to ovalbumin vaccinations was linearly increased by yeast product. Treatments quadratically increased fecal IgA concentrations, suggesting that 30 and 60 g/d doses enhanced mucosal immunity. We also observed that supplementation of yeast product at 30 and 60 g/d decreased uterine *IL-6* mRNA abundance, whereas the abundance of *neutrophil myeloperoxidase*

and *elastase* was linearly increased by treatments. Although yeast product exerted inconsistent effects on different uterine inflammatory signals and the implications are unclear, our findings nevertheless indicate that certain nutritional strategies are effective in modulating inflammation.

There are many intriguing questions that would be valuable to explore in future research. Does immunosuppression in the transition period contribute to elevated inflammation, or *vice versa*? What is the prevalence and duration of metabolic inflammation in transition cows? What are the most important sources of metabolic inflammation in dairy cows? How does metabolic inflammation increase the risk of certain transition cow disorders? Does metabolic inflammation directly affect mammary gland function and subsequently milk synthesis? Given the necessary role of inflammation in normal physiological function, should we inhibit inflammation in early lactation? How can we help cows to strike a balance to promote a healthy degree of inflammation while minimize the pathological aspects of inflammation? What are the mechanisms underlying the modulatory effects of nutrients (such as chromium and yeast products) on immunity? How to develop nutritional strategies that selectively inhibit excessive inflammation while enhancing immune function?

Overall, a greater understanding of the role of metabolic inflammation in the transition period and the nutritional strategies that could modulate these signals may improve the production and health of dairy cows.

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Chapter 2: TNF α altered inflammatory responses, impaired health and productivity, but did not affect glucose or lipid metabolism in early-lactation dairy cows

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Chapter 3: Effects of supplemental amino acids and chromium propionate on nutrient metabolism, neutrophil activation, and adipocyte size in dairy cows during peak lactation

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