Interaction Among Energy Status, Immune Status, Retinol-Binding Protein And Retinoids Status In Periparturient Dairy Cows

A Thesis

Presented in Partial Fulfillment for the Requirements of the

Degree of Master of Science

with a

Major of Animal Science

in the

College of Graduate Studies

University of Idaho

by

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April 2016

Authorization to Submit Thesis

This thesis of Kirk Christen Ramsey, submitted for the degree of Master of Science with a major in Animal Science and titled **"Interaction Among Energy Status, Immune status, Retinol-Binding Protein And Retinoids Status In Periparturient Dairy Cows,"** has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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Abstract

The objectives of this study were to determine the effect of feeding various amounts of dietary vitamin A (0 or 110 IU/kg BW), protein (10% or 12%), and an ionophore (monensin at 0 or 400 mg/d per head) on performance measures, retinoid metabolism, immune function, and metabolic response. Multiparous cows (n = 80) were studied from day -35 to +21 post-parturition. Milk samples were obtained and processed for components, somatic cell count (SCC), retinol, α-tocopherol, and β-carotene. Serum samples were collected and processed for NEFA, BHBA, haptoglobin, and Thiobarbituric acid reactive species (TBARS) and retinol binding protein. Neutrophils, peripheral blood mononuclear cells (PBMC), hepatic, and adipose tissue were used to measure gene expression of selected products related to inflammation and energy metabolism. Overall, these observations show that dietary vitamin A, monensin and protein affect retinoid have an effect on immune status without compromising performance measures.

Acknowledgments

Benjamin Franklin said “Either write something worth reading, or do something worth writing.” My sincere hope is that this project accomplished both, and my failure to acknowledge those who made this possible would be a complete disservice to the whole work. Dr. Pedram Rezamand has providing unceasing, patient, motivating, and kind mentorship. Without his patience on my behalf, and his willingness to provide me with this opportunity none of this would have been possible. My wife Rebecca, the one who had to deal with the real me, she has always been there to help me keep motivated, and remind me that there is light at the end of the tunnel. Her love and friendship is really an anchor to my life. Dr. Mark McGuire, and Dr. Larry Fox for being patient with me and their willingness to provide great lifelong mentorship. I have to acknowledge all the students that helped with this project through the year, Dr. Rezamands lab is always bustling with new students and so many were amazingly dedicated to the work. Cynthia Scholte and Chia-yu Tsia went from lab mates, to some of my very best friends, and they did so much, and worked so hard on my behalf. It is also important to recognize the two students that ran this project before me, Amanda Betker, and Jeff Blickenstaff. Elanco Animal Health, the United Dairymen of Idaho, and the University of Idaho College of Agriculture, and all others who provided funding and service on behalf of this project. Last of all, to recognize God’s role in my life in providing me with the abundant opportunities I have be given during my life.

Dedication

This thesis is dedicated to the families who tirelessly work to promote agriculture in the great state of Idaho.

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Introduction

Managing dairy cows during the transition period presents many challenges to the dairy industry. The transition period has been defined as 3 weeks prior to parturition to approximately 3 weeks after parturition (Drackley et al., 1999). It is estimated that several metabolic disorders and diseases a dairy cow may experience will occur during the transition period (Goff et al., 2002). These include ketosis, milk fever, retained placenta and displacement of the abomasum (Goff et al., 2002). It has also been shown that during this period, there is an impairment of the immune functions, which may lead to other diseases such as mastitis (Goff et al., 2002). Depressed dry matter intake (DMI) and severe metabolic changes are associated with diseases and metabolic disorders during the transition period.

The primary problem during the transition period is the massive increase in nutrient requirements in order to initiate and maintain lactation. This increase in nutrient requirements typically can't be met by nutrient intake. It has been demonstrated that at 4 d postpartum a healthy cow's intake of net energy and metabolizable protein only accounts for 65% and 75%, respectively, of her total requirements. Furthermore, calculated net energy and metabolizable protein utilization by the mammary gland accounted for 97% and 83%, respectively (Bell, 1995). In order to compensate for this increase in demand a cow must mobilize available body stores including adipose, and protein stores. The extent of this negative energy balance during the transition period may play a critical role in the success of future lactation performance.

During the transition period a significant decrease in immune function has been demonstrated by many researchers. The immune suppression has been linked to many

metabolic and hormonal factors. These results include; an increase in glucocorticoids around the time of calving (Burton et al., 1995), changes in estrogen and progesterone concentrations (Weber et al., 2001), extended negative energy balance has also been shown to play a role in the extent of immunosuppression. Others have demonstrated that major decreases in essential vitamins occur during this period. Vitamins such as vitamin A, vitamin E, and vitamin D have all been shown to be significantly lower at this time (Weiss, 1998; Sordillo, 1997).

Dietary factors have been heavily researched for many years to understand how improvements could be made to help bolster cattle health during the transition period. Many major advancements have been made in understanding the profile and metabolism of key vitamins such as vitamin A, β-carotene, and vitamin E. Vitamin A and its derivatives have been heavily researched to understand its role in this critical period. Retinol, the most abundant form of vitamin A, is essential to growth, vision, immunity, and epithelial cell differentiation and proliferation (Chew, 1987; Van Merris et al., 2004a). It has been shown however, that during the transition period plasma retinol concentrations decrease dramatically during the transition period and do not regain pre-partum levels until after 4 weeks postpartum (Chew, 1993).

Literature Review

Transition Cow Management

During the transition period cattle will have finished fetal development, prepared the mammary gland for lactation, delivered a calf, and initiate lactation. To accomplish these tasks significant strain will be imposed on her both physically and metabolically. Proper management and nutrition during this period are essential for obtaining health, reproductive capacity, and optimum milk production during the following lactation (Bachman et al., 2003). Most metabolic disorders occur during this time due to multiple compounding factors, and immunosuppression makes the transition dairy cow more susceptible to infections diseases such as mastitis.

Metabolic disorders and health

The main health related disorders and diseases common to transition dairy cattle are ketosis, displaced abomasum, milk fever, retained placenta, metritis, and mastitis. Each of these disorders can have devastating impacts on cattle health and future lactations. For example, Rajala-Schultz et al. (1999) found that during a 305 d lactation ketosis decreased milk yield by 535 kg for cows in 4 parity or greater. They also found that milk fever affected milk yield for a period of 6 wk post parturition. Wallace et al. (1996) studied the effect of health disorders during the transition period and concluded that cows with any health disorder around calving produced 7.2 kg less milk per day during the first 20 d postpartum when compared with healthy cows during the same period. It was also found that cows with metritis and retained placenta had a milk yield loss of 8.2 kg/d during this period. The projected 305 d 2x mature equivalent milk yield was 8573 kg for cows with displaced

abomasum and ketosis compared to 9426 kg for healthy cows. These disorders have significant and lasting effects on milk yield but the effects of health disorders during this period can also impact overall cattle performance including future reproductive success (Drackley, 1999). To manage health disorders during the transition period it is necessary to begin with proper dry period management.

Dry period

The length of the dry period is an essential element to proper management. Historically, a 50 - 60 d period is advised, which is critical for optimal production during the following lactation (Coppock et al., 1974, Sorenson and Eneveoldsen, 1991). In recent studies a window of 40 days or less has been suggested (Bachman, 2002). Regardless of the exact time period, it is crucial that the dry cow has proper time for mammary epithelial tissue differentiation and development (Bachman et al., 2003), and proper rumen development in preparation for the nutrient dense diet that will be required for optimal lactation postpartum. The dry period is typically divided evenly into two time periods, close up and far off. Far off and close up energy requirements are typically around 1.4 and 1.6 Mcal/kg DM respectively. Crude protein (CP) requirements also differ for far off and close up periods at 12.8% to 15.9% CP respectively.

Crude protein concentrations during the dry period have also been shown to have significant impact on mRNA expression of hepatic gluconeogenic enzymes in transition dairy cattle. The change in these enzymes impacts the transition dairy cows' ability to produce glucose in order to meet demands of the gravid uterus. It is estimated that the gravid uterus consumes 46% of the maternal daily glucose needs and lactation consumes 85% (Greenfeild et al.,

2000). Greenfeild et al. (2000) fed cattle varying levels of crude protein (CP) and rumen undegradable protein (RUP) from d 28 before calving to the day of calving and determined the mRNA gene expression of phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate carboxylase (PC). Pyruvate carboxylase and PEPCK are possible rate-limiting enzymes in hepatic gluconeogenesis from precursors that enter the gluconeogenic pathway (Lucas et al., 1990; Greenfeild et al., 2000). They concluded that prepartum dietary protein had little effect of gene expression of PC and PEPCK. Even though dietary crude protein played no effect it was observed that the abundance of PC mRNA increased 7.5 fold the day after calving when compared with -28 d before calving then declined by +28 DIM. Phosphoenolpyruvate carboxykinase abundance was unchanged during the day after calving but was increased at d 28 postpartum. These changes in gluconeogenesis during the transition period indicate an increased capacity for the production of glucose from amino acids, lactate and a greater capacity for fatty acid oxidation. The increased capacity for gluconeogenesis is crucial for successful lactation; however, it is often not sufficient to compensate for the demands placed on cattle during lactation.

Transitioning from pregnancy to lactation

One of the key factors in the health issues associated with the transition period is an increased nutrient demand for fetal development and milk production. Bell et al., (1995) estimated that the net energy required for a female cow at 250 d of gestation and the lactating mammary gland 4 d post-partum increases dramatically. They calculated a threefold increase in demand for glucose, and twofold increase in the demand for amino acids, and nearly a fivefold increase in the fatty acid requirements. This increase in nutrient demand typically cannot be met by the animal's dry matter intake (Overton and Waldron

2004) and must be compensated for by hepatic gluconeogenesis created by nonesterified fatty acid (NEFA), and amino acid mobilization from body reserves (Reynolds et al., 2003). Mobilized NEFA are taken up by the liver and either oxidized to ketone bodies, completely oxidized for energy, or re-esterified to form triacylglycerides (TG) (Drackley et al., 1999). Ruminants are relatively slow to export the newly esterified TG (Grummer, 1993) leading to accumulation of TG in liver. The lipid accumulation disrupts liver function further exacerbating the situation. Ketosis is often associated with fatty-liver disease and is typically diagnosed by the abundance of ketones, namely β-hydroxy buterate (BHB) in the blood. Mobilization of body stores are a direct result of the negative energy balance that occurs because of the increase in energy requirements from lactation that is not met by energy intake. The increase in negative energy balance places extreme stress on the body's ability to mount an immune response, which may provide evidence toward a decrease in immune function during the transition period (Kimura et al., 1999).

Metabolic Disorders

The transition period is often fraught with production diseases, diseases often associated with her inability to cope with significant increases in energy demands during this period. Production diseases include hypocalcemia, ketosis, displaced abomasum, retained placenta, laminitis, and mastitis (Mulligan et al., 2008). Although, many of these diseases can occur at any time during the lactation it is most common to see them occur during the first few days following parturition before cows reach their maximal milk production. Furthermore, the risk of disease is concurrent with a high rate of acceleration in milk yield (Ingvartsen, 2006).

Ketosis is a metabolic disorder characterized by abnormally high levels of ketone bodies in the blood. Ketone bodies such as acetoacetate, β-hydroxybutyrate, and acetone are elevated during periods of negative energy due to elevated lipid mobilization and low glucose concentrations (Drackley, 1999). Ketosis is often categorized as primary ketosis and secondary ketosis. Primary ketosis is known to occur as glucose demands exceed production by liver gluconeogenesis. This stimulates lipid mobilization from the body reserves and absorption of fatty acids in liver mitochondria. Glucogenic structures like oxaloacetate are low leading to the production of ketone bodies in blood, milk, and urine (Ingvartsen, 2006). Secondary ketosis is defined as ketosis occurring as a result of another disease. The disease often causes decreased feed intake leading to increased lipid mobilization, however, the etiology of primary and secondary ketosis is the same (Ingvartsen, 2006).

Displaced abomasum is a common disease to transition dairy cows. It occurs as gas builds up in the abomasum and dislocates to the right or the left. This dislocation leads to partial or total occlusion of the passageway for feed to the intestines (Shaver et al., 1997). Occurrence of displaced abomasum is typically a result of changes in the diet often from a high forage low concentration ration to a low forage high concentrate ration typical of an early lactation ration. Coppock et al., (1972) fed diets varying in forage percentage. Diets included 75, 60, 45, and 30% forage. Cows were fed from 4 wks prepartum through 4 wk postpartum. Left displaced abomasum incidences were 0, 16.7, 40, and 36% respectively. Displaced abomasum occurrence is highest in high-yielding dairy cows in early lactation, but is not limited to that group (Ingvartsen, 2006). Displaced abomasum is a multifaceted disease that is not fully understood. Other hypothesized factors leading to the displacement

include; increased abdominal space postpartum allowing easier abomasal movement, hypocalcemia leading to decreased abomasal contractility, and a dip in dry-matter intake in the immediate days pre and postpartum (Ingvartsen and Anderson, 2000; Constable et al., 1992; Massey et al., 1993).

Hypocalcemia is characterized by low or inadequate blood calcium concentrations leading to inability to rise to her feet due to Ca requirements in muscle and nerve function (Goff., 2008). Blood Ca levels for cattle are maintained around 2.1 and 2.5mmol/L, during parturition and early lactation Ca is shunted toward milk production dropping the concentration to well below 2.0mmol/L. Dairy cattle and most mammals are programed to mobilize Ca from bone in order to meet lactational Ca requirements. (Goff, 2008). This will result in bone loss of 9-13% in the first month of lactation (Ellenberger et al., 1932). Metabolic alkalosis predisposes cows to hypocalcemia and blunts the cows response to parathyroid hormone (Goff et al., 1991; Craige and Stoll, 1947). This lack in parathyroid hormone decreases the response of bone tissue decreasing mobilization of Ca into blood (Goff, 2008). Hypocalcemia has multiple effects beyond cattle mobility, hypocalcemia reduces feed intake, reduces teat sphincter function allowing bacterial invasion, and impairs immune cell response to an antigen (Goff, 2006; Kimura et al., 2006).

These diseases and the others mentioned are all critical players in the challenge dairy cattle face during the early lactation. Feed intake and metabolic adequacy are crucial to cattle health and a successful lactation.

Immune Suppression

Immune suppression is often seen at or around the time of calving. This suppression is not isolated to a single variable; rather it is observed in multiple cell types (Sordillo and Streicher, 2002). Cows become hyposensitive or hyporesponsive to antigens and are therefore more susceptible to infectious diseases such as mastitis (Mallard et al., 1998). In a study conducted by Wilson et al.(2004) that studied the effect of clinical mastitis on the lactation curve it was concluded that all cows, both primiparous or multiparous, were more likely to contract mastitis in the first wk post calving than at any other point along the lactation curve. It has also been shown that some metabolic disorders have been associated with mastitis. For instance, Curtis et al, (1985) showed that cows with that were diagnosed with milk fever were 8.1× more likely to contract mastitis. Although milk fever is a metabolic disorder rather than a disease there is other data suggesting that the metabolic state may play a role in the immune suppression. For instance, Burton et al. (1995) showed that both cortisol and dexamethasone dramatically down-regulated neutrophil L-selectin and CD18. Down-regulation of these molecules would decrease neutrophil emigration to the infected tissue. These results indicate that during the high stress transition period glucocorticoids may participate in the immune suppression observed during the transition period.

Dry Matter Intake

A depression in dry matter intake is a hallmark characteristic of the transition period, and the severity of depression plays a role that will determine success during milk production. On average, DMI decreases by 30% one to two d prior to calving and does not recover until one

to two d post parturition (Bertics et al., 1992). Although a dramatic drop in DMI occurs during those days prior and post calving, a significant decrease is manifested over the whole transition period (Bertics et al., 1992) The decrease in DMI can occur as early as 16 weeks prior to calving and does not recover to prepartum levels until nearly 6 d postpartum (Invartsen et al., 2000). The exact mechanism behind the drop in DMI is not fully understood; however, reticulo-rumen distention is generally considered the main site at which DMI is most often regulated. Intake is therefore limited to the passage rate of the feed (Allen, 2000). If distention and passage rate are key determinants of DMI then the concept of physical compression by a growing fetus and uterine size could likely play a role in the decrease in DMI during the transition period (Lagerlof, 1929). However, reticulo-rumen compression is not thought to be the only regulator of DMI. Ingvartsen et al, (2000) suggested that multiple factors affect DMI including fat mass, blood metabolites, corticosteroids, leptin, insulin, cytokines, and neuropeptides.

As discussed earlier, a decrease in DMI and severe negative energy balance during the transition period may be central to immune suppression and susceptibility to metabolic disorders. Therefore, it is safe to conclude that cows exhibiting less mastitis and greater overall health are less likely to go off feed and further exacerbate the complications associated with the transition period. Fat soluble vitamins such as vitamin A, E, and D are essential to physiological function and have received major interest in recent years (Weiss, 1998). Ensuring that these vitamins are present in rations and that they meet the requirements of cattle is becoming a necessity as cattle increase in productivity.

Innate Mammary Gland Immunity

The mammary gland is an essential organ to providing nourishment to neonates through milk. Milk provides nourishment and also disease resistance to offspring and is often a staple product to the human diet. Through genetic selection and technological improvements the modern milk cow‘s production has increased dramatically and far surpass the production rates of her predecessors. The mammary gland requires specific and innate immune factors to protect it from infection diseases and damage, without these factors injury and bacterial invasion would certainly lead to mastitis. Mastitis prevention and treatment is a billion dollar industry and crucial the health of the cow and her success productively. It is estimated by the National Mastitis Council that mastitis affects one-third of all dairy cow (Sordillo et al., 1997). Extensive research has provided further insight into understanding the immunobiology of the mammary gland and how to help protect the gland from infection, but more understanding is needed.

Mastitis occurs as a result of tissue damage from injury or when bacterial invasion ensues. The mammary gland has many defensive strategies to protect against invasion and infection using both innate and adaptive immune responses to keep infection at bay. The innate response uses the physical barriers of the teat, along with macrophage, neutrophils, and other portions of the innate response to recognize and destroy bacterial invasion at the first response. Adaptive immunity is acquired through the priming of naïve immune cells which instills memory and makes antibodies making an adaptive response much faster upon future invasion (Sordillo et al., 1997).

Bacterial invasion typically occurs as bacteria enter the mammary gland through the teat end into the teat canal. This canal leads to both mammary lumens and ducts where mammary epithelial cells are producing milk. To combat bacterial invasion the teat has developed certain anatomical features in order to protect from invasion. This includes a teat sphincter at the opening of the teat canal which closes post milking. Also, the teat canal is also lined with keratin that entraps invading bacteria and has bacteriostatic properties (Treece et al., 1966). Each of these mechanisms provides initial protection from invasion. If the bacterial invasion overwhelms the physical barriers, bacteria are met with a warm, moist environment, and milk is a nutritious product that allows for optimal bacterial growth. Macrophages are the predominant cell type found in mammary tissue and milk in both lactating and non-lactating animals (Lee et al., 2009). Macrophages and other sentinel cells react to bacterial invasion by signaling the response of neutrophils to the sight of infection. They also act as phagocytic cells and ingest bacteria, cellular debris, and accumulated milk products (Sordillo et al., 1988). Macrophages recognize pathogen associated molecular patterns (PAMPS) and damage associated molecular patterns (DAMPS) and produce cytokines, prostaglandins, and leukotrienes that signal neutrophil migration and chemotaxis, and induce an inflammatory response (Bianchi, 2007; Kehrli et al., 1999). Macrophages utilize phagocytosis, proteases, and reactive oxidative species (ROS) to destroy pathogens, however in the mammary gland macrophage numbers tend to decrease during inflammation and possess less phagocytic ability when compared to neutrophils (Niemialtowski et al., 1988). Macrophages also are important for the development of the adaptive immune response through antigen processing and presenting via MHC II complex to lymphocytes (Fitzpatrick et al., 1992)

Neutrophils circulate in the blood and are found in the bovine in concentrations over 100 billion circulating, mature cells (Paape et al., 1979). Neutrophils respond to cytokines produced by macrophages and endothelial cells at the sight of infection such as interleukin 8 (IL-8). IL-8 is a chemotactic factor that binds to a receptor on neutrophils in order to recruit neutrophils toward the sight of infection (Bergin et al., 2010). In order to migrate toward the infected mammary tissue neutrophils express a series of cell surface receptors known as L-selectin that bind to ligands on endothelial cells and facilitate entry into the tissue (Wang et al., 1997). However, during times of parturition is has been shown that bovine L-selectin may be shed from neutrophil surface potentially compromising neutrophil function during early lactation (Lee and Kehrli, 1998). Burton and Kehrli (1995) showed that cows treated with cortisol and dexamethasone had decreased L-selectin expression in milk neutrophils which lead to an increased bacterial infection during experimental mastitis challenge. Regardless, L-selectin is a critical component for neutrophil migration through blood endothelial cells and into the mammary tissue.

The mammary gland has developed many factors to help protect from bacterial invasion. However, during the transition period these factors maybe suboptimal (Aitken et al., 2001a). Although activation of the innate immune system to induce inflammation during mastitis is critical, aggressive inflammation during this period may cause undue damage to the host and add significantly to the disease (Sordillo, 2005). As discussed earlier, neutrophil migration occurs in a response to cytokine release such as tumor necrosis factor alpha (TNG-α), IL-8, and IL-4 (Aitken et al., 2011a). At the site of infection leukocytes such as neutrophils and macrophages phagocytize the pathogen and destroy it utilizing reactive oxidative species (ROS) (Sordillo and Aitken, 2009). In severe mastitis, activation of the immune system is

very significant resulting in massive migration of neutrophils to the sight of infection. This may lead to uncontrolled recruitment of leukocytes leading to toxic levels of cytokines, ROS production, and other mediators that can damage milk synthesizing tissue, compromise vascular health, or death may ensue (Aitken et al., 2011a). Vascular damage may result from excessive exposure of the endothelium to TNF-α and Intracellular adhesion molecule (ICAM) resulting from enhanced leukocyte migration, increased ROS production, and nitrogen species production, potentially leading to cellular apoptosis and can contribute to further and potentially an excessive inflammatory response (Madge and Pober, 2001, Aitken et al., 2011b). Maintaining adequate ROS production to decrease pathogenic damage, but also reducing ROS production to maintain vascular health is critical in maintaining mammary gland inflammation (Sordillo et al., 2009). The presence of vitamins A and E will ameliorate potential damage induced by ROS (Weiss et al., 1997).

Retinoids

Retinol is the most abundant form of retinoids in blood circulation. Retinol is a long chain unsaturated alcohol form of vitamin A that is essential to growth, vision, immunity, and epithelial cell differentiation (Chew, 1987; Van Merris et al., 2004a). This fat soluble vitamin is typically found in three forms; retinol, renitaldehyde, and retinoic acid. Vitamin A consists of a hydrophobic head, a conjugated isoprenoid side chain, and a polar terminal group. The terminal group can be oxidized to a carboxylic group to yield retinoic acid or modified to yield retinal (Chew, 1987). Green plants typically contain little to no vitamin A, rather, they contain carotenoids the most abundant being β- carotene. These carotenoids are precursors to vitamin A in the body. β-carotene is converted to retinol in the intestinal mucosa by a series of enzymes. Not all β-carotene is however converted to retinol, in many

species including the bovine and human β-carotene can be absorbed and stored in large quantities in adipose tissue (Chew 1987). Retinyl-esters (RE) and carotenoids are hydrolyzed first to retinol in the intestinal wall then absorbed into the mucosal cell. In the mucosal cell, retinol is re-esterified back to RE and carotenoids and then incorporated into chylomicrons and transported in the lymphatic system. These RE are taken up by the liver and stored in parenchymal cells and liver adipose cells. The stored RE can then by mobilized in the form of retinol by a specific protein called retinol binding protein (RBP). RBP is responsible for the transport of retinol to extrahepatic tissue throughout the body (Ross, 1993).

During the transition period, plamsa concentrations of retinol gradually decrease as parturition nears and plummets at parturition (Goff et al., 2002). Plamsa concentrations of retinol may not return to normal concentrations until more than 4 wk postpartum (Chew et al., 1982). In a report by Goff et al., (2002) in which the effects of the mammary gland on plasma vitamin A concentrations were studies, mastecomized cows (n=10) observed no significant decrease in plasma vitamin A concentrations during the transition period, in contrast to the non-mastectomized cows (n=8) whose plasma vitamin A concentrations decreased dramatically. These results highlight the severe demand placed on circulating vitamin A status during periparturient period as vitmain A is shunted toward colostrum and milk production. The decrease in plasma vitamin A concentration can also be attributed to feeding lower quality forages (Michal et al., 1990) and reduced feed intake during the last 2 wk prior to parturition (Grummer, 1995).

Vitamin A has been shown to be extensively involved in normal immune function in both human and bovine subjects. In human medicine it has been noted that serum concentrations

of retinol are dramatically decreased during an acute phase inflammatory response, and that the greater the severity of infection the greater the decrease in serum retinol concentrations (Mitra et al., 1998). Vitamin A involvement in immunity is very extensive but starts at the barrier, the skin. Vitamin A deficiency has been shown to compromise mucosal epithelial barriers found in the conjunctiva of the eye, along with barriers in the gastrointestinal, urogenital, and respiratory tract (Stephensen, 2001). This loss in mucosal cell linings reduces the resistance to infectious pathogens that would be typically trapped in and removed by mucosal linings (Stephensen 2001). Vitamin A deficiency has also been associated with decreased neutrophil and macrophage function. In rats, vitamin A deficiency disrupts normal neutrophil development and can result in a decrease phagocytosis and killing of bacteria (Twining et al., 1997). In transition dairy cattle, it has also been shown that supplemental vitamin A had a significant effect on lymphocytes proliferation and increased phagocytosis and intracellular killing indices by milk PMN against *Staphylococcus aureus* (Daniel et al., 1986; Tjoelker et al., 1986). Similar results have also been observed using the rat model, Weidermann et al (1996) concluded that vitamin A deficiency predisposes subjects to *Staphylococcus aureus* infection. These results indicated a decrease in phagocytic activity and bacterial killing of peritoneal macrophage for *Staphylococcus aureus*. One of the main reasons neutrophil function might be diminished during vitamin A deficiency is that neutrophils and other granulocytes develop from myeloid stem cells in the bone marrow that are mediated by retinoic acid binding to retinoic acid receptor in order to stimulate gene expression (Lawson et al., 1999).

Mastitis is an extremely challenging disease common to dairy cattle in the early postpartum period. Early studies have shown that during this time increasing dietary retinol and β-

carotene intake resulted in a decrease in IMI incidence and severity (Chew et al., 1982; Chew and Johnston, 1985; Dahlquist and Chew, 1985). A study by Johnston and Chew (1985) concluded that by feeding a diet supplemented with retinol (173,000 IU/d) from wk 2 to 8 cows had lower milk somatic cell count (SCC) as compared with control cows only fed (53,000 IU/d). In a later study by Daniel et al. (1986), it was concluded that diets fed retinol or β-carotene enhanced milk PMN killing and phagocytosis. In contrast, Oldham et al. (1991) fed three groups of cattle from 2 wk before dry off and ended on the 6 week of lactation. Groups were fed supplemental retinol at 50,000 IU/d, 170,000IU/d, or 50,000 IU/d plus 300 mg β-carotene/d. Results concluded that there was no beneficial effect on immune function or SCC by feeding these diets. The disparity in this data set leads one to question the efficacy of supplemental retinol during the transition period in alleviating mastitis incidence. However, most reports indicate that cattle receiving adequate vitamin A had better health and production.

Retinol transport in circulation is contingent upon a single binding and transport protein known as retinol binding protein (RBP) which carries it to tissues in the body (Heller, 1975). Without RBP retinol will not reach tissue thus lead to a retinol deficiency (Eldaim et al., 2009).

Retinol Binding Protein

Retinol binding protein (RBP) is a relatively small (21kDalton) single polypeptide chain with one binding site for retinol. RBP is primarily produced in the rough endoplasmic reticulum of hepatocytes in the liver (Heller, 1975b, Nonnecke et al., 2001). Retinol binding protein is the main transport mechanism by which retinol is transported in circulation.

Plasma retinol is bound to RBP and transthyretin (TTR) in the form of *all-trans* retinoic acid (Heller 1975a).

Plasma RBP status greatly fluctuates during the transition period. It has been demonstrated that plasma RBP declines immediately following parturition and begins to rebound following partition (Abd Eldaim et al., 2010). Plasma RBP has also been shown to be dependent on plasma retinol in that a decrease in plasma retinol is associated with a decrease in plasma RBP status (Goodman, 1980; Rask et al., 1980). Also, Lindberg et al; (1999) demonstrated that export proteins such as RBP are dependent upon amino acid availability. Using 14 multiparous and 16 primiparous Ayrshire dairy cows fed varying levels of CP during the late dry period until parturition authors (Linberg et al., 1999) concluded that protein supplement had a positive effect on synthesis and release of RBP from the liver.

In addition to RBP's function as the main transport system for retinol, it has also been shown to be involved in many metabolically important pathways including glucose metabolism, insulin resistance, and inflammatory processes (Graham et al., 2006; Yang et al., 2005). The acute inflammatory response has been associated with a decrease in plasma retinol concentration along with its transporters RBP and TTR (Rosales et al., 1996). Rosales et al. (1996) induced an inflammatory response using lipopolysaccharide (LPS) in rats adequate in vitamin A status. At 24 h post-treatment, plasma retinol was significantly lower in LPS treated rats as compared with control rats. This result was similar for plasma, liver, and kidney RBP and TTR concentration after LPS injection. It was concluded that the reduction in plasma retinol concentration was related to a reduction of hepatic synthesis of RBP and secretion of the retinol-RBP complex and that plasma retinol concentration is a poor indicator of vitamin A status during inflammation (Rosales et al., 1996). Furthermore,

Rezamand et al. (2007) studied the effect of new intramammary infection (IMI) and the impact on plasma vitamin concentration and other acute phase proteins. Authors (Rezamand et al., 2007) concluded that cows with a new IMI had significantly lower plasma RBP that cows without a new IMI. It has also been reported that RBP and TTR concentration are reduced in cows in hyperketonemia and fatty-liver disease (Grohn and Lindberg, 1985; Murata et al., 2004). Each of these results may help explain the reduction of plasma retinol concentration during the transition period and the negative impact associated with a decrease in plasma retinol.

In human and murine studies RBP has been heavily researched in type 2 diabetic patients. Type 2 diabetes is caused by a resistance to insulin leading to failure of pancreatic beta cells to produce insulin to compensate for hyperglycemia (Graham et al., 2006). In type two diabetics insulin sensitive glucose transporters (GLUT4) are down regulated in adipose tissue, but not is skeletal muscle (Yang et al., 2005). Multiple studies in mice and humans have shown an increase in serum RBP leading to insulin resistance (Yang et al., 2005; Wang et al., 2000). In a studied published in 2005 by Yang et al. it was concluded that RBP is elevated in obese and insulin-resistant mice. The authors also concluded that serum RBP might be a link to why GLUT4 transporters in adipose tissue are down regulated.

Monensin

Monensin is a carboxylic polyether produced by *Streptomyces cinnamonensis* and works by creating a flux of ions across bacterial cell membranes (Russell et al., 2003). Monensin creates an efflux of cellular potassium and an influx of hydrogen, this increase in hydrogen ion concentration must be removed in order to maintain cellular equilibrium. This is

accomplished either by the expenditure of cellular adenosine triphosphate though active transport or via passive transport with cellular sodium. This increase in energy expenditure results in reduced bacterial growth or death of the bacterium (Bergen et al., 1984). Monensin's effects act on bacterial cell membranes, therefore, gram negative bacteria tend to be more resistant to the effects of monensin than gram positive bacteria. This is due to the more complex outer membrane on gram negative bacteria thus shifting rumen microbial populations (Duffeild et al., 2000).

The shift in microbial populations is accountable for the effects of monensin on energy metabolism in the rumen. Monensin increases propionic acid in the rumen and decreases both butyric and propionic acids thus improving gluconeogenesis (Schelling, 1984). Mullins et al. (2012) studied the effects of monensin in transition dairy cows by feeding (400mg/cow daily) thirty-two multiparous transition cows starting 21 d prepartum to 21 d postpartum. Authors reported that treatment cows had significantly lower BHBA over the course of the study than the control group. It was reported however, that monensin did not significantly affect plasma glucose, insulin or NEFA concentrations. Others have also reported similar results regarding serum glucose concentrations (Arieli et al., 2001). These results are however, in contrast to what was reported in a meta-analysis by Duffield et al. (2008a). Results indicated a significant decrease in serum BHBA and NEFA, and an increase in serum glucose concentration (Duffield et al., 2008a). No significant difference however, was detected on insulin concentration across the studies. These results collectively highlight the efficacy of monensin on decreasing the severity of negative energy balance during the transition period. Monensin and other ionophores have a significant impact on dry matter

intake, rumen volatile fatty acid production, gluconeogenesis, rumen methane production, and even acting as a coccidiostat (Duffeild et al., 2000).

Monensin has been shown to increase rumen propionate production (Prange et al., 1978), and rumen propionate accounts for 50 to 60% of total glucose flux in dairy cows (Reynolds, 1988). This may be explained by monensin's affect in gene expression of specific gluconeogenic enzymes during the transition period. Karcher et al. (2007) studied the effects of prepartum feeding of monensin on gene expression of liver PC and PEPCK in transition dairy cows. The authors concluded that expression of PC was not altered because of monensin; however, PEPCK mRNA abundance was significantly greater at calving with prepartum monensin feeding (Karcher et al., 2007). During gluconeogenesis, carbon from propionate, lactate, and amino acids are metabolized through PEPCK. It has been demonstrated that the activity of PEPCK is greater in lactating dairy cows than in pregnant and non-pregnant cows (Mesbah and Baldwin, 1983). The increase in abundance of PEPCK mRNA in the liver of transition dairy cows because of monensin feeding could provide extensive help in mitigating the effects of negative energy balance.

Monensin has also been shown to have potential effects on the immune system in its ability to decrease serum haptoglobin in lactating dairy cows (Crawford et al., 2004). Haptoglobin is an acute phase protein that is elevated during inflammation and has been used as a potential biomarker for determining inflammation in cattle. An increase in serum haptoglobin has been associated with mastitis, metritis, and retained placenta in lactating dairy cattle (Crawford et al., 2004). Haptoglobin has also been shown to be increased in feedlot cattle during respiratory infection (Young et al., 1996).

Haptoglobin is expressed in hepatocytes, and functions to bind hemoglobin to form a complex, which enhances hemoglobin peroxidase and reduces the loss of hemoglobin and iron after the destruction of erythrocytes (Langlois and Delanghe, 1996). Hepatic haptoglobin synthesis is activated by cytokines such as TNF-α resulting in an increase in haptoglobin concentrations during an inflammatory event (Murata et al., 2004).

Crawford et al. (2004) collected sera from 897 lactating dairy cows from wk 1 to wk 6 postpartum. Cows received either a controlled release capsule containing monensin or a capsule placebo at approximately 3 wks prior to parturition. Results indicated that at week 1 serum haptoglobin was increased in cows with mastitis, metritis, and retained placenta. Authors concluded that controlled release capsule of monensin decreased haptoglobin concentrations in diseased heifers, and tended to decrease haptoglobin concentrations in non-diseased cattle at week 6. These results indicate a potential influence of monensin on inflammation.

Hypothesis and Objectives

Hypothesis:

Prepartum dietary vitamin A, protein, and monensin will have an effect on immune function measures, milk somatic cell, and milk retinol concentrations without negatively impacting production measures and metabolic response. Vitamin A, protein, and monensin will increase serum retinol binding protein (RBP) concentration, and increase RBP gene expression in both hepatic and adipose tissue.

Objectives:

- Determine the effect of feeding various amounts of dietary vitamin A (0 or 110 IU/kg BW), protein (10% or 12%), and an ionophore (monensin at 0 or 400 mg/d per head) prepartum, on performance measures, retinoid metabolism, immune function, and metabolic response.

- Determine the interaction of vitamin A, an ionophore (monensin), and crude protein on RBP metabolism and production.

- Understand the interaction among dietary treatments on performance measure, retinoid metabolism, immune function, and metabolic response.

Methods and Materials

Eighty multiparous Holstein cows were monitored from day -35 relative to expected calving date to day 21 post calving. Cows were assigned to a treatment group in a 2×2×2 factorial arrangement of treatments in a completely randomized block design. Starting at -35 d relative to expected calving date animals were fed twice daily one of eight dry cow ration combinations. The rations had two protein contents (10% or 12%), each with vitamin A (0 or 110 IU/kg BW), and monensin (0 or 400 mg/d per head). After parturition all cows were placed on an identical lactation ration containing 16% crude protein, no monensin, and 72000 IU/head per day of vitamin A in the form of a liquid supplement. The control was designated as low protein, no monensin, no vitamin A. Ninety four total multiparous cows were used during the research period; however 14 were removed due to severe metabolic disorders, or extreme calving difficulty.

Daily dry matter intakes were recorded, along with periodic body weights and body condition scoring. Liver samples were taken between the 12^{th} and 13^{th} rib and adipose tissue samples were taken near the tail head of the animal on days -35, -3, +3, +10, and +21. Tissue samples were immediately frozen in liquid nitrogen and stored at -80˚C until processing. Median coccygeal venipuncture blood samples were collected on days -35, -21, -14, -7, +1, +2, +3, +6, +9, +12, +15, +18, and +21. Blood samples were collected using 10 ml BD vacationer tubes (BD Diagnostics, Franklin Lakes, NJ) and stored at 4˚C for 24 hours before being centrifuged for serum separation. After centrifugation, samples were stored at -80˚C until needed for processing. Composite milk samples were obtained at the 1st, 2nd, and 3rd milking post calving, and every three days thereafter. Milk samples were

analyzed for fat, protein, lactose, total solids non-fat, and somatic cell count (SCC ; Washington DHIA).

Postpartum energy balance was calculated using data collected at d 3, 10, and 21. EB was calculated by determining NEL consumed (DM intake × NEL/kg DM) minus NEL required (NEL for maintenance + NEL for milk) (NRC, 2001).

Serum Vitamin Analysis: All vitamin analyses were determined by reversed-phase HPLC (Waters e2695 Separation Module, Waters Corp., Milford, MA) with a photodiode array detector (Waters 2998, Waters Corp.). To determine Serum *13 cis* retinoic acid and *all-trans* retinoic acid concentrations 400µL serum samples were mixed with 420 µL of acetonitrile + 20 µL of acetic acid, and 20 µL of retinol acetate (Sigma-Aldrich, St. Louis, MO) as the internal standard. Samples were extracted twice with 1.5 mL of a mixture of 6.5:1.5 hexane: isopropanol and 0.2 mL of HPLC-grade water, and the hexane layer was removed via evaporation under a light flow of nitrogen gas. The residue was dissolved in 200 µL of a 50:50 mixture of the mobile phase solutions and 100 µL was applied to the instrument. Separation of retinoids was performed using an isocratic gradient on a Waters C18 CSH 3.5 µm column (4.6 × 75 mm, Waters Corp.). The mobile phase consisted of two solvents. Solvent A contained a 10mM ammonium acetate solution in HPLC grade water, and Solvent B contained acetonitrile (450 mL), isopropyl alcohol (450 mL), and tetrahydrofuran (100 mL) at a flow rate of 1 mL/min was used.

Serum retinol, α-tocopherol, and β-carotene concentrations were determined in a similar manner as serum *13 cis* retinoic acid and *all-trans* retinoic acid except retinol palmitate was used as the internal standard and samples were measured using a non-isocratic method. A

single mobile phase consisting of acetonitrile (720 mL), dichloromethane (120 mL), methanol (80 mL), and n-butanol (0.8mL) and a flow rate of 1.25 mL/min was used.

Milk retinol, α-tocopherol, and β-carotene were isolated similar to serum retinol α-tocopherol, and β-carotene, milk samples, however were processed in the following manner; 2.5 mL of milk was mixed with 5 mL of 1% Pyrogalic acid solution dissolved in 200 proof HPLC grade ethanol, then mixed with 10 mL of a 50% potassium hydroxide solution. Samples were placed in a water bath for 7 min at 70˚C, once samples are cool 10 mL of HPLC grade Petroleum Ether was added and samples were mechanically shaken on high for 10 min. 15 mL of HPLC grade water were then added to each sample and then the samples are centrifuged for 10 min. Once complete 5 mL of the upper layer was extracted and evaporated under a light flow of nitrogen gas. Samples were dissolved in 100 µL of HPLC grade methanol then 400 µL of starting mobile phase (below), and 100 µL was applied to the instrument.

Serum Metabolites: Serum concentration of β-hydroxy butyric acid (BHBA) was determined via enzymatic-colorimetric assay (Wako Chemical, Dallas, TX) using 2.3 µL serum. Serum was mixed with 150 µL reagent 1(mixture of β-thionicotinamide adenine dinucleotide and a phosphate buffer) and incubated for 5 min at 37˚C. Next, 50 µL of reagent 2 (mixture of 3-hydroxybutyrate dehydrogenase in a provided buffer) was added and the mixture was incubated for 2 more minutes at 37˚C. The initial readings were recorded every 30 seconds for 2 minutes at 405 nm. Serum concentration of BHBA was determined using a linear standard curve according to manufacturer instructions. Concentrations of non-esterified fatty acid (NEFA) concentration were determined using an enzymatic assay (Wako Chemical). The assay used two reagents and one solvent provided by the

manufacturer; solvent A (phosphate buffer) was added to the company-provided color reagent A (acyl-CoA synthetase, adenosine triphosphate CoA, adenosine monophosphate, and pyrophosphate) then 100 µL of the mixture was added to 2.5 µL of serum and the mixture was incubated for 5 min at 37˚C before absorbance read at 550 nm. When finished, 50 µL of color reagent B (acyl-coenzyme A oxidase and peroxidase) was added to each sample and left to incubate for 5 min at 37˚C. The absorbance was again read at 550 nm and the concentration was determined using a linear standard curve as instructed by the manufacturer. Serum concentration of haptoglobin was determined using an enzymatic assay (Tridelta, Maynooth, Co. Kildare, Ireland) using 3.25 µL of serum. Fifty µL of the first reagent (stabilized hemoglobin) was added to the sample before 70 µL of the second reagent (chromogen) was added. Samples were incubated for 5 min at room temperature before absorbance was measured at 600 nm. Concentrations were determined using a linear standard curve according to manufacturer's instructions. Serum RBP concentration was determined using a commercially available ELISA kit (BioSource, San Diego, CA) according to manufacturer's instructions. Briefly, 100 µL of sample was added to a pre-coated 96-well plate and then 50 µL of the conjugate (RBP4-HRP) was added to each well. The plate was then incubated for 1 h at 37˚C. Upon completion of the incubation, each well was washed 5-times using a 1× wash solution. Then 50 µL of substrate A (substrate for HRP enzyme) and B (stop reagent) were added to each well and the plate was covered and incubated for 15 min at 37˚C. After incubation 50 µL of stop solution was added and the optical density was determined at 450nm. The standard curve was fit to an exponential model using SAS and concentration was determined for each sample. Serum concentration of malondialdehyde (MDA) was determined via colorimetric assay testing for thiobarbituric

acid reactive species (TBARS; Caymen Chemical Company, Ann Arbor, MI) according to the manufacturer's instruction. Fifty µL of serum was mixed with 50 µL of sodium dodecyl sulfate (SDS) solution then 2 mL of color reagent (thiobarbituric acid, acetic acid, and sodium hydroxide) were added to each sample. Samples were boiled for 1 hour then immediately cooled on ice for 10 minutes. Samples were centrifuged, transferred on a 96 wall plate, and absorbance recorded at 532 nm. A linear standard curve was created and MDA concentration was determined according to the standard curve. All kits were read on a Synergy 2 microplate spectrophotometer (BioTek, Winooski, VT).

Real Time Polymerase Chain Reaction: Real-time reverse transcribed PCR was carried out in a 7500 Fast real time PCR system (Applied Biosystems, Foster City, CA) using custom designed TaqMan MGB probes on targeted genes (liver RBP, TNF- α, PEPCK, PC, adipose RBP, TNF- α, PPAR-γ, and PBMC IL-1β, IL-6, TNF-α, and ICAM) using bovine RPS9 and GAPDH as endogenous controls. Reaction mixture included 2 µL of cDNA, 10 µL of Applied Biosystems Taqman Universal PCR Mastermix (Applied Biosystems), 1 µL of Applied Biosystems 20X custom primer probe mixture, and 7 µL of water.

Polymorphonuclear Leukocytes (PMN), Peripheral Blood Mononuclear Cells (PBMC) Isolation and Neutrophil Killing Assay: Peripheral blood mononuclear cells (PBMC) and neutrophils (PMN) were isolated from 50 mL of blood by gradient centrifugation using a mixture of Histopaque 1077 and 1119 (Sigma Aldrich, St. Louis, MO). After centrifugation at 456 × g for 1 hour at room temperature, plasma was discarded and the buffy coat and red blood cells collected. Red blood cells were lysed using a 10% NaCl solution and water then further washed with phosphate buffered saline (PBS). Samples were separated using the Histopaque gradient and PBMC were stored at -80˚C for further processing for gene

expression. Neutrophils were used for the neutrophil killing assay; cells were counted using a hemocytometer and checked for viability. Stock of Esherichia coli were diluted and mixed with a dilution of the isolated neutrophils at a ratio of 3:1 bacteria to neutrophils and incubated for 10 minutes. Samples were then plated on tryptic soy agar plates and left at room temperature for 24 h. Colony forming units were then counted and percent killed established.

Statistical Analysis: The experimental design was a 2×2×2 factorial arrangement of treatments in a completely randomized block design. Preplanned orthogonal contrasts were used to compare individual dietary main effect (protein, vitamin A, and monensin) on the response variables measured. Secondarily, treatments were compared over time to highlight trends over time. Response variables were analyzed as repeated measures ANOVA using the MIXED model procedure (Version 9.3, SAS Institute Inc., Cary, NC). Sources of variation in the model included effects of treatment and time as well as 2- and 3-way interactions amongst the main effects. Significance was declared at $P \leq 0.05$ with trends toward significant when $P < 0.1$. Separation of least squares means (LSM) was accomplished using the diff option within SAS to perform a pair wise test between means. Intercept was designated as the random effect with the subject being cow. Several covariate structures including compound symmetry, unstructured, autoregressive(1), variance components, and toeplitz matrix were tested; however, compound symmetry fit the model best likely because sampling periods were not equidistant. Samples taken on periods previously listed in ”sampling” section, were used for the analysis of treatment and time. Data are presented as least square means (LSM) ± standard errors of the mean (SEM).

Gene expression data were analyzed using delta Ct values (Ct values normalized to the average of the endogenous control genes, GAPDH and RPS9) and are graphically presented as fold change ($2^{-\Delta\Delta Ct}$) relative to the control treatment (low protein, no supplemental vitamin A, no monensin).

Results and Discussion

Production

Production measures were evaluated including milk yield, milk fat percent and yield, protein percent and yield, lactose percent and yield, energy corrected milk, somatic cell count (SCC), linear somatic cell score, as well as dry matter intake (DMI) pre- and postpartum.

Understanding the exact physiology of DMI during the transition period has been extremely challenging for researchers interested in the critical time period. It is well documented that DMI decreases significantly during the last few weeks prepartum, and often does not return to adequate intakes until later in early postpartum. Dry matter intakes for lactating cows can decrease nearly 52% during the final 2 and 3 wks prior to parturition (Marquardt et al., 1977). The physiology of the DMI decrease during this period is complex and many have hypothesized that the decrease is associated with anatomical space constraints, reproductive hormones, metabolic hormones, stress, and even inflammatory mediators (Ingvartsen, 2000). It is likely that multiple factors are involved in the decrease in DMI during this period, however a better understand of the physiologic mechanisms of DMI will greatly aid in understanding how to compensate for the negative energy balance experienced during this period. This research looked mainly at the effects of vitamin A, monensin, and crude protein on multiple physiologic, metabolic, and immune factors including their effects on DMI. Our research found that none of the dietary treatments (CP, monensin or vitamin A) acted solely or interactively to affect pre- or postpartum DMI (Figures 1 and 2, respectively). However, the DMI data are in agreement with what is typically found during the transition period indicating that at approximately d -7 relative to calving, DMI begins to decrease, reaching intakes of about two-thirds of the previous 28 d prepartum intakes. After

calving postpartum DMI doubles in the first few wks as cows need to meet the energy demands of milk production. These results are in agreement with those reported by Janovick et al. (2010) who studied the effects of prepartum dietary energy intake on postpartum intake and performance of Holstein dairy cows. Although our results provide no evidence toward an effect of treatments on DMI, Petersson-Wolfe et al. (2007) found that cows fed controlled-release monensin capsules tended to have a decreased DMI during the transition period. Similarly, Sauer et al. (1989) reported a decrease in DMI in cattle receiving 33 ppm monensin during the early postpartum. However, others have found that the prepartum supplementation of monensin had no effect on prepartum or postpartum DMI (Fairfeild et al., 2007; Chung et al., 2008). Monensin is known to increase propionate production in the rumen by altering microbial population. This alteration in flora allows for better utilization of feed components, increasing efficiency. Monensin administration during the postpartum period has been shown to decrease DMI (Anil and Forbes, 1988; Allen, 2000, Duffeild et al., 2007).

Research also suggests an effect of dietary protein on DMI during the transition period. Hayirli et al. (2002) concluded that dietary rumen undegradeable protein (RUP) tended to decrease DMI. Several groups (Oldham, 1984; Allen, 2000) reported that DMI is related positively to CP content in diets of lactating dairy cattle. Crude protein concentration in the current study did not differ during the lactation period, which may explain why prepartum dietary crude protein did not have a detectable effect on DMI during the lactating period. During the dry period, CP was formulated to meet requirements which may also explain why no effect of dietary CP on DMI was found.

Little research has been done to identify if vitamin A has a specific impact on DMI in transition dairy cows. However, DMI is often used to monitor animal health, and adequate DMI is crucial for proper nutrient delivery. In many studies it has been concluded that disease (mastitis, retained fetal membrane, displaced abomasum, for example) are often preceded by a decrease in DMI (Lukas et al., 2007; Zamet et al., 1979). Vitamin A has been shown to be a vital nutrient in prevention of disease and could have an effect on DMI. Interestingly, our data provides evidence that prepartum vitamin A supplemented cows tended (table 5) to have greater DMI than cows not receiving vitamin A. Ultimately, more research is needed to fully understand the impact of vitamin A on DMI.

Dry matter intake is a key component to calculating energy balance (NRC, 2001). Therefore, it is conceivable to note that a decrease in DMI may be directly correlated to a decrease in energy balance. In the current study postpartum energy balance was measured to evaluate the effects of treatments on energy balance during the first 3 weeks postpartum. No significant difference among dietary treatments on energy balance was determined. However, a significant difference was detected at d 10 postpartum for cows receiving HP and LP diets. Cows receiving the LP diet were in greater energy balance than cows receiving the HP diet ($P = 0.03$). It is important to note that at the time points tested (d3, 15, and 21), cows were in negative energy balance. This is consistent with the decrease in DMI shown in figure 1 and the gradual increase in DMI shown in figure 2. Energy balance in the current study is also similar to what others have reported in postpartum dairy cows (Grummer et., 2004; Drackley, 1999). The significant difference detected on d 10 postpartum among protein treatments might be explained in part by another observation where cows receiving prepartum HP had significantly greater milk fat percent at d 9

postpartum than cows that received the LP ration. Milk fat is a critical component to the NEL calculation (NRC, 2001) so cows that received the HP diet would likely be in greater energy deficit than the LP cows. Milk production increases dramatically during the weeks following parturition driving the need for increased feed intake. Our research found no significant dietary effect on milk yield (Table 1 and 2), which followed a typical early postpartum milk yield curve (Figure 3). Cows used in this study were high producing and many reached approximately 40 kg/d by d 21 postpartum. Means of milk yield over the first 21 d of lactation without the first 3 milkings (colostrum) ranged from 33.2 to 36.6 kg/d across the dietary treatments (Table 2). Even though our finding failed to detect a significant difference among treatments, other studies have shown an effect of dietary vitamin A on milk yield. For instance, Oldham et al. (1991) reported 12% greater milk yield over the first 6 wk of lactation for cows supplemented with 170,000 IU/d vitamin A starting 2 wk before dry off to 6 wk postpartum. Cows in the current study consumed 72,000 IU/d of vitamin A, which may in part, explain the lack of a detectable effect on milk yield. Monensin has also been shown to increase milk yield. For example, in a meta-analysis performed by Duffield et al. (2008), monensin fed during the lactation period increased milk production by 2% while decreasing DMI by 2% on average across the studies. However, in other studies where monensin was only fed during the dry period no significant difference was detected (Fairfield et al., 2007; Karcher et al., 2007) Similarly, cows on the current study only received monensin during the prepartum period and no significant change in milk production was observed. The proposed pathway by which monensin may increase milk production is through altering the rumen bacterial profile by selecting for more propionate producing bacteria (Duffield et al., 2008). The increased propionate production provides

more substrate for gluconeogenesis in the liver of ruminants thus supplying more glucose to be utilized for maintenance and milk production (Beckett et al., 1998; Phipps et al., 2000). Duffield et al. (1999) reported an increase in milk yield for cows receiving monensin in the form of a controlled release capsule starting at 3 weeks prior to calving and continuing for 95 d.

Prepartum dietary CP concentration did not impact milk production in the current study. Similarly, others have reported that altering prepartum dietary CP above NRC (2001) requirements for dry cows has shown to have little effect on milk production (Greenfield et al., 2000; Dewhurst et al., 2000). Therefore, it is conceivable to conclude that the effect of prepartum crude protein on milk production postpartum is negligible as long as it is fed to meet requirements for rumen digestible protein and rumen undegradeable protein.

A significant difference was not detected among dietary treatments over the 21 d postpartum period with a mean of 3.92% (Table 2). However, a significant diet × time interaction was detected ($P = 0.02$) for milk fat percent (Figure 4). Overall, milk fat percent declined from d 3 until reaching a minimum on d 15. When data were analyzed without the 1st, 2nd, and 3rd milking's (colostrum), an interaction of dietary CP × time was detected (Figure 17). These results show that at d 9 postpartum, cows that received high crude protein had significantly greater milk fat percent ($P = 0.02$) than cows that received low crude protein. Significant difference was not observed at any other time point during the study. No other main effects of diet or their interactions were detected on milk fat percent. In a study performed by Santos et al. (2001), different dietary protein concentrations (12.7 vs 14.7% CP) fed prepartum to primiparous and multiparous cows did not affect milk yield, fat corrected milk, or milk fat percent, which agree with our main effect observations in the current study.

However, the importance of the significant difference detected early postpartum for cows receiving HP diet is not completely understood and further investigation is required to determine its significance.

Milk fat yield did not differ by treatment over time (Figure 5) with a mean of 1.36 kg/d. When data were analyzed without the 1st, 2nd, and 3rd milking (colostrum), an interaction of monensin × vitamin A was observed ($P = 0.04$). Milk fat yield did not significantly differ for cows fed monensin with or without vitamin A. However, cows receiving no monensin had greater milk fat yield when vitamin A was not supplemented (figure 14). Monensin has been shown to decrease milk fat (Duffield et al., 2008) but this was not observed under the current experimental conditions. Other studies also have reported no reduction in milk fat for cows receiving monensin (Duffeild et al., 1999; Lean et al., 1994). According to the theory that milk fat depression may be associated with a decrease in acetate and butyrate production in the rumen, the effect of monensin on milk fat depression may be partially explained by the shift toward propionate production and deviation from acetate production in the rumen. According to that theory, acetic acid and butyric acid acts as the primary precursor for milk fat production (Sutton et al., 1988). However, this theory may not be an adequate explanation for milk fat depression. Baumen and Griinari (2001) proposed an alternate explanation called the biohydrogenation theory. This theory concludes that under certain dietary conditions rumen biohydrogenation produces unique fatty acid intermediates that may inhibit milk fat synthesis. Further research is needed to understand the effect that monensin might have on milk fat synthesis. In the current study, milk fat yield was determined by multiplying milk fat percent by milk yield and no significant effect of dietary

treatments was detected on either of those observations. Thus, understanding the reason for the significant effect on milk fat yield is not fully explainable.

Milk protein percent in the first 21 d was found to be significantly different (P =0.05) when vitamin A was fed prepartum (Table 2); supplementation of vitamin A reduced milk protein percent to 3.4% as compared with cows not receiving vitamin A. No reports were found that provide evidence toward a significant difference in milk protein percent by supplementing vitamin A. Interestingly, milk protein yield tended to also be reduced for cows that received vitamin A supplementation (Table 2). Milk protein percent was not found to be significantly different among the other dietary factors. Similarly, Santos et al. (2001) found no difference between cows fed 12.7 versus 14.7 % CP prepartum on milk protein percent during the first 120 d postpartum. Furthermore, fairfield et al. (2007) fed monensin prepartum and reported no significant difference in milk protein percent for cows receiving monensin. No diet × time interaction was detected for milk protein percent over the 21 d studied with milk protein declining from d 3 until approximately d 18 (Figure 6).

Milk protein yield was not affected by dietary treatment (Table 2) with a mean of 1.2 kg/d, although there was a trend ($P < 0.08$) for supplementation of vitamin A prepartum to reduce milk protein yield during the first 21 d postpartum when excluding the first 3 milkings. No diet x time interaction was detected for milk protein yield (Figure 7). The effect of vitamin A on milk protein percent and yield is not well understood and has not been reported in the literature. More research is required to provide further evidence toward this finding.

Milk lactose percent did not differ by dietary treatment (Table 2) and no interactions were found to be significantly different. On average milk lactose percent increased from about 4%

on d 3 to 4.5% on d 21 of lactation (Figure 8). No main effect or interactions among dietary treatments were detected for milk lactose yield (Table 2 and Figure 9).

Milk somatic cell count and linear somatic cell score were found to be significantly affected by dietary treatments. Milk somatic cell count was lower ($P = 0.001$) for cows that received vitamin A supplementation prepartum compared with those that did not receive vitamin A, when the 1st, 2nd, and 3rd milkings were included in the analysis (Figure 20). This observation is similar to the results seen when the 1st, 2nd, and 3rd milking's were removed (Figure 16). A trend ($P<0.10$) for a similar vitamin A effect was demonstrated in SC linear score when the 1st 2nd, and 3rd milkings were removed from the analysis. These results are in agreement with those reported by Chew et al. (1985) in which serum content of vitamin A were correlated to a lower somatic cell score on a California mastitis test. Milk somatic cell count and linear score were not affected by prepartum dietary treatments when analyzed over time (Figures 11 and 12). In general, SCC and SC linear score declined over the 21 d postpartum, as expected. Monensin and vitamin A fed prepartum interacted ($P = 0.04$) to alter SCC over the postpartum period when the first 3 milkings were excluded to compensate for the high SCC normally found in colostrum (Table 2). In transition dairy cows, it has also been shown that supplemental vitamin A had a significant effect on lymphocyte proliferation and increased phagocytosis and intracellular killing indices by milk PMN against *S. aureus* (Daniel et al., 1986; Tjoelker et al., 1986). Similar results have also been observed using a rodent model, Weidermann et al, (1996) concluded that vitamin A deficiency predisposes subjects to *S. aureus* infection. Weidermann et al, (1996) also indicated a decrease in phagocytic activity and bacterial killing of peritoneal macrophage for *S. aureus*. One of the main reasons neutrophil function might be diminished during vitamin

A deficiency is that neutrophils and other granulocytes develop from myeloid stem cells in the bone marrow that are mediated by retinoic acid binding to retinoic acid receptor in order to stimulate gene expression (Lawson et al., 1999). This understanding may highlight that cows not receiving vitamin A supplement had greater SCC because of a decreased ability to mount an adequate immune response.

In the current study milk SCC was not detectably affected by monensin or crude protein. This is contradictory to some of the reports in literature. For instance, Heuer et al. (2001) showed that cows receiving monensin during the transition period had significantly lower SCC. The overall effect was that the proportion of cows over 250,000 cells/mL was reduced from 31% to 18%. The authors concluded that this observation was related to the effect of monensin on decreasing circulating ketone bodies providing evidence that the cows on monensin were in a better energy balance than control cows. Our data do not show a direct effect of monensin on SCC but a significant effect of monensin on BHBA concentration was observed, providing further evidence that plane of nutrition during the transition period has a significant impact on immune status.

Milk Vitamins

Milk sample were tested on five time-points (d 1, 3, 9, 15 and 21) during early lactation. Samples were processed using HPLC to determine serum concentrations of retinol, β-carotene, and α-tocopherol vitamin concentrations.

Figures 22, 23, and 24 represent milk vitamin concentrations over time by diet. Milk retinol concentrations did not differ ($P = 0.3$) among treatments and were similar to what has been observed by other research groups. For example, Johnston et al. (1984) observed a decrease

in milk vitamin A and β-carotene concentrations in early postpartum. Our results also indicate a similar decrease in milk retinol and β-carotene concentrations (Figures 22 and 24, respectively). The decrease in milk vitamin concentration occurring during the early postpartum period is likely related to the high concentration of milk components observed in colostrum. Overtime, the vitamin concentration in the milk decreases as milk production increases. Furthermore, cows receiving low protein and no monensin had the lowest (P = 0.03) concentration of milk retinol when compared with all other treatment groups (Figure 26). However, milk retinol was affected by dietary crude protein over time. Cows receiving high crude protein had greater (P = 0.015) milk retinol than cows receiving low crude protein (Figure 25). Little research has been performed investigating the relationship of milk retinol and prepartum dietary crude protein; however, Lindberg et al, (1999) reported a significant difference in serum RBP concentration among cows receiving low crude protein diets during the prepartum period. Potentially decreasing amino acid availability to tissues RBP concentrations may be limited thus limiting retinol delivery to the mammary gland.

Milk α-tocopherol concentration was not affected by dietary treatments when observed over time (P = 0.8) as shown in figure 24. However, an interaction (P = 0.04) between dietary protein and monensin on milk α-tocopherol concentration was observed (Figure 27). Milk samples were also analyzed for β-carotene concentration yet no differences (P > 0.05) were detected among dietary treatments. Milk α-tocopherol and β-carotene did follow an expected trend over time similar to what was observed with milk retinol concentrations (figures 23 and 24 respectively). Milk α-tocopherol concentrations were similar to what was reported by Weiss et al. (2003) (Table 3). Furthermore, milk β-carotene also followed what was observed by others (Lindqvist et al. (2011); Table 3).

Serum Metabolites

Serum samples were collected and analyzed for metabolites shown in figures 28, 29, 30, and 31. No significant difference was detected for the effect of dietary treatments was detected on serum NEFA concentrations ($P = 0.6$) (Figure 28). Serum NEFA does however follow a typical trend over time during the transition period where NEFA concentrations decrease in late prepartum and begin to increase postpartum (McCarthy et al., 2015). The results are also in agreement with observations reported by Karcher et al. (2007) in which cows were fed monensin prepartum in combination with a varying fiber source. Dohorst et al. (2002) fed close-up prepartum diets that varied in crude protein concentration (9.1% and 11.2%) and studied the effect on rumen digestibility until the day of calving. It was concluded that prepartum dietary crude protein had no significant effect on serum NEFA concentrations prepartum. Serum retinol concentration has been shown to be inversely related to serum NEFA concentration during the transition period (LeBlanc et al., 2004). However, LeBlanc (2004) concluded that the change in retinol and NEFA was small enough that it did not represent a major significance. Our results also indicate no evidence toward an effect of prepartum protein, vitamin A or monensin supplementation on serum NEFA concentrations.

Serum β-hydroxy butyrate (BHBA) concentrations are shown in figure 30; no significant difference was detected among diets. However, a monensin × time effect was detected indicating that cows receiving monensin had a significantly lower ($P = 0.02$) serum BHBA concentration at d 9 postpartum than cows not receiving monensin (Figure 34). Serum BHBA is a ketone body that is elevated during negative energy balance (Duffield et al., 1997). Duffield et al. (1997) reported cows receiving monensin in the form of a controlled released capsule during the transition period had lower BHBA concentrations than control

cows. The effect of monensin on BHBA is commonly attributed to the increased production of propionate in the rumen. This shift increases glucose production in the liver and decreases the effect of negative energy balance as more energy is available for metabolic needs (Duffeild et al., 2001; Karcher et al., 2007). It is important to recognize that in the current study monensin was only fed during the close-up period. This indicates that monensin may have a residual effect even after it is removed from the diet. This may be attributed to the change in rumen microflora and the time required for the rumen to return to a microflora consistent with no monensin supplementation. This provides evidence that the use of monensin during the close-up period to reduce the effects of negative energy balance in early post-partum is warranted. A significant difference was not detected among the other dietary treatments.

No significant difference was detected on serum haptoglobin ($P = 0.2$) in a diet × time comparison as shown in figure 29. Haptoglobin is an acute phase protein that is increased during inflammation and acts to bind free hemoglobin in the body. This action sequesters iron found in hemoglobin and keeps bacteria from using the iron. Furthermore, free hemoglobin promotes oxidation so haptoglobin also acts indirectly as an antioxidant (Langlois and Delanghe, 1996). Our observations follow similar trends observed in serum haptoglobin during the transition period where serum haptoglobin concentrations increase near the time of parturition (Huzzey et al., 2010; Trevasi et al., 2011). Figure 32 depicts a monensin × time interaction ($P = 0.01$) on serum haptoglobin concentrations. Cows not receiving monensin had greater haptoglobin concentrations on d 3 postpartum than cows receiving monensin. Similar results were observed by Crawford et al. (2005) using controlled release capsules of monensin fed prepartum. The decrease in serum haptoglobin

concentration for cattle receiving monensin may be related in part to the effect monensin has on changing rumen microflora to promote propionate production, thus promoting glucose synthesis in the liver through gluconeogenic enzymes (Karcher et al., 2007; Arieli et al., 2001). The increase in glucose production may lead to less subclinical metabolic disorders such as hyperketonemia and fatty liver syndrome by decreasing the effect of negative energy balance during the transition period (Crawford et al., 2005). The effect of monensin on haptoglobin and the immune response may also be attributed to a decrease in NEFA and BHBA as reported by Stephenson et al. (1997) and may not be associated with glucose metabolism at all. In the current study, BHBA and haptoglobin are reported to be decreased post-partum for monensin supplemented cows during similar time periods postpartum. These results provide evidence that monensin supplementation prepartum positively affects metabolism and immune status. Furthermore, cows receiving low crude protein had greater ($P = 0.03$) haptoglobin concentration than cows receiving high crude protein (Figure 33). No research reports could be found on the effects of crude protein and haptoglobin so further research is needed. It is important to recognize that haptoglobin is an acute phase protein that is elevated during inflammation. Haptoglobin was measured to evaluate the effect of specific dietary factors that might improve the immune status of the animal; therefore, a decrease in haptoglobin would be an indication of less immunologic stimulation. In that regard, both monensin and crude protein might be providing cows with a better plane of nutrition, which would improve the overall immune status.

Lipid oxidation measured by TBARS assay via testing the presence of malondialdehyde (MDA) in the serum was performed. An interaction of dietary treatments by time is shown in Figure 31. No significant difference was detected ($P = 0.7$) among diets. Results

however demonstrate an effect of dietary vitamin A concentration on serum MDA concentration (Figure 35). This result indicates that cows receiving vitamin A had a tendency ($P = 0.06$) to have greater concentrations of serum MDA than cows that did not. Although Vitamin A has been shown to have antioxidant capacities, it has been demonstrated that the vitamin A precursor, β-carotene, has a greater antioxidant capacity (Olson et al., 1996). In the current study serum β-carotene concentration may have been adequate enough to provide sufficient antioxidant capabilities. Others have reported that retinol supplementation increases lipid oxidation. For instance, Dal-Pizzol et al. (2001) reported an increase in lipid oxidation assessed through tests such as TBARS, superoxide dismutase, and others in rat sertoli cells and concluded that retinol supplementation may cause this oxidation. Others have shown similar results with retinol supplementation, concluding that retinol activated catalase and increased catalase production (Gelain et al., 2008). The current study does provide evidence that retinol supplementation may have deleterious effect on cellular oxidation; however, further investigation is required because serum MDA was the only product used to determine antioxidant capacity in the cows. Other products such as the catalase enzyme might provide further evidence toward a reduction in the anti-oxidant capacity of retinol.

Serum Vitamins and Retinoid Concentrations

Serum samples were analyzed using HPLC for retinol, *13 cis* retinoic acid, *all- trans* retinoic acid, α-tocopherol, and β-carotene.

No significant difference was detected among diets on serum retinol concentrations ($P = 0.89$), as shown in Figure 36. Furthermore, significant difference was not detected in other

interactions tested for serum retinol concentrations. Under normal physiological conditions, serum retinol decrease during the transition period especially as parturition nears (Chew et al., 1982). Our data follow the trend reported by others (Chew et al., 1982; Rezamand et al., 2007; Oldham et al., 1991) in that serum retinol concentration did decrease during the transition period. Hydrolyzed Retinyl-esters (RE) and carotenoids are taken up by the liver and stored in parenchymal cells and liver adipose cells. Stored RE are mobilized in the form of retinol by a specific circulating protein called retinol binding protein (RBP). Retinol binding protein is responsible for the transportation of retinol to extrahepatic tissue throughout the body (Ross, 1993). Retinol initially is absorbed by the lymphatic system and excess is stored in the liver: this may explain why an increase in retinol was not observed in serum during or immediately after supplementation. The mean serum retinol concentrations (Table 4) are in agreement with values reported by others (LeBlanc et al., 2004; Chew et al., 1993; Oldham et al., 1991).

Similar to serum retinol concentration, no significant difference was observed in serum α-tocopherol or serum β-carotene concentrations. The interactions of dietary treatments on serum α-tocopherol and β-carotene concentrations by time are shown in figures 37 and 38. No significant difference was detected for either α-tocopherol or β-carotene ($P = 0.96$ and $P = 0.98$, respectively over time). As reported by others, both serum α-tocopherol and β-carotene decrease as parturition nears and then recovers postpartum (Chew et al., 1982; Goff et al., 1990; Rezamand et al., 2007). The dramatic decrease in serum vitamin concentration occurs because of the large requirements needed for colostrogenesis. This occurs as both dietary and stored lipid soluble vitamins such as retinol are shunted toward the mammary gland (Abd Eldaim et al., 2010).

No detectable difference was demonstrated for the effect of diets on serum *13 cis* or *all-trans* retinoic acid concentrations over time ($P = 0.3$ and $P = 0.8$, respectively), as shown in figures 39 and 40, respectively. However, specific dietary factors did contribute to changes in serum concentrations of 13 *cis* and *all-trans* retinoic acid. The interaction of dietary vitamin A and protein on concentrations of *13 cis* retinoic acid was greatest ($P = 0.02$) for cows receiving vitamin A and low crude protein (Figure 41). Furthermore, on d 3 and d 9 postpartum, cows receiving vitamin A supplementation had greater ($P = 0.004$) concentrations of *13 cis* retinoic acid than cows that did not receive a vitamin A supplement (Figure 42). Van Merris et al. (2004) demonstrated that nulliparous cows with experimentally induced *Eschericia coli* mastitis had significantly reduced serum concentrations of *13 cis* retinoic acid. It is possible that the increase in *13 cis* retinoic acid at d 3 and d 9 for cows supplemented with vitamin A may be related to the decrease in SCC for cows supplemented with vitamin A. Van Merris et al. (2004) showed that cows with mastitis had reduced concentrations of *13 cis* retinoic acid. The current study also provides evidence towards a link between vitamin A supplementation and *13 cis* retinoic acid on mastitis, likely through the conclusion made by Van Merris et al. (2004) that cows with mastitis had lower 13 *cis* retinoic acid. In another study, Linberg et al. (1999) showed that increased dietary protein content improved retinol transport protein RBP. However, in the current study serum RBP concentration was not significantly affected by dietary crude protein providing evidence that increased dietary protein may have little effect on retinol transport in the body. It is also possible that the increase in *13 cis* retinoic acid is strictly a result of dietary vitamin A supplementation as signified in the interaction of vitamin A supplement and time (Figure 42). Cows receiving no vitamin A had greater ($P = 0.009$) *all-*

trans retinoic acid concentration than cows receiving the vitamin A supplement (Figure 43). Because *all- trans* retinoic acid was reported to be increased in experimentally induced *E. coli* mastitis in nulliparous cows by Van Merris et al (2004), the increase in *all- trans* retinoic acid in cows not receiving vitamin A may also be an observation related to SCC, where cows not receiving vitamin A supplementation had significantly greater milk SCC (Fig 11 & 12). This connection however, is unlikely because the difference in *all-trans* retinoic acid is only significant at d 35 prepartum and not at any other time point analyzed. Somatic cell count is used to monitor mammary gland inflammation, and interestingly the increase in SCC seen in the current study was observed with cows not receiving vitamin A supplementation. These results provide further evidence that not only does vitamin A play a role in the immune function; supplementation of vitamin A improves immune status.

Gene Expression Analyses

An effect of high crude protein on PBMC expression of TNF-α is demonstrated in figure 47: cows receiving high crude protein had greater ($P = 0.04$) TNF-α gene expression in PBMC. The interaction of protein by vitamin A on gene expression of ICAM in PBMC is shown in Figure 48: cows receiving high crude protein and vitamin A supplement had a greater ($P = 0.05$) expression of ICAM than that for cows receiving vitamin A and low crude protein. Intracellular adhesion molecule (ICAM) expression is mediated by the expression and binding of both TNF-α and 9-*cis* retinoic acid, this was demonstrated by Chadwick et al (1998) by inducing expression of ICAM in immortalized human aortic endothelial cells. It was concluded that both TNF-α and 9-*cis* retinoic acid acted synergistically to aid in the expression of ICAM. This may explain why cows receiving vitamin A had greater expression of ICAM in PBMCs when they were isolated on d 7 postpartum. The increase in

TNF-α for cows receiving crude protein may also provide evidence toward the increase in ICAM expression because ICAM is induced by TNF-a. Interestingly, ICAM has been shown to be upregulated when retinoic acid is used as a treatment for cervical cancer in humans (Santin et al., 1998). Furthermore, ICAM expression is upregulated by both TNF-α and lipid polysaccharide (LPS: Stratowa et al., 1995). This suggests that by upregulating expression of ICAM in neutrophils of periparturient dairy cows, retinol may enhance cellular emigration to sights of inflammation. This understanding may provide evidence as to why cows receiving retinol supplementation had lower SCC than cows not supplemented: because ICAM expression may have aided neutrophil diapedesis into the mammary gland more effectively removing potential infection and damage. It is important to recognize that greater ICAM expression was a result of both vitamin A supplementation and the higher crude protein: This effect was not related to vitamin A alone. It is speculated that the greater crude protein diet may have aided immune status by improving the plane of nutrition for cows as parturition neared. This may also in part explain the increase in TNF-α expression for cows receiving the higher crude protein diet. Interleukin 1-β and IL-6 were also measured for gene expression; however, no significance was detected among dietary treatments.

Gene expression was tested in hepatic tissue samples: no differences were detected among dietary treatments on any of the genes tested. The lack of effect among dietary treatments, specifically monensin, on the PEPCK and PC is in contrast to what other studies have shown. For instance, Karcher et al. (2007) showed that cows fed monensin during the prepartum period had greater gene expression of PEPCK in hepatic tissue. However, this was not reported in PC mRNA expression, which is in agreement with PC gene expression

in the current study. The difference in expression in our study is not easily explained because Karcher et al (2007) also fed monensin during the dry period. The difference may likely be explained by other factors, possibly the varying fiber source. This may have impacted the monensin effect on mRNA gene expression in hepatic tissue because of the lower energy concentration of soybean hulls. Despite the difference in PEPCK gene expression, our data still provide evidence that monensin does affect energy metabolism through the effect on lowering BHBA serum concentration, as discussed earlier. Retinol bind protein hepatic gene expression was also not detectibly influenced by dietary treatments and will be discussed in further detail later.

Neutrophil killing assay

Neutrophil killing assay was performed on blood-derived peripheral mononuclear cells (PMN) from all study cows on day 7 postpartum. Peripheral mononuclear cells from cows receiving the low crude protein diet had greater percent bacterial kill compared with cows fed high crude protein (Figure 49). This observation should be viewed cautiously as there were many missing observations in this data set. However, some evidence has been reported by Raboisson et al. (2014) indicating a decrease in neutrophil function in response to excess dietary nitrogen fed to cattle. The authors concluded that excess dietary nitrogen negatively impacted neutrophil function in cattle fed chronic levels of excess nitrogen in the form of crude protein. The crude protein concentrations used in this study are not in significant excess of what is recommended (NRC 2001) for high producing dairy cattle during the close up period. It is unlikely that our results may provide evidence toward the effect of excess dietary nitrogen on neutrophil function but more information is required before conclusions could be drawn.

In transition dairy cows, it has also been shown that supplemental vitamin A has a significant effect on lymphocyte proliferation, increased phagocytosis, and intracellular killing indices by milk PMN against *S.aureus* (Daniel et al., 1986; Tjoelker et al., 1986). Similar results have also been observed using a rodent model; Weidermann et al. (1996) concluded that vitamin A deficiency predisposes subjects to *S. aureus* infection. These results indicate a decrease in phagocytic activity and bacterial killing of peritoneal macrophage for *S. aureus*. One of the main reasons neutrophil function might be diminished during vitamin A deficiency is that neutrophils and other granulocytes develop from myeloid stem cells in the bone marrow that are mediated by retinoic acid binding to retinoic acid receptor in order to stimulate gene expression (Lawson et al., 1999). Our observations did not detect any significant difference in neutrophil response for cows receiving or not receiving the prepartum vitamin A supplement. However, the other factors used in the current study to measure immune status including ICAM mRNA, SCC, and the increase in serum *13 cis* retinoic acid discussed earlier do indicate that neutrophil function may have been altered but the neutrophil killing assay was likely not sensitive enough to detect the changes.

No detectable difference was found on the role of monensin on neutrophil function in the current study. Interestingly, monensin was shown to increase production and/or release of haptoglobin, which has a direct influence on neutrophil stimulation and antioxidant capabilities during the acute phase response (Quaye, 2008). Again, monensin likely plays a significant role in the immune process through its modulation of haptoglobin as observed in the current study, but the killing assay was likely not sensitive enough to detect the significant difference.

Serum RBP and RBP Gene Expression

Serum retinol binding protein was measured using a commercially available polyclonal antibody ELISA. Retinol binding protein gene expression was also determined in adipose and hepatic tissue (Tables 8 and 9). Serum retinol binding protein concentrations were much lower than what has been reported (4.16 μg/ml) by others. Abd Eldaim (2010) reported an average concentration in early postpartum lactating dairy cows of 45 μg/ml, using a Western blot analysis. The significantly lower RBP concentrations observed in the current study might be explained by the different methods used to determine RBP concentrations. Furthermore, Lindberg (1999) also reported significantly greater serum RBP concentrations in prepartum multiparous and primiparous cows and heifers using a monoclonal antibody ELISA developed in their laboratory. The polyclonal antibody ELISA used in our determination of serum RBP may not be as sensitive as needed. Limited commercially available RBP ELISA are currently being produced and further product development with greater sensitivity and specificity is required. Despite the lower serum RBP concentrations, a similar trend in serum RBP concentrations is observed (figure 50) when compared with other reports (Abd Eldaim et al., 2012; Lindberg et al., 1999). Serum RBP concentration is reduced significantly as parturition nears (Lindberg et al., 1999; Abd Eldaim et al., 2010; Rezamand et al., 2007). Abd Eldaim et al. (2010) reported that serum RBP concentration drops prior to parturition, and that RBP concentration in colostrum is elevated. This indicates that RBP is passed from serum into colostrum following other proteins passed from dam to offspring (Abd Eldaim et al., 2010). Rezamand et al. (2010) reported that hepatic gene expression of RBP follows that of serum during the periparturient

period, as serum RBP concentration decreased liver gene expression of RBP also downregulated.

Our results indicate that there was a time × protein × monensin interaction ($P = 0.004$) on retinol binding protein, in which cows that received high crude protein and monensin had significantly greater serum concentration of RBP at day -35 than all other treatments (figure 50). Those differences were only observed at day -35 (the day treatments started) and were not observed at any other time points. Lindberg et al. (1999) demonstrated that primiparous cows that received a greater crude protein ration had increased concentration of serum RBP. Our results provide little evidence toward an interaction between dietary crude protein and serum RBP concentration. The difference in serum concentration and method of determination may account for the difference in results.

Hepatic gene expression of RBP was also determined; however, no significant difference was detected. Rezamand et al. (2010) reported that gene expression of RBP declined as parturition neared and spiked following parturition. The lack of increased hepatic mRNA expression provides little evidence toward an interaction between protein, dietary retinoids, and monensin on hepatic RBP gene expression; however, this is in agreement with the lack of significant difference determined in RBP serum concentration. Retinol binding protein has been associated with hepatic production of gluconeogenic enzymes in the liver such as PEPCK. Yang et al. (2005) determined that adipose tissue production of RBP acted as a signaling pathway to increase PEPCK expression in a rat model. It was also concluded that lipid associated RBP production is a potential biomarker for type II diabetes in the human patient. Therefore, RBP expression in the adipose tissue might provide more evidence toward hepatic gluconeogenesis modification than hepatic RBP expression.

Retinol binding proteins and other export proteins such as haptoglobin have been shown to be altered during inflammation and are part of the acute phase response (Humbelt et al., 2006). Furthermore, TNF-α and other inflammation mediators such as IL-1, IL-6 are known pro-inflammatory markers that are elevated during a LPS-induced inflammatory response, while RBP and TTR are decreased (Rosales et al., 1996). Rezamand et al. (2012) examined the relationship between TNF-α and RBP gene expression and concluded that hepatic RBP expression was positively correlated with TNF-α as a pro-inflammatory mediator. However, when rbTNF-α was injected no correlation between cows injected with rbTNF-α and hepatic RBP expression. Our results also provide little evidence toward a potential interaction between hepatic gene expression of TNF-α and RBP.

Conclusion

Managing the health and production status of periparturient dairy cows is especially challenging. Energy demands increase as parturition nears and further increase dramatically during the initiation of lactation and colostrogenesis. This extremely elevated energy demand often cannot be met by dietary intake alone and thus postpartum cows enter into a phase of negative energy balance. As cows partition most of their energy toward milk production immunosuppression occurs, leaving the periparturient cows susceptible to infection diseases such as mastitis. Furthermore, negative energy balance in conjunction with immunosuppression predisposes cows to a myriad of metabolic disorders such as hepatic lipidosis, hyperketonemia, and displaced abomasum. These events during such a short window explain why a large number of lactating cows are culled during this period. It also places economic burdens on producers, it is estimated that subclinical mastitis alone cost the industry over $1 billion annually. The current study was initiated to help provide a better understanding of how prepartum supplementation of vitamin A, monensin, and increased dietary crude protein may help promote immune function, improve energy status, and alter retinol transport proteins during the transition period (-35 through +21, relative to expected calving).

In the present study, results provide evidence that immune status was altered because of dietary interventions. For example, cows receiving prepartum dietary retinol supplementation had lower SCC and linear somatic cell score, indicating that prepartum retinol supplementation may promote a lasting effect on mammary gland immune function. Our results also provide evidence that may explain why the somatic cell parameters were decreased. First, dietary crude protein, interactively with retinol supplementation enhanced

PBMC gene expression of ICAM, which aids in neutrophil diapedesis into infected tissue. Second, cows receiving greater concentrations of crude protein prepartum had greater milk retinol concentrations, providing evidence that delivery of retinol into milk may have been enhanced. Third, vitamin A supplemented cows had greater serum concentrations of *13 cis* retinoic acid, a retinol derivative shown to be decreased during mastitis. These results drive the conclusion that vitamin A supplementation when paired the higher crude protein concentrations may act synergistically to promote neutrophil entry into the mammary gland and increase mammary gland retinol concentrations. Further evidence of dietary nutrients altering the immune response was observed in the observation that prepartum monensin supplementation reduced serum haptoglobin concentration during the first few days of postpartum period. This indicates that prepartum monensin supplementation can have lasting effects on the immune system.

Another key element of this study was determining the effects of vitamin A, protein, and monensin on energy metabolism and function, particularly through gene expression of PEPCK, PC, and RBP in liver, PPAR-γ and RBP in adipose tissue, and serum concentrations of BHBA and NEFA. However, only dietary monensin was found to have a significant effect on the energy parameters measured. Monensin supplemented cows had decreased serum BHBA concentrations in early postpartum indicating that cows supplemented with monensin may have been in a better plane of nutrition (i.e. reduced ketogenesis and lipomobilization) than cows not receiving monensin.

Each of these results provide evidence that vitamin A, crude protein, and monensin fed during the late prepartum period acted solely and synergistically to enhance health during the transition period. This greatly influences how late prepartum nutrition should be

managed, and how we can influence health status of the dairy cows postpartum. Further research is required to better understand what mechanisms these individual factors are using to manipulate the immune system, and how they can be used to decreases the incidences of both metabolic disorders and infectious diseases.

Figures and Tables

Figure 1. Least square means of prepartum dry matter intake of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21.

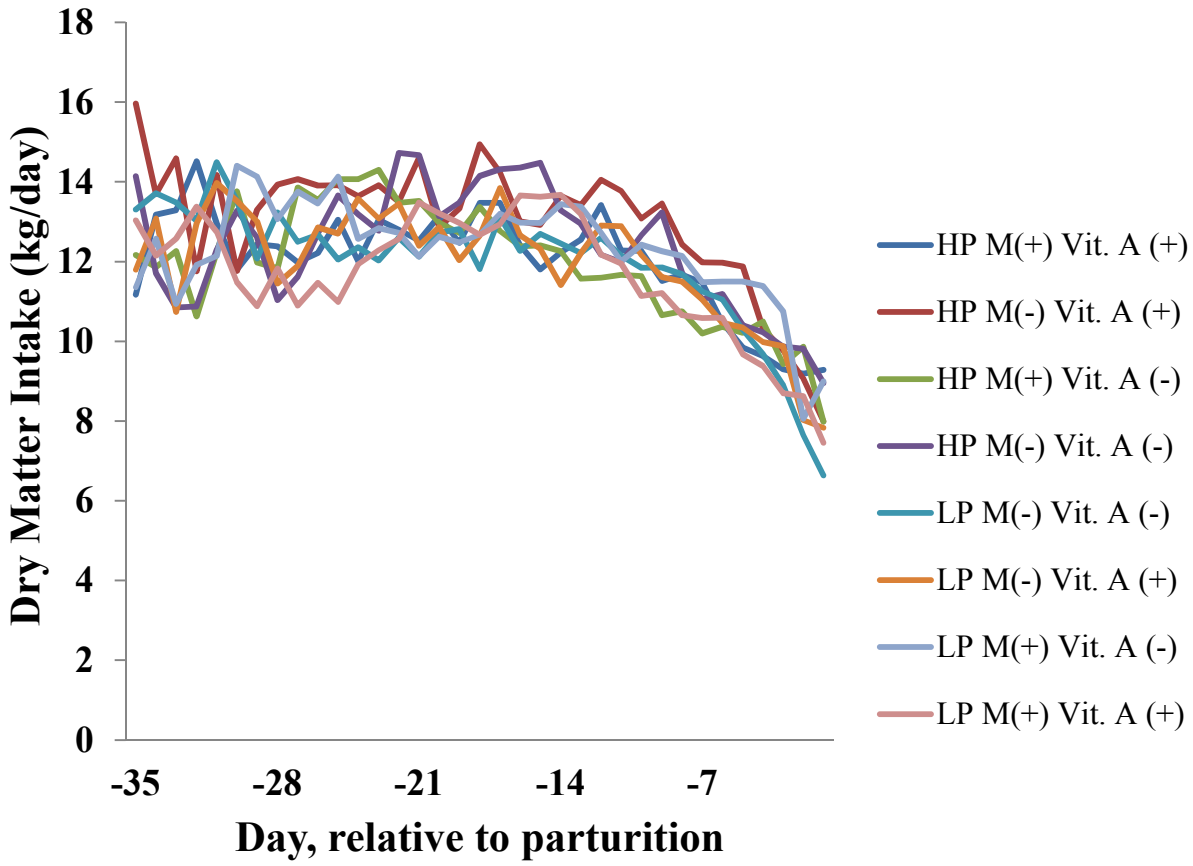

Figure 2. Least square means of postpartum dry matter intake of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21.

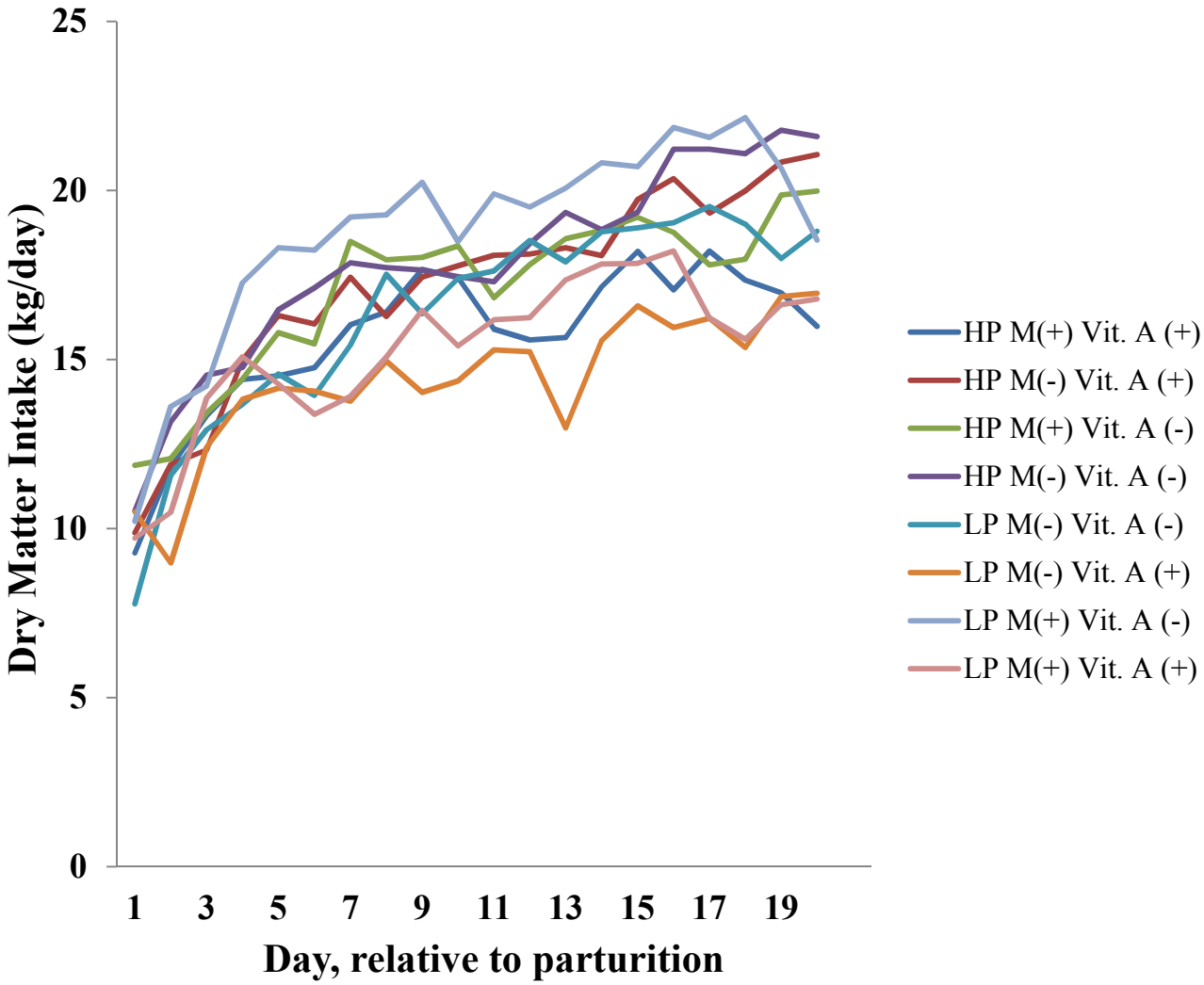

Figure 3. Least square means of milk yield of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Samples from the 1st, 2nd, and 3rd milking's were removed.

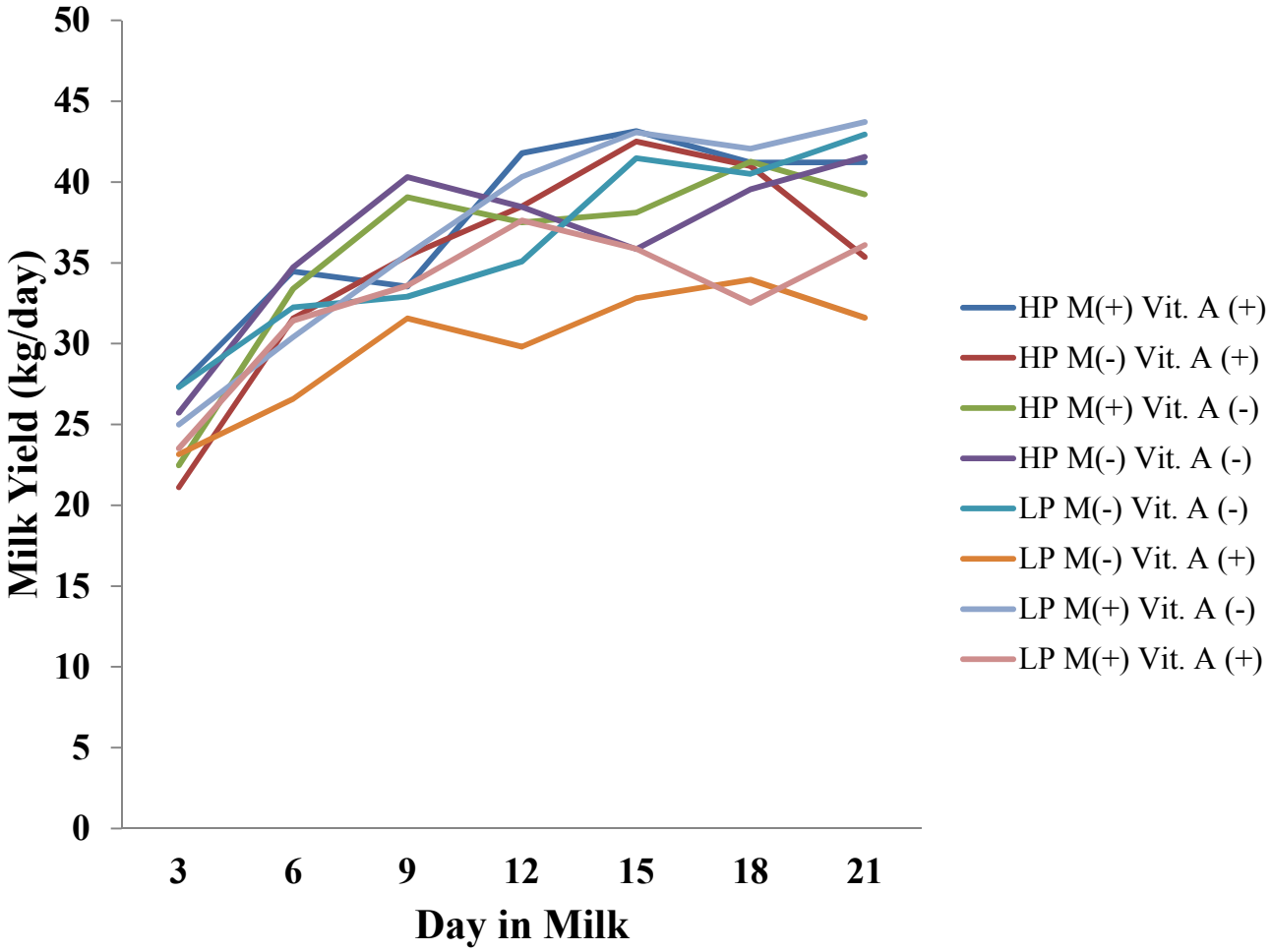

Figure 4. Least square milk fat percent of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Samples from the 1st, 2nd, and 3rd milking's were removed. Treatment by time interaction ($P = 0.02$)

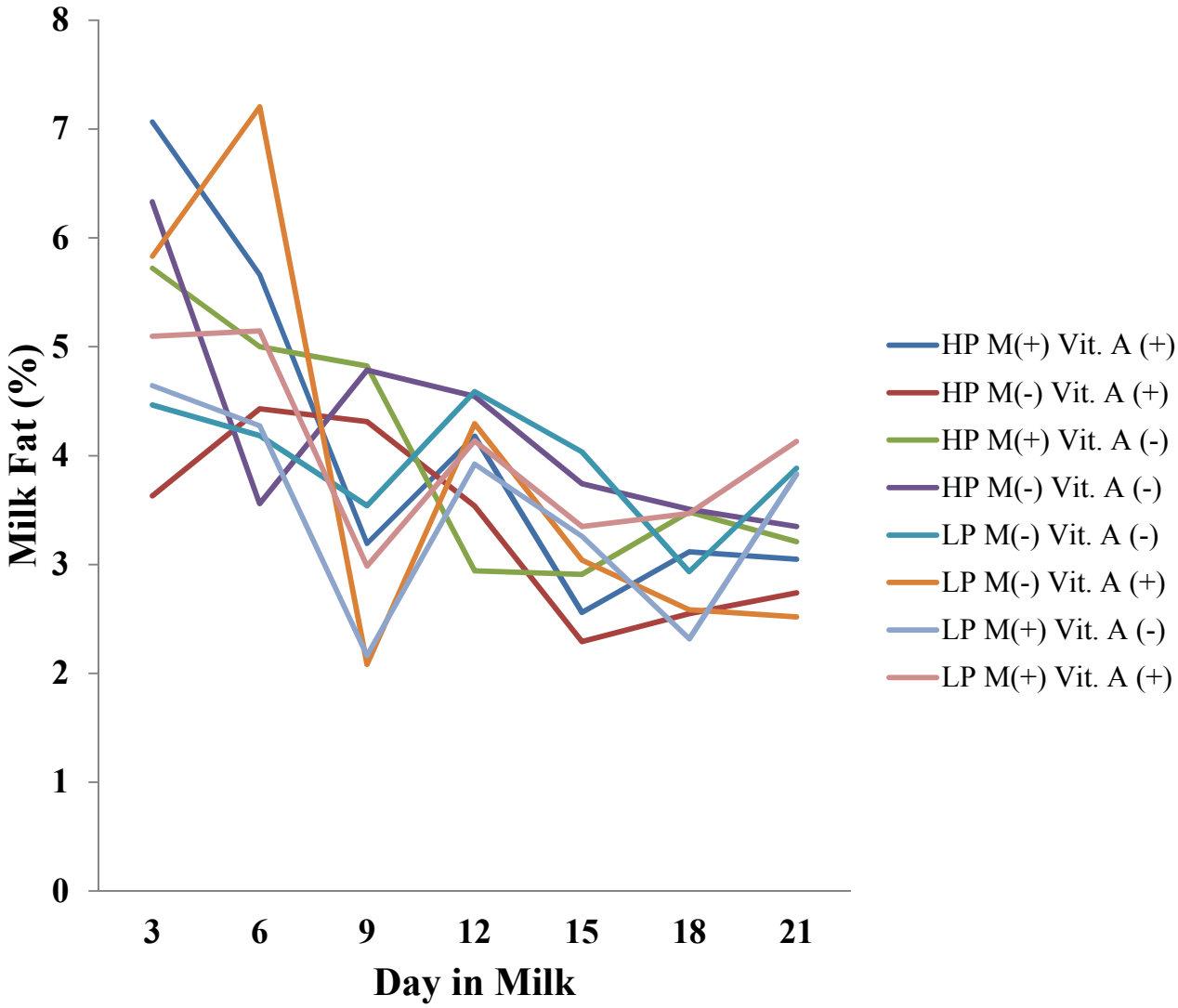

Figure 5. Least square means of milk fat yield of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Samples from the 1st, 2nd, and 3rd milking's were removed.

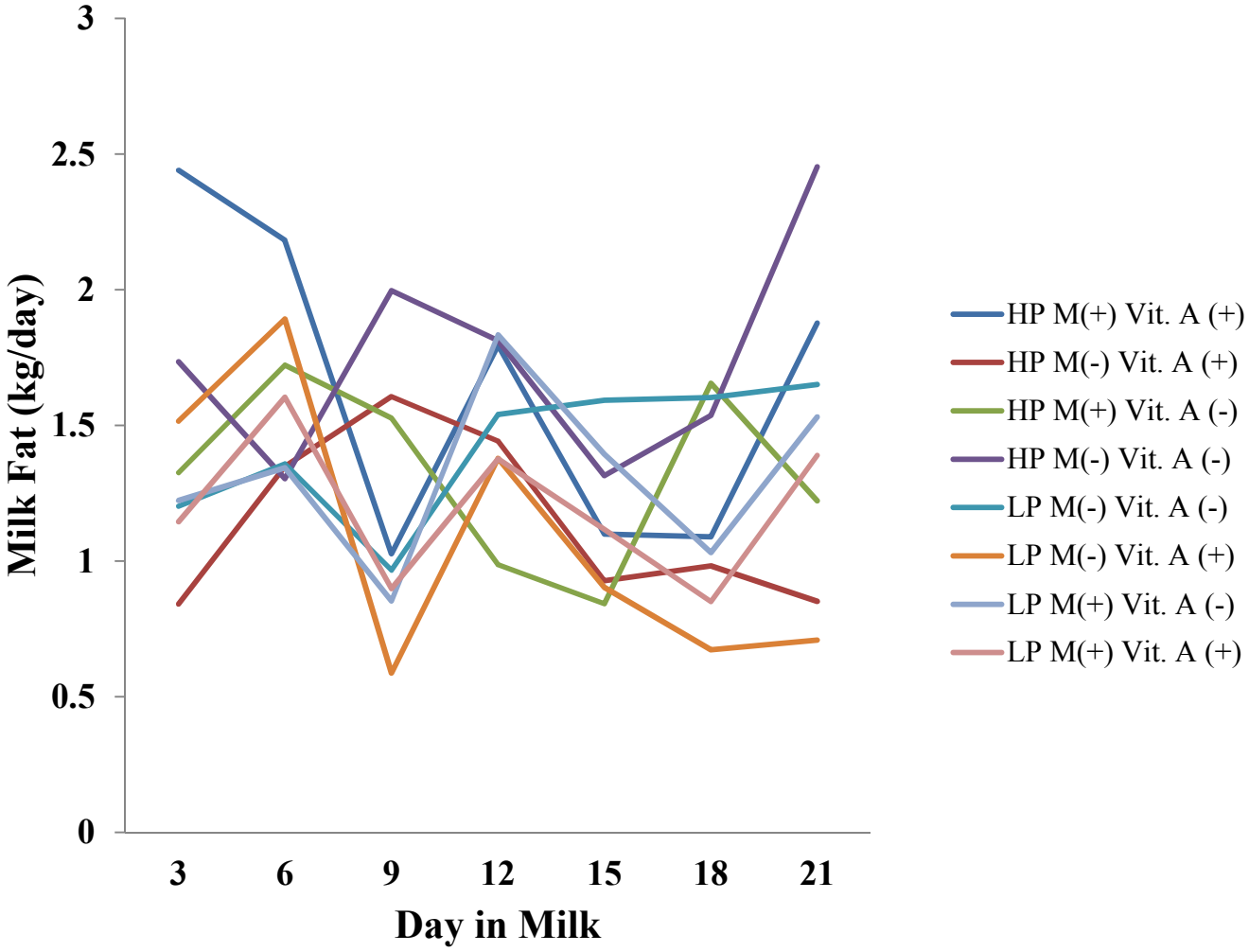

Figure 6. Least square means of milk protein percent of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Samples from the 1st, 2nd, and 3rd milking's were removed.

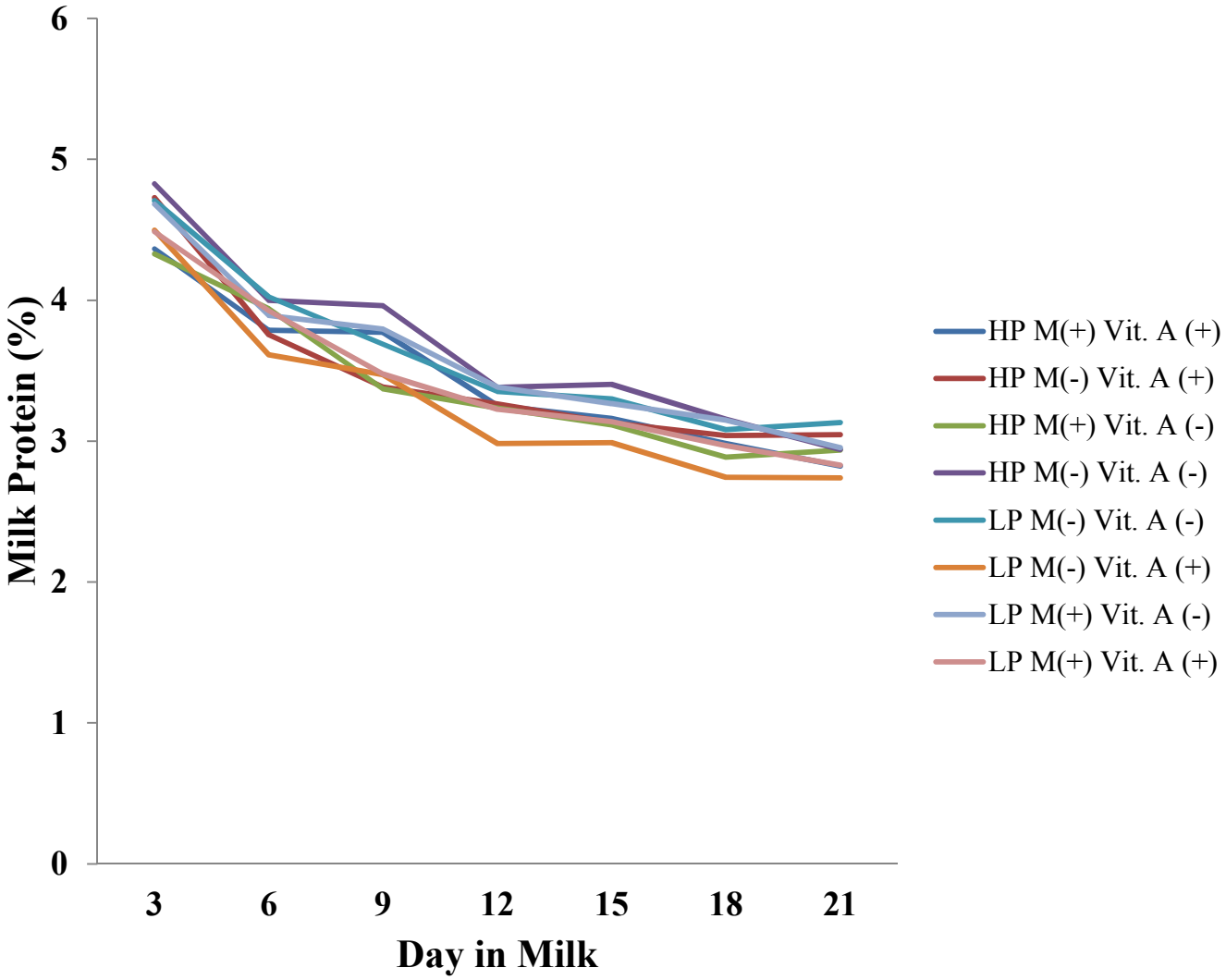

Figure 7. Least square means of milk protein yield of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Samples from the 1st, 2nd, and 3rd milking's were removed.

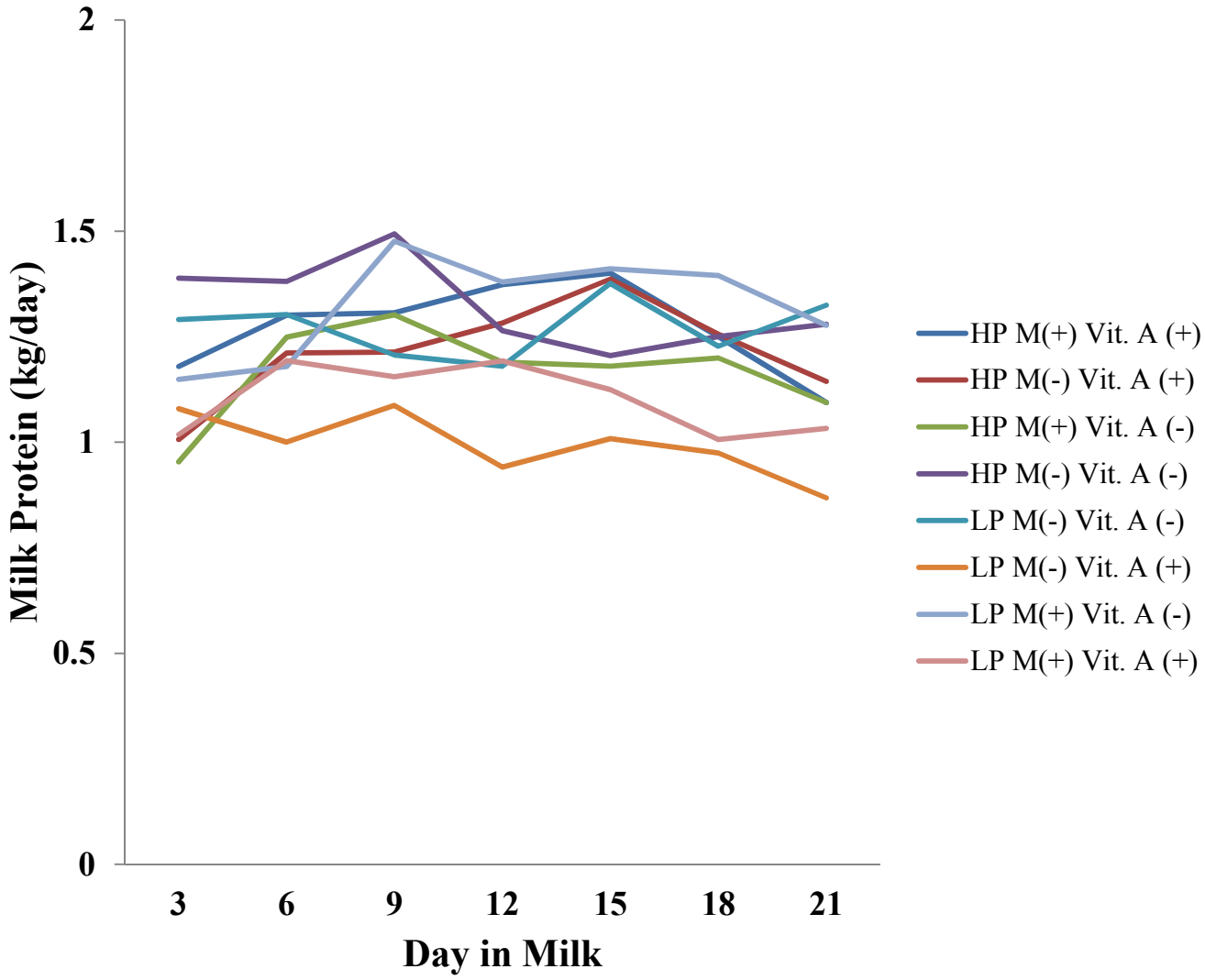

Figure 8. Least square means of milk lactose percent of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Samples from the 1st, 2nd, and 3rd milking's were removed.

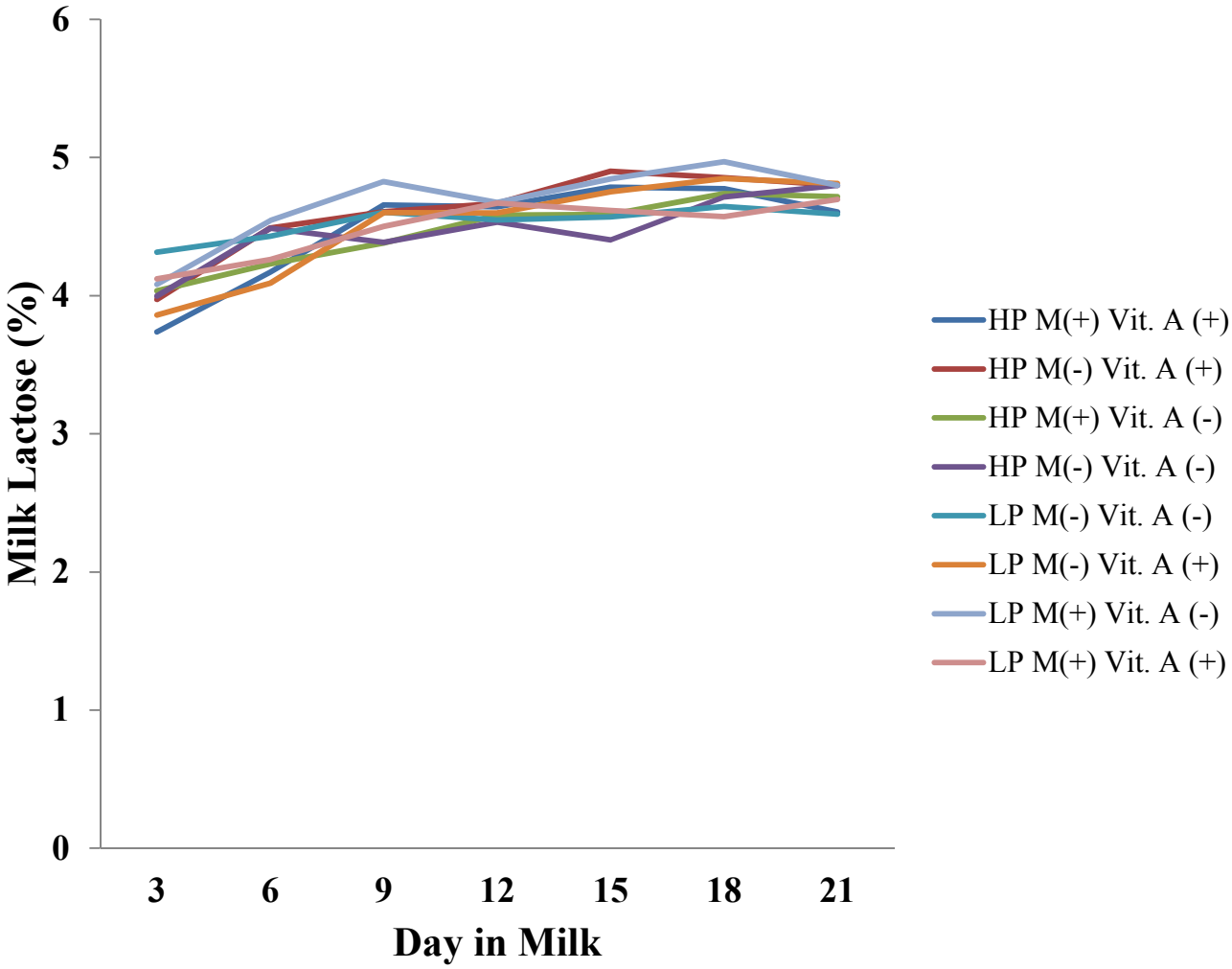

Figure 9. Least square means of milk lactose yield of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Samples from the 1st, 2nd, and 3rd milking's were removed.

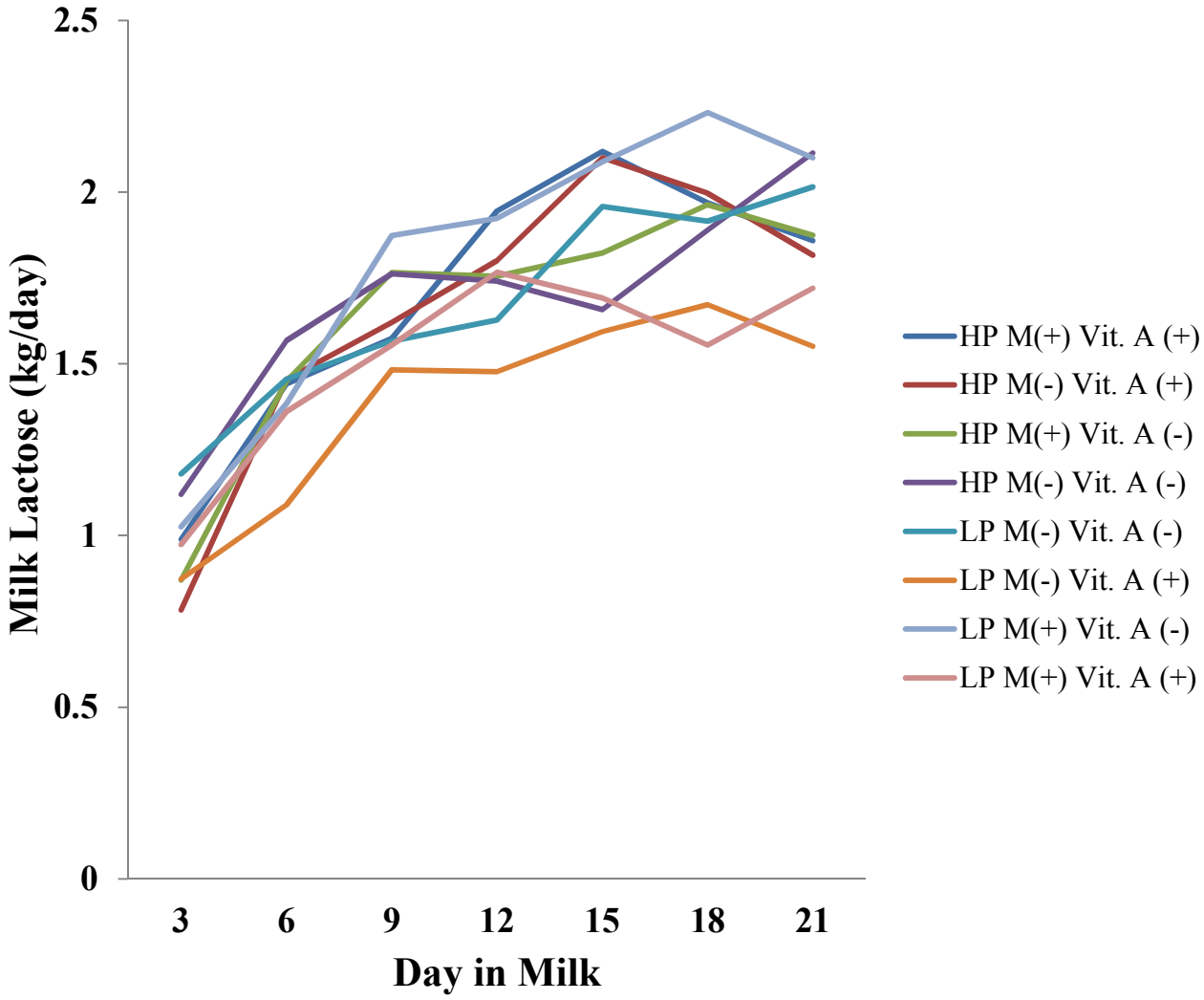

Figure 10. Least square means of milk solid non-fat percent of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Samples from the 1st, 2nd, and 3rd milking's were removed.

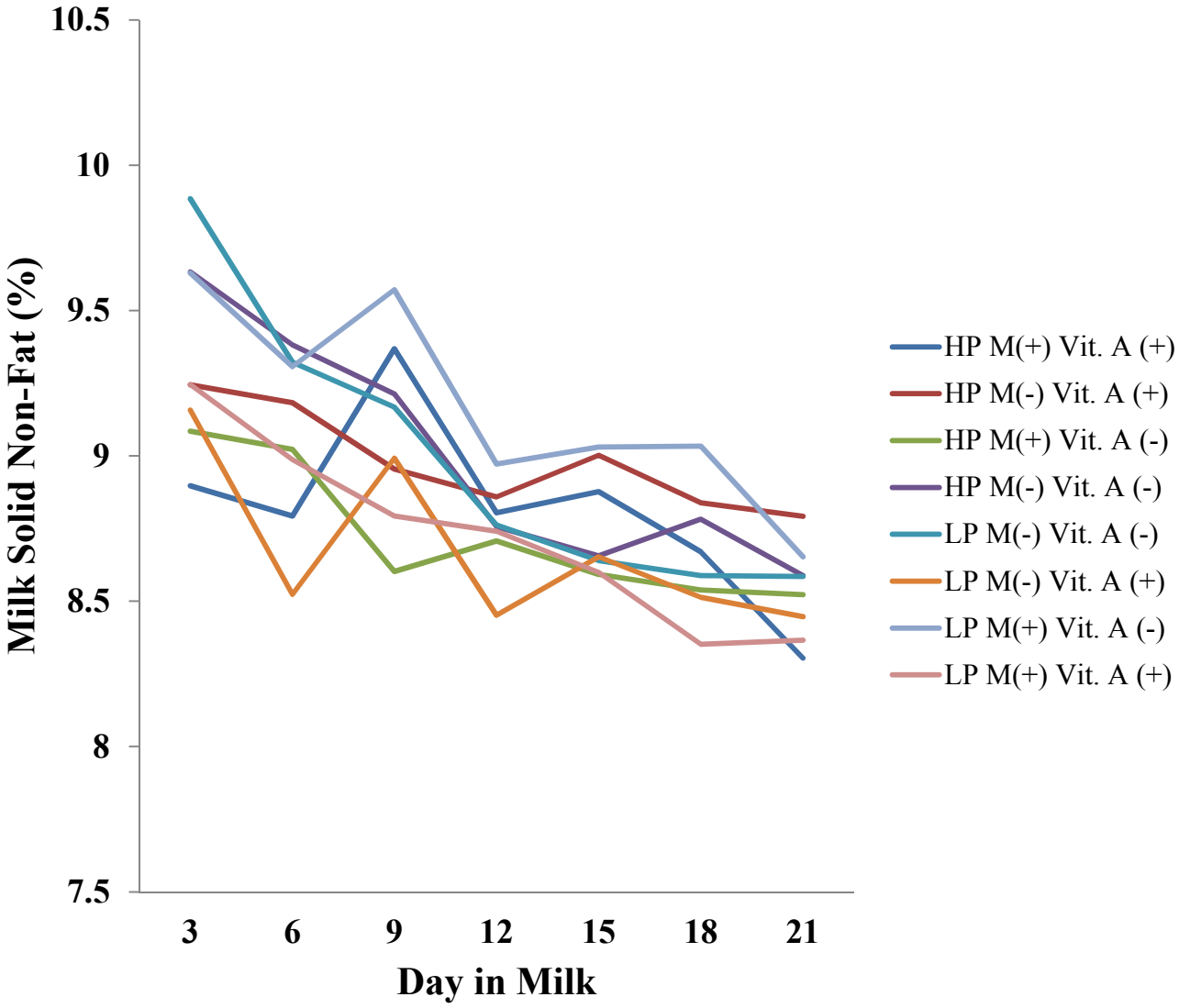

Figure 11. Means of somatic cell count of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Samples from the 1st, 2nd, and 3rd milking's were removed.

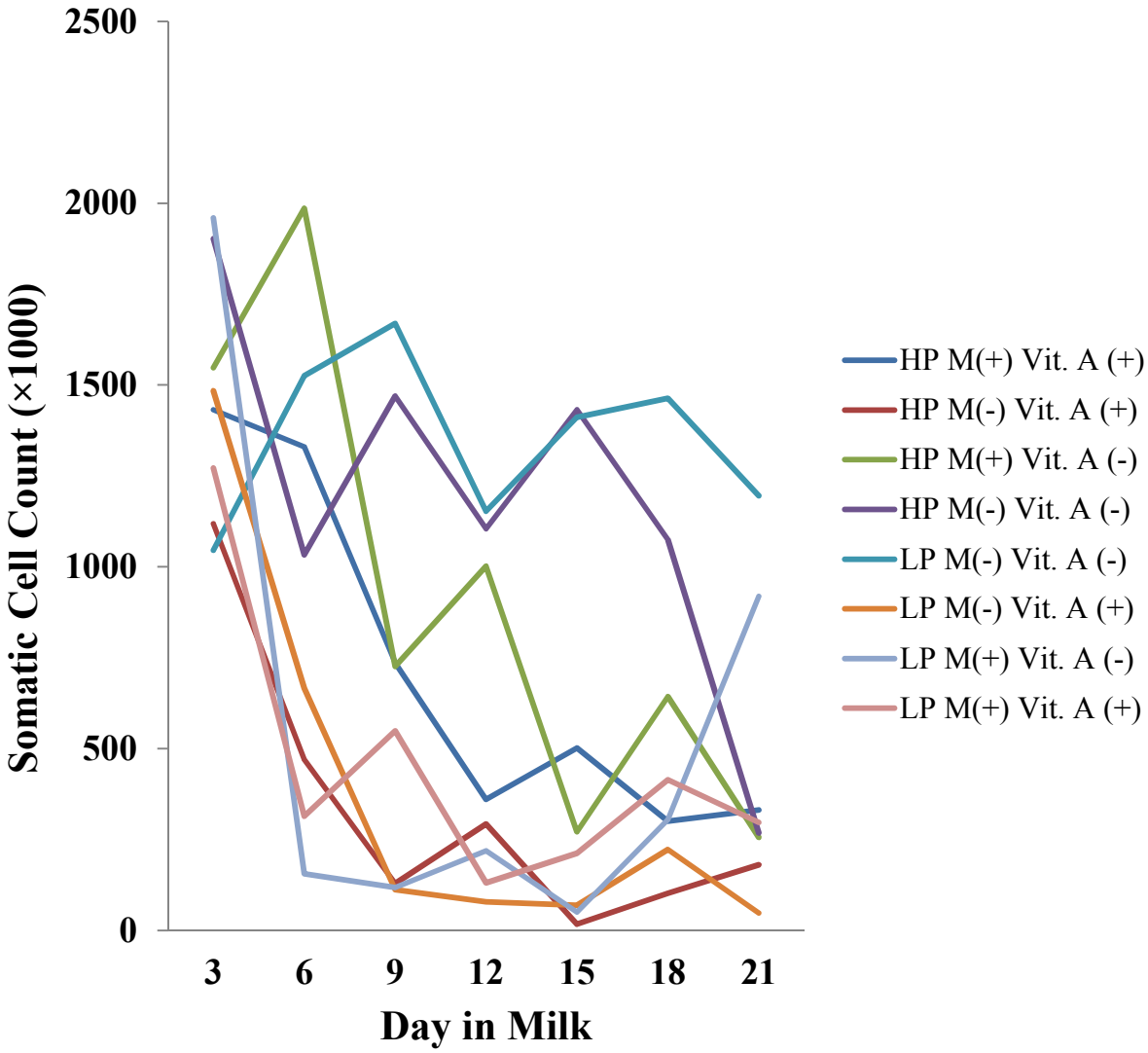

Figure 12. Least square means of milk linear somatic cell score of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Samples from the 1st, 2nd, and 3rd milking's were removed.

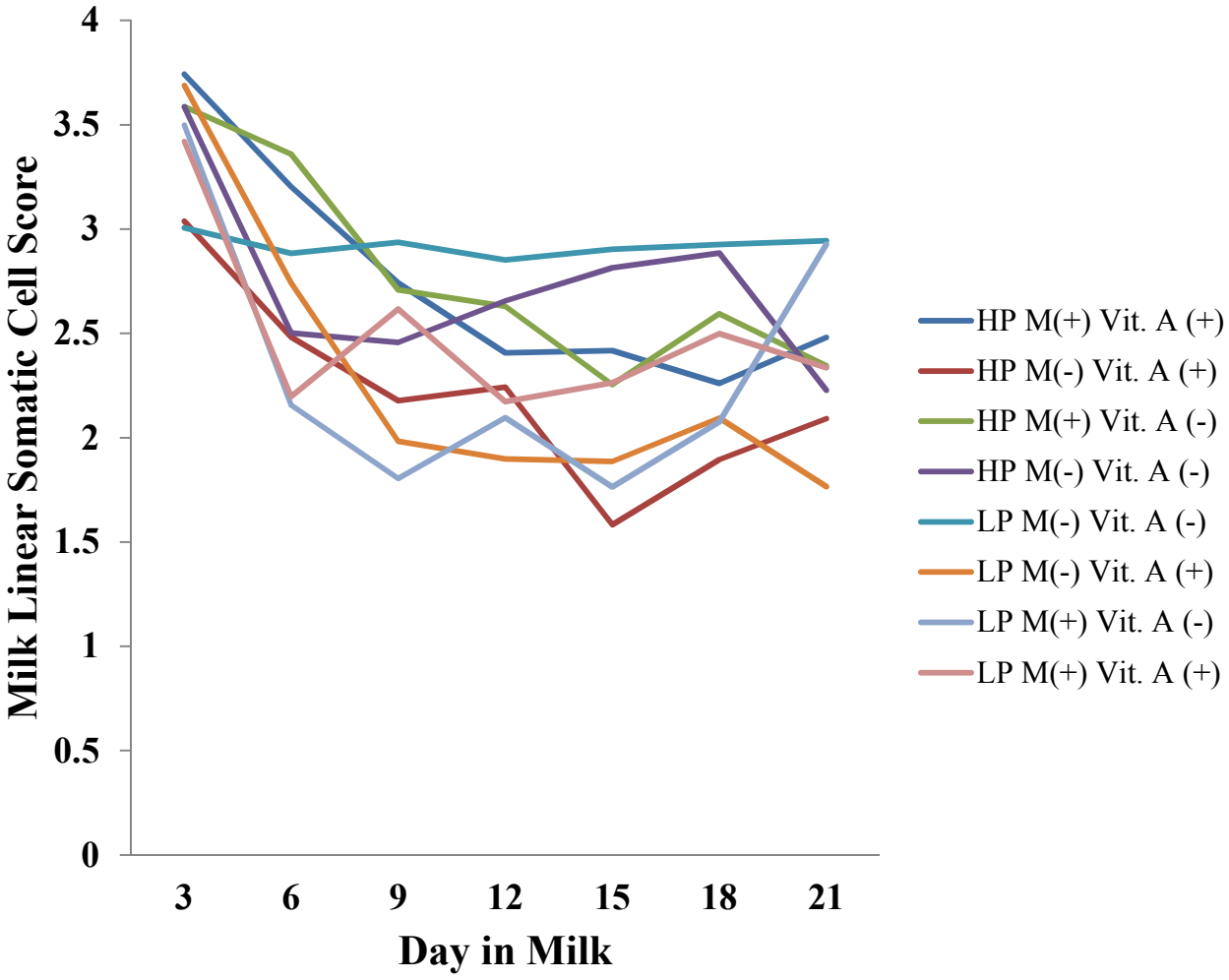

Figure 13. Least square means of energy corrected milk of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Samples from the 1st, 2nd, and 3rd milking's were removed.

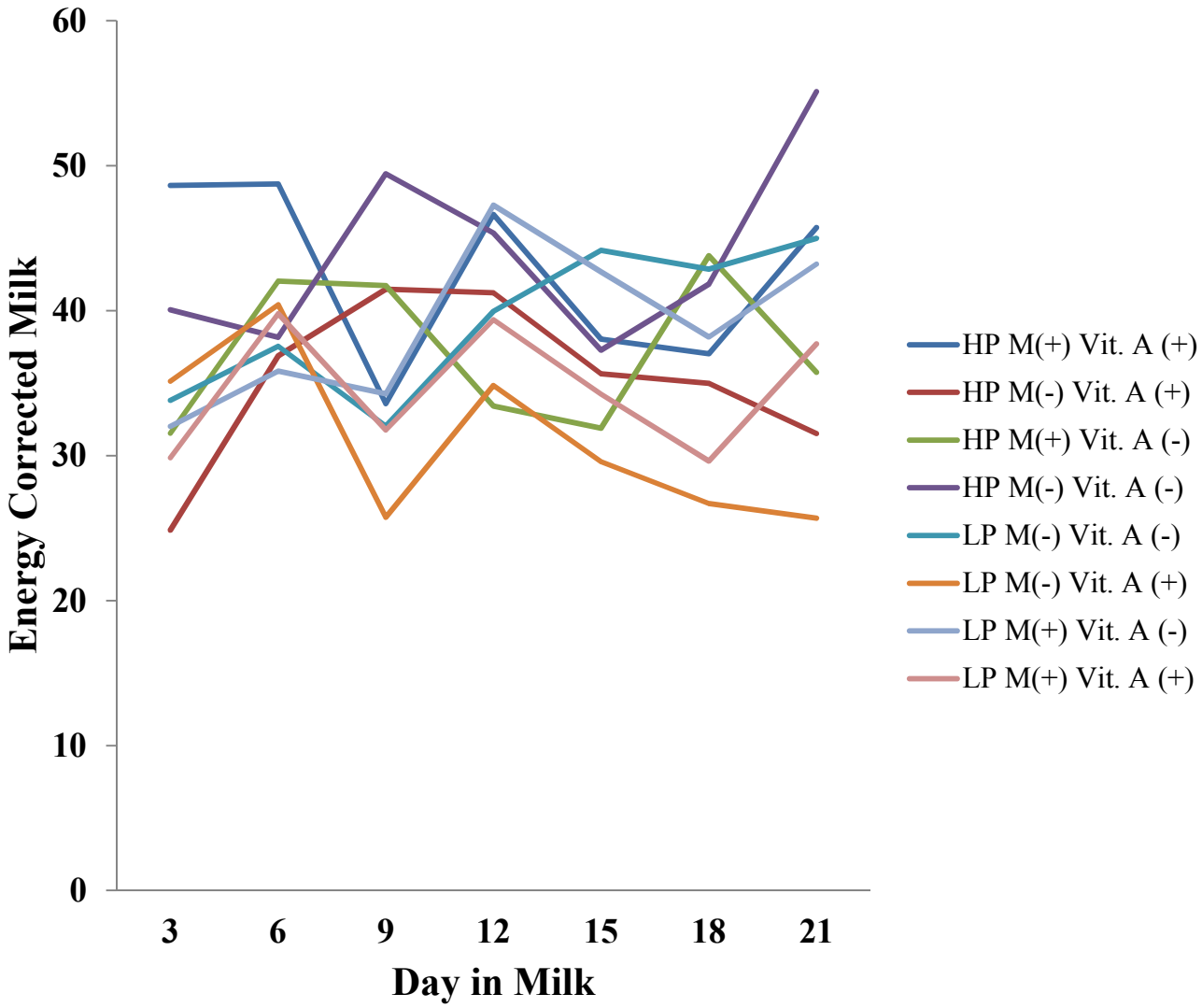

Figure 14. Least square means of milk fat yield of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Samples from the 1st, 2nd, and 3rd milking's were removed. Dietary monensin × vitamin A interaction ($P = 0.04$); cows that received monensin but did not receive vitamin A (closed bar), cows that did not receive monensin and did not receive vitamin A (open bar), cows that received monensin and vitamin A (grey bar), and cows that did not receive monensin nor vitamin A (slashed bar).

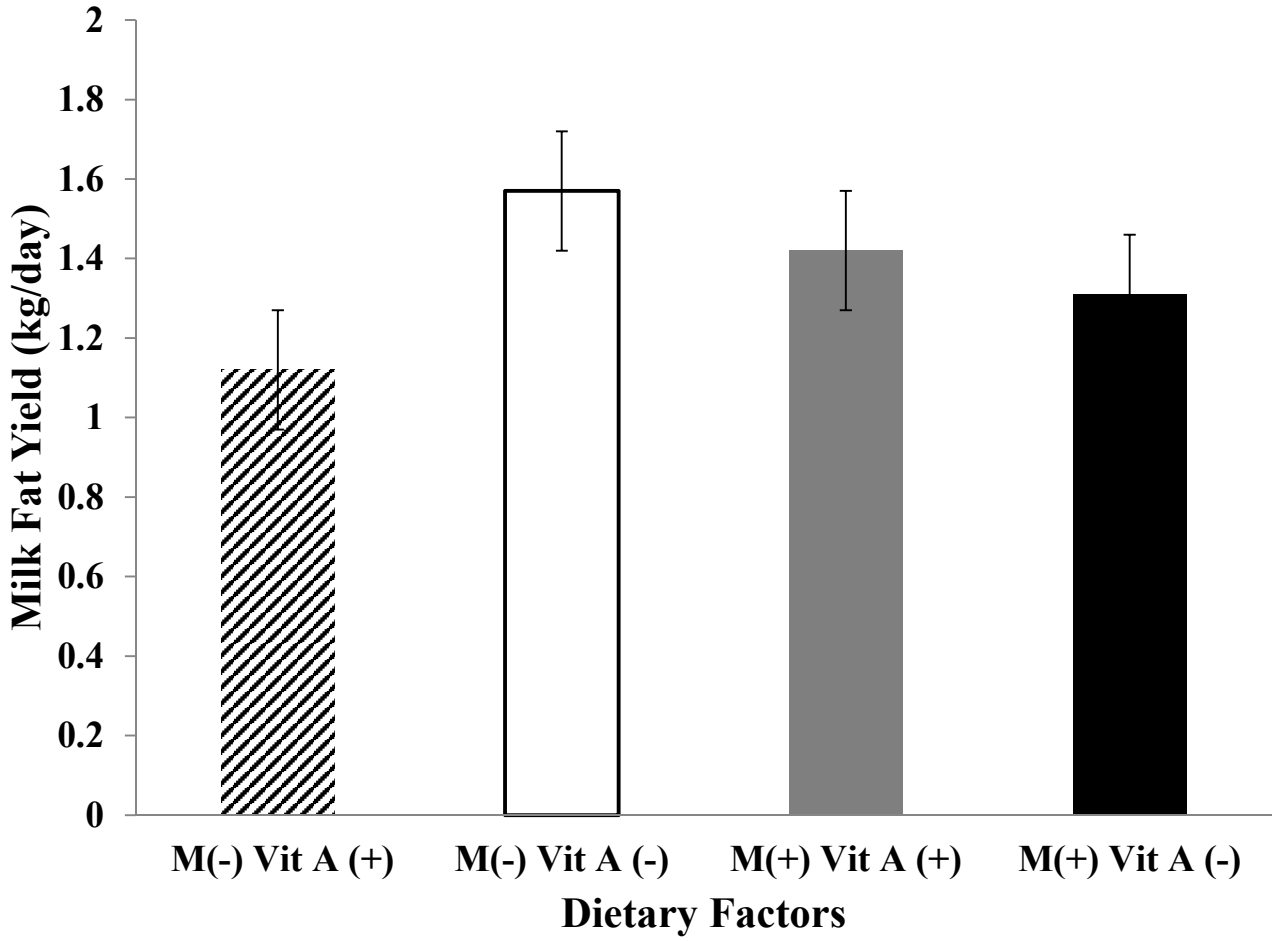

Figure 15. Least square means of milk solid non-fat percent of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Samples from the 1st, 2nd, and 3rd milking's were removed. Dietary vitamin A × protein interaction ($P = 0.05$); cows that did not receive vitamin A but received high crude protein (closed bar), cows that received vitamin A and received high crude protein (open bar), cows that did not receive vitamin A and received low crude protein (grey bar), and cows that received vitamin A and received low crude protein (slashed bar).

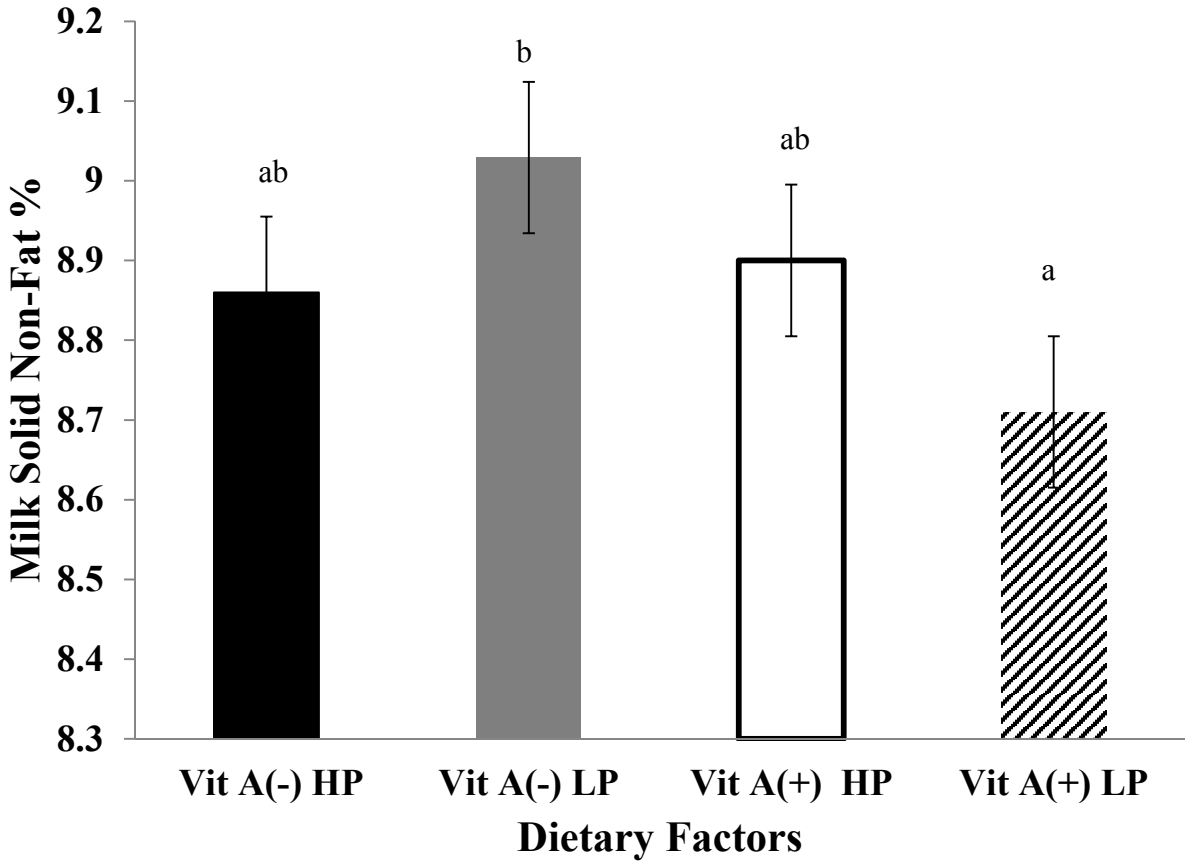

Figure 16. Least square means of milk somatic cell count of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Samples from the 1st, 2nd, and 3rd milking's were removed. Dietary vitamin A interaction (*P* = 0.001); cows received vitamin A (closed bar), no vitamin A (open bar).

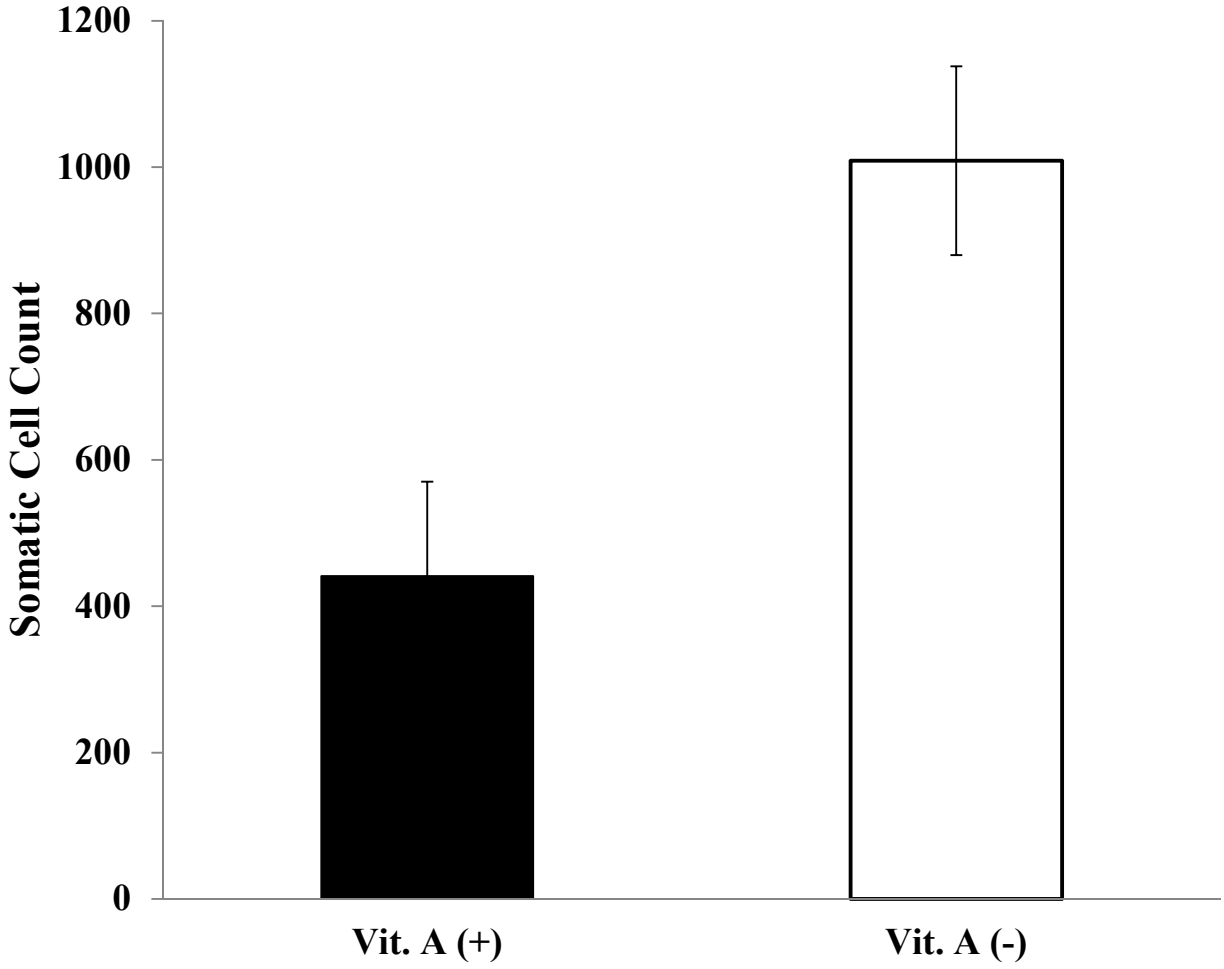

Figure 17. Least square means of milk fat percent of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Samples from the 1st, 2nd, and 3rd milking's were removed. Dietary time × protein interaction (P = 0.02)

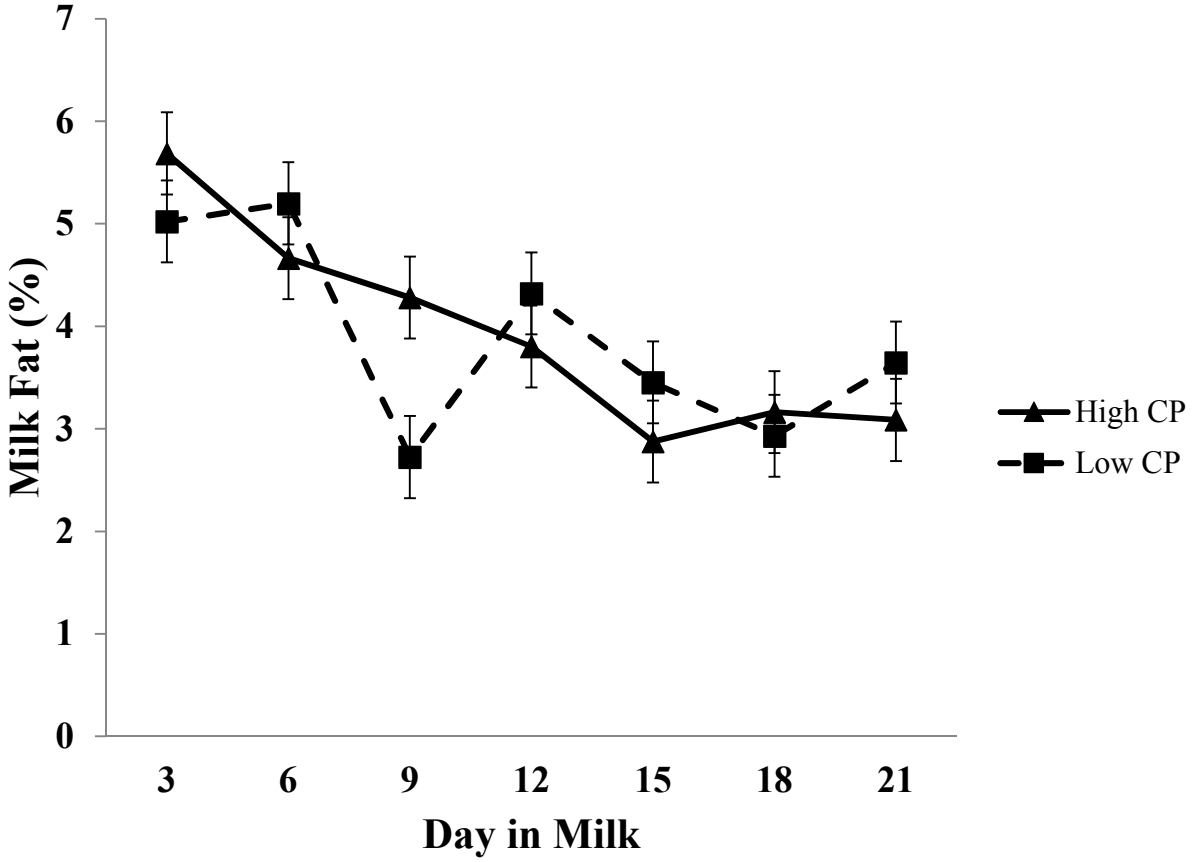

Figure 18. Least square means of milk protein percentage of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Dietary monensin × protein interaction ($P = 0.001$); cows that did not receive monensin but received high crude protein (closed bar), cows that did not receive monensin but received low crude protein (open bar), cows that received monensin and high crude protein (grey bar), and cows that received monensin and received low crude protein (slashed bar).

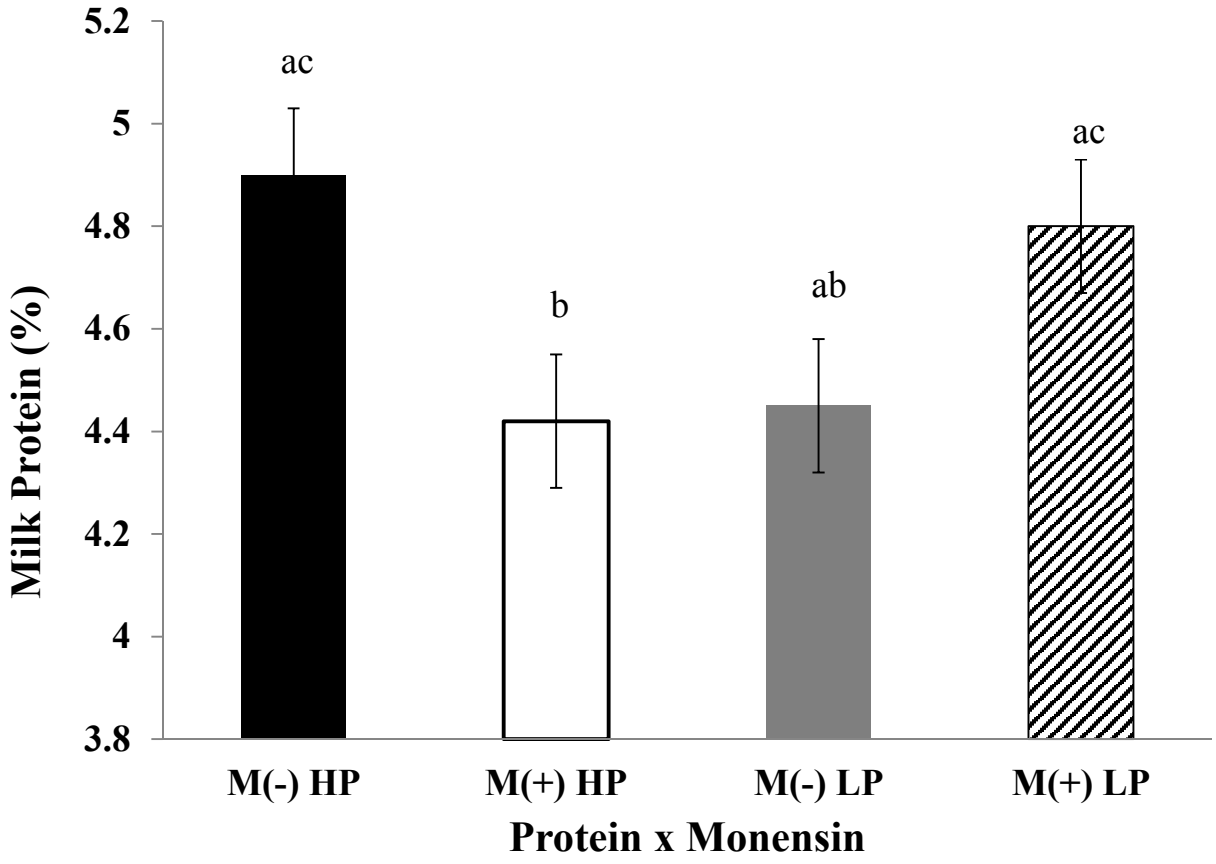

Figure 19. Least square means of milk lactose percentage of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Dietary protein × vitamin A interaction ($P = 0.05$); cows that did not receive vitamin A but received high crude protein (closed bar), cows that did not receive vitamin A and low crude protein (open bar), cows that received vitamin A and high crude protein (grey bar), and cows that received vitamin A and low crude protein (slashed bar). Significant difference among factors could not be determined.

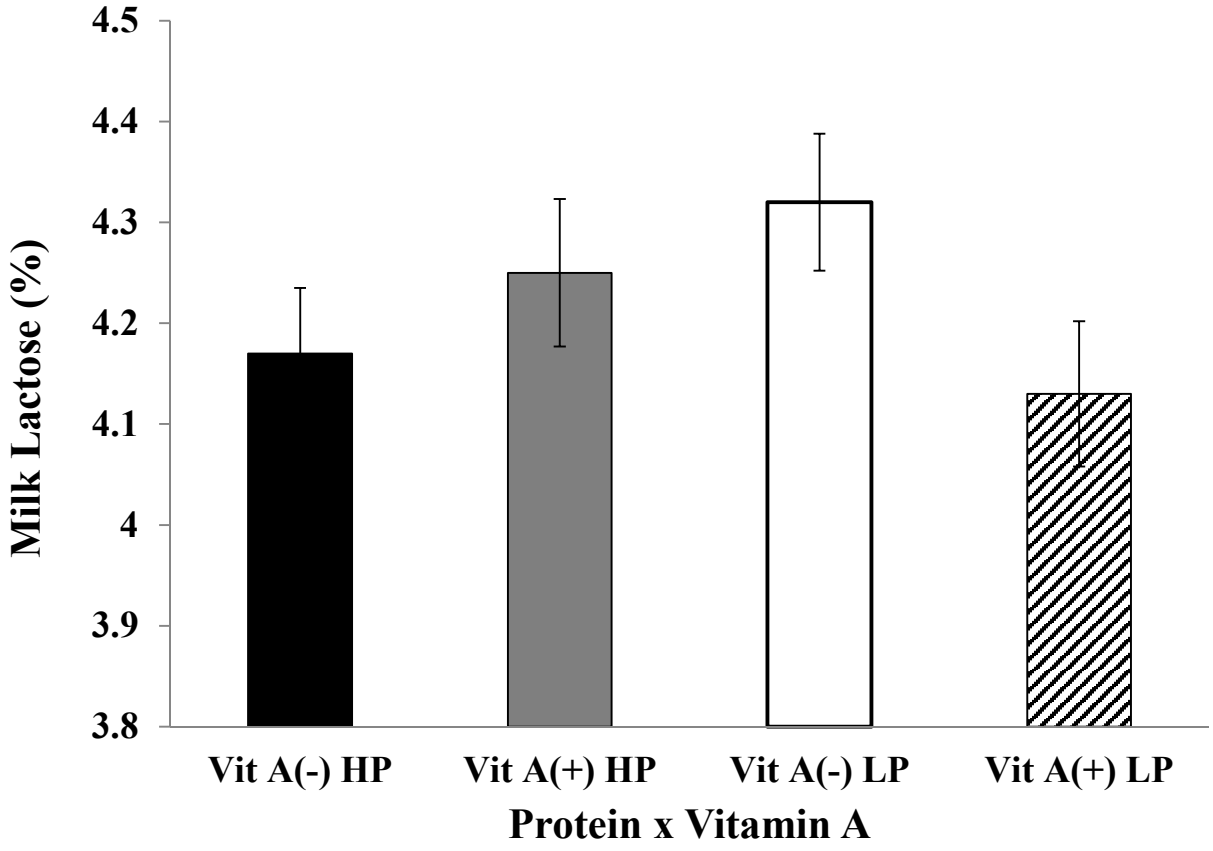

Figure 20. Least square means of somatic cell count (SCC) of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A = 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Dietary vitamin A interaction ($P = 0.04$); cows received vitamin A (closed bar), no vitamin A (open bar).

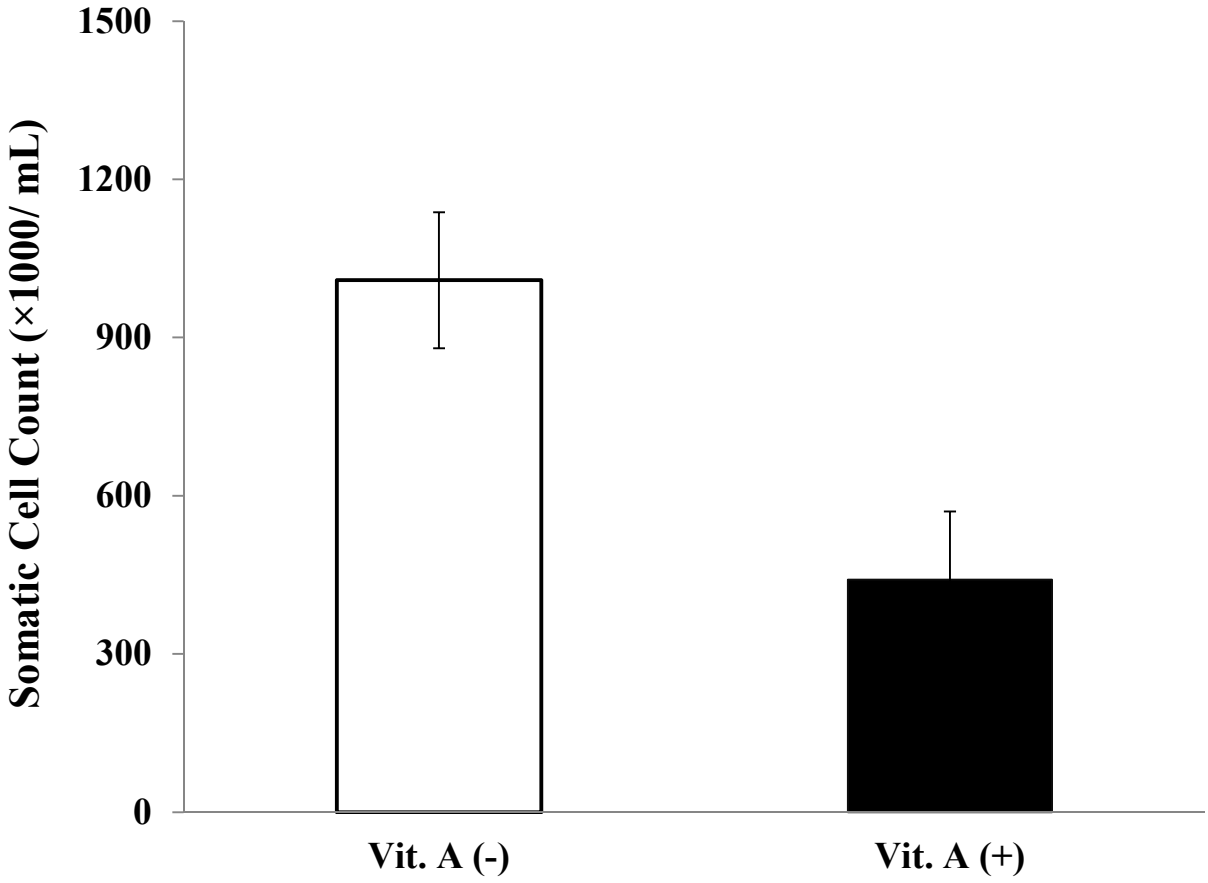

Figure 21. Least square means of the linear somatic cell score of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Dietary vitamin A interaction ($P = 0.07$); cows received vitamin A (closed bar), no vitamin A (open bar).

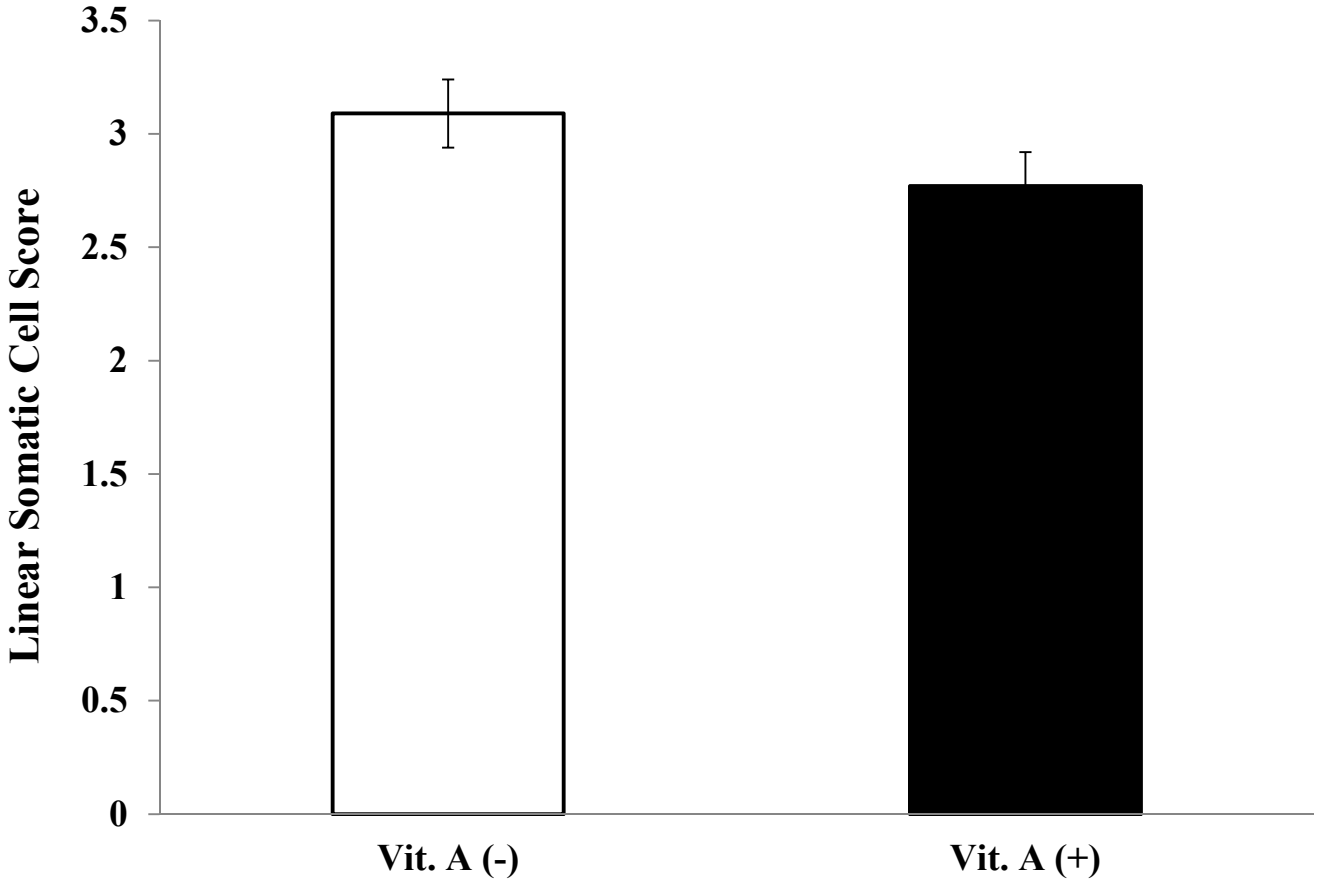

Figure 22. Least square means of milk retinol concentration of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Diet × time interaction ($P = 0.3$).

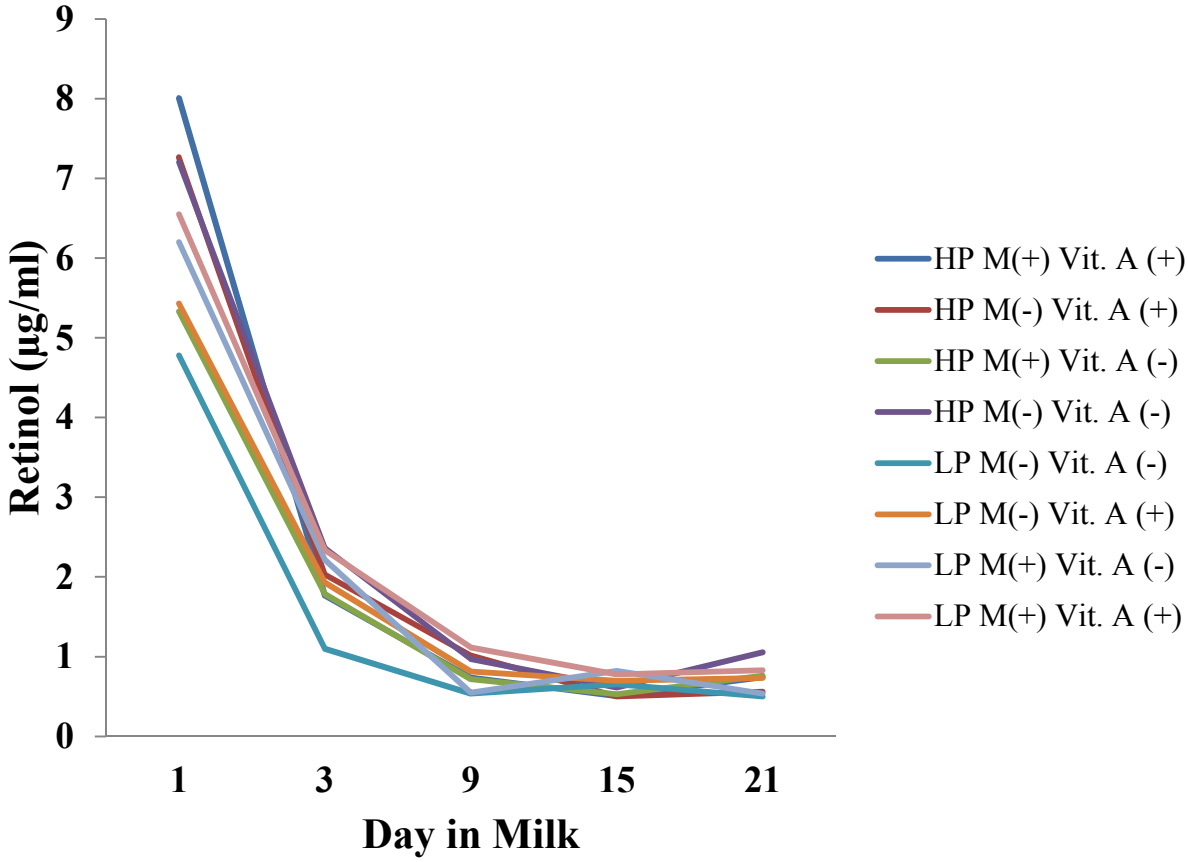

Figure 23. Least square means of milk α-tocopherol concentration of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Diet × time interaction ($P = 0.4$).

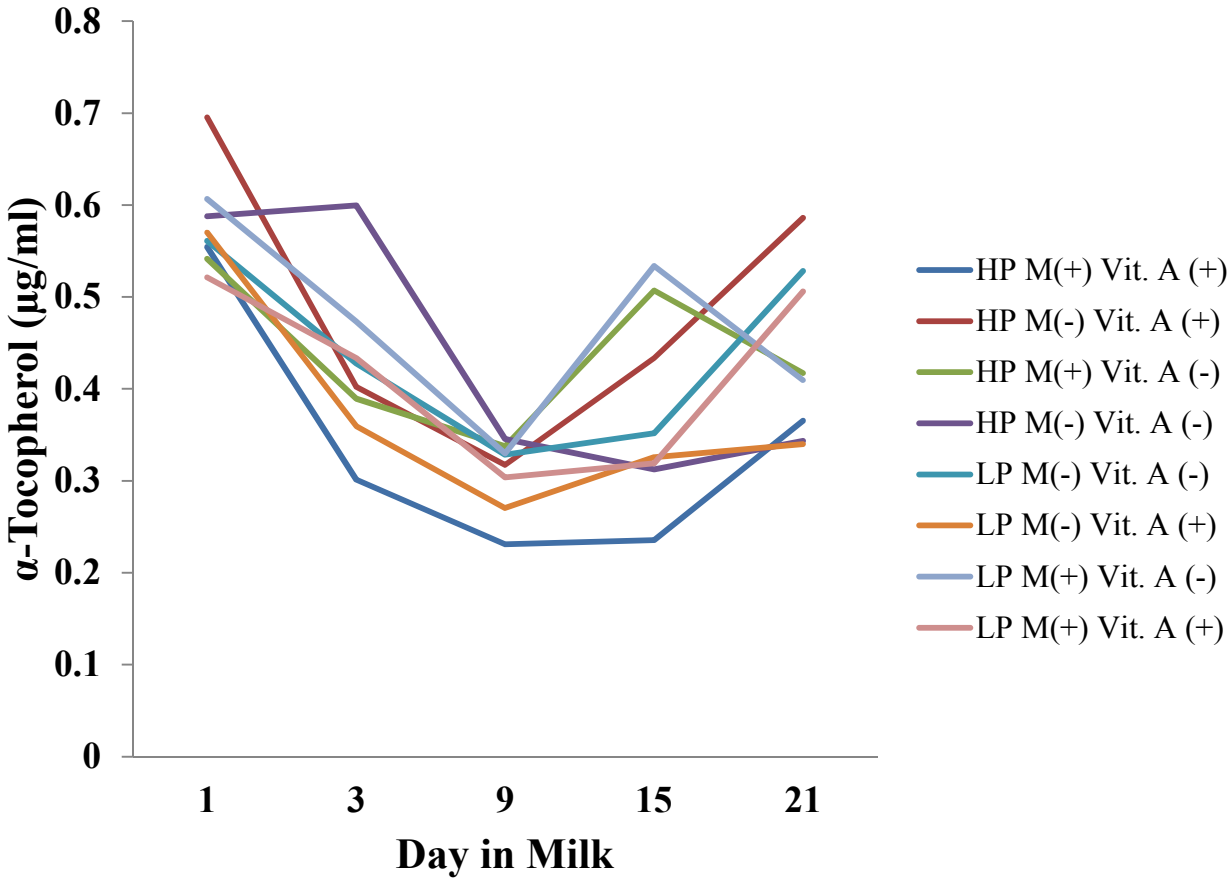

Figure 24. Least square means of milk β-carotene concentration of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Diet × time interaction ($P = 0.83$)

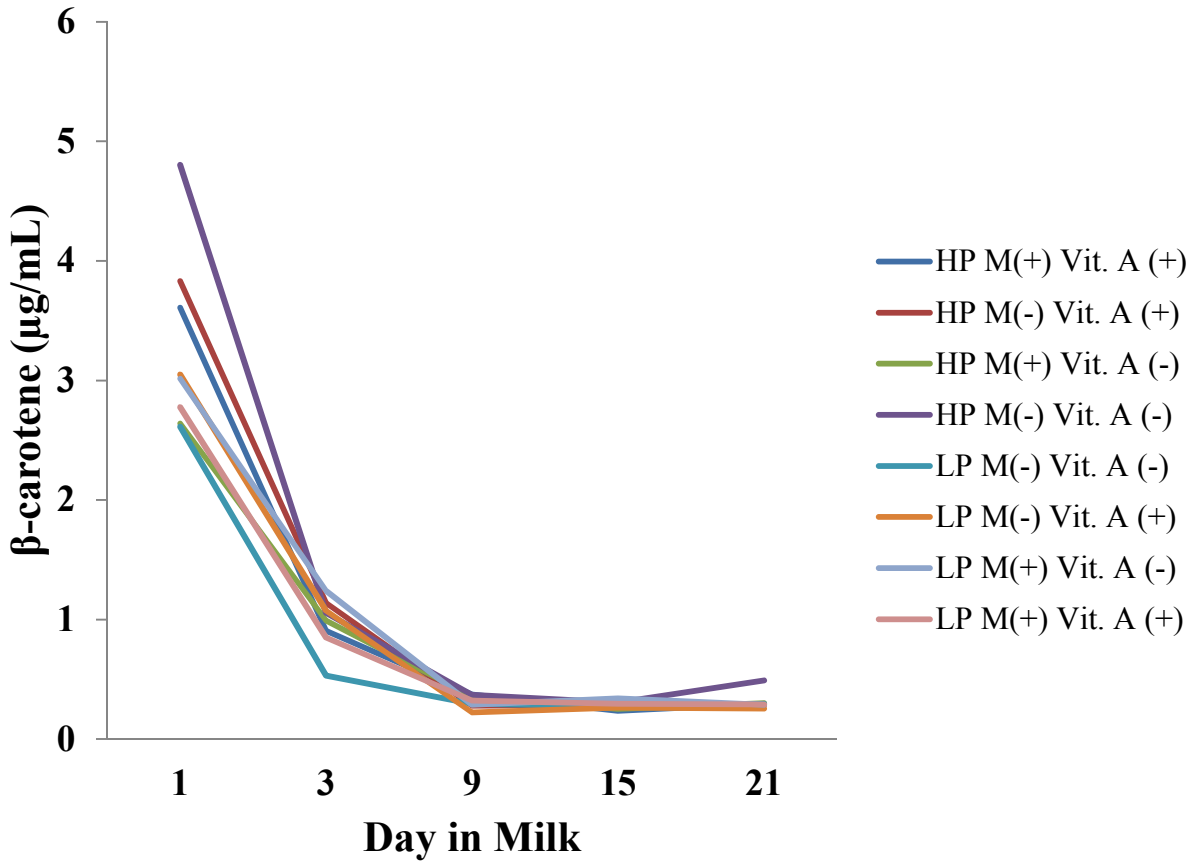

Figure 25. Least square means of milk retinol concentration of dairy cows (n=80) throughout the transition period in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Dietary crude protein × time interaction ($P = 0.04$); cows received high crude protein (black line), low crude protein (dashed line). Asterisks show significant difference at time point specified.

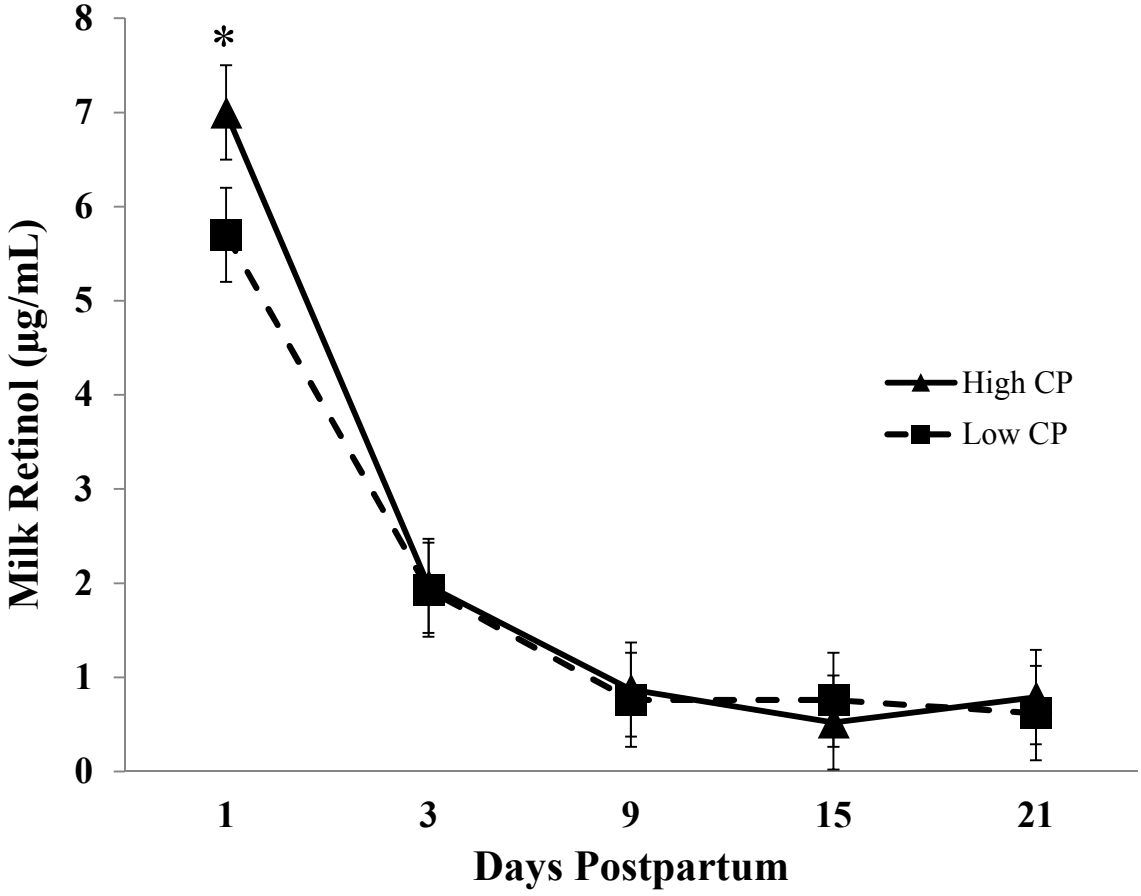

Figure 26. Least square means of milk retinol concentration (µg/mL) of dairy cows (n=80) throughout the transition period in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Dietary monensin × protein interaction ($P = 0.01$); cows that did not receive monensin but received high crude protein (grey bar), cows that did not receive monensin and received low crude protein (closed bar), cows that received monensin and high crude protein (open bar), and cows that received monensin and received low crude protein (slashed bar).

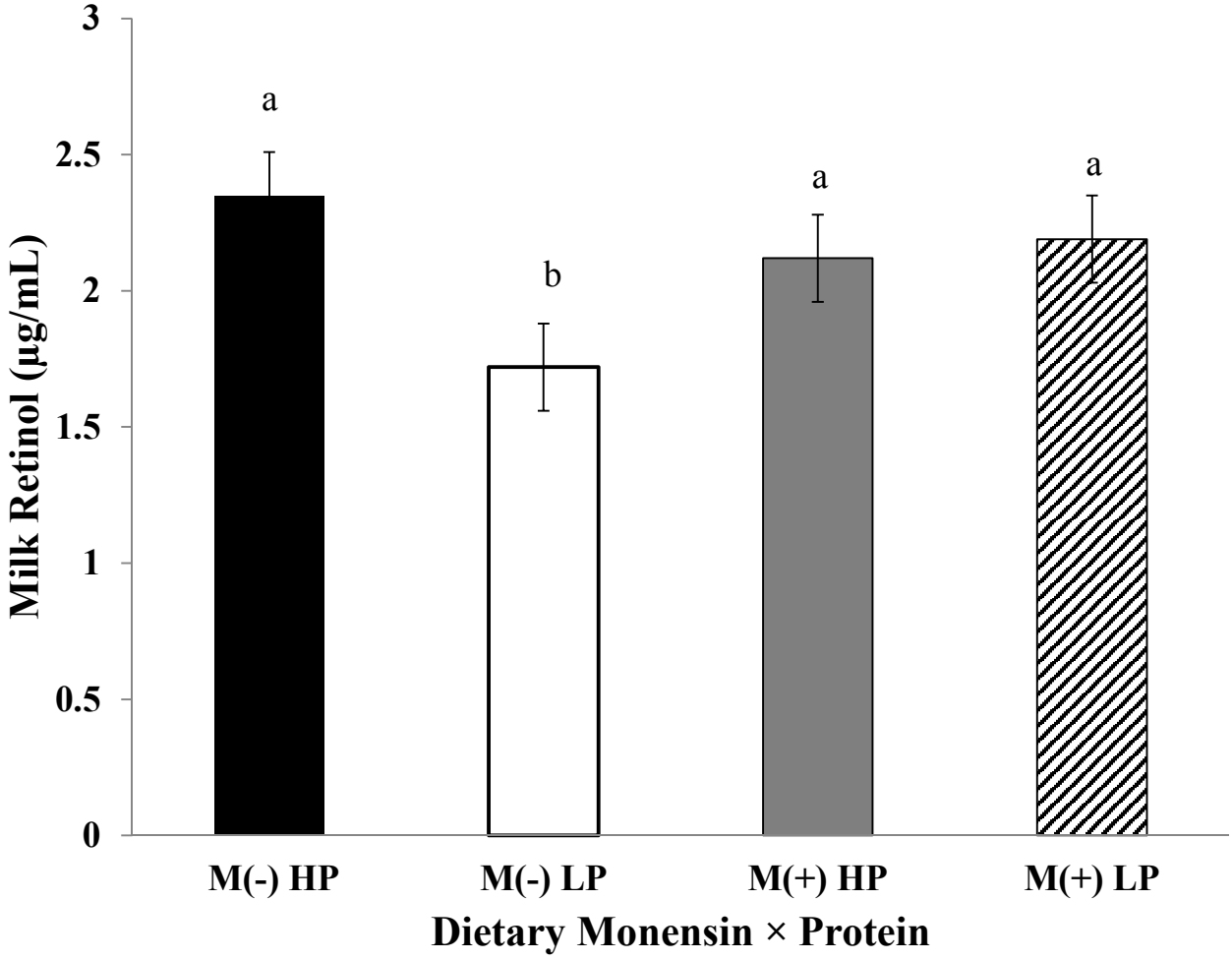

Figure 27. Least square means of milk α-tocopherol concentration (µg/mL) of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Dietary monensin × protein interaction ($P = 0.04$); cows that did not receive monensin but received high crude protein (closed bar), cows that did not receive monensin but received low crude protein (open bar), cows that received monensin and high crude protein (grey bar), and cows that received monensin and received low crude protein (slashed bar). Significant difference among treatments could not be determined.

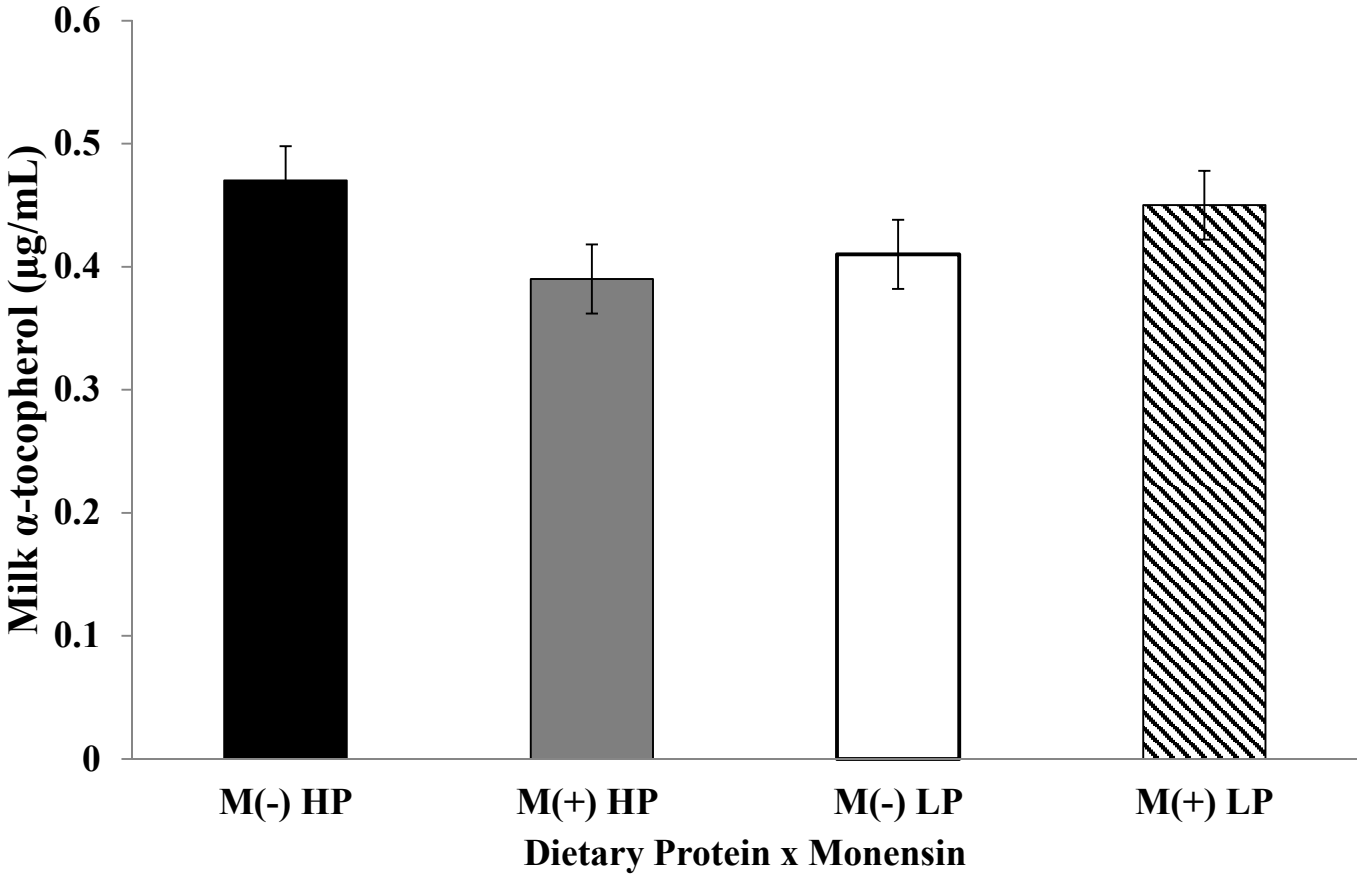

Figure 28. Least square means of serum nonesterified fatty acid (NEFA) concentration of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Diet × time interaction ($P = 0.6$)

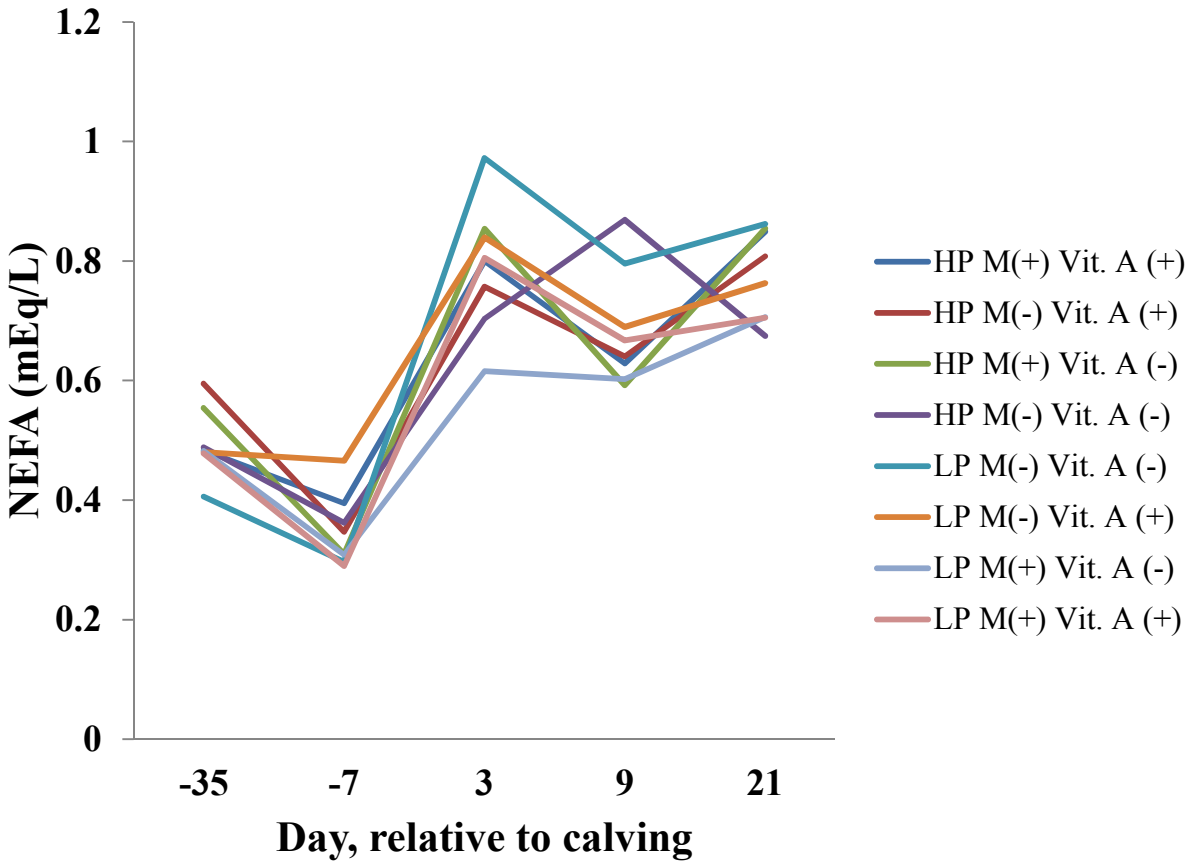

Figure 29. Least square means of serum haptoglobin concentration of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Diet × time interaction ($P = 0.2$)

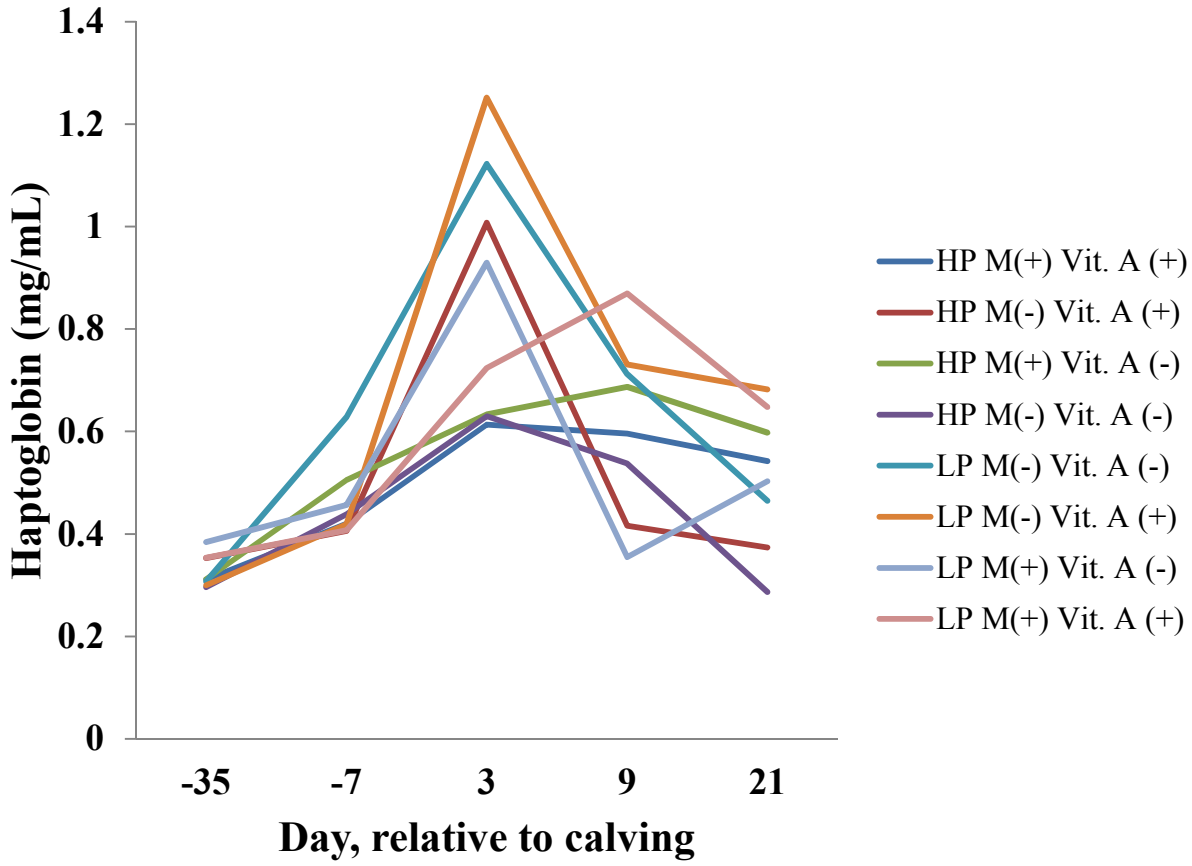

Figure 30. Least square means of serum β-hydroxybuterate concentration of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Diet × time interaction ($P = 0.8$)

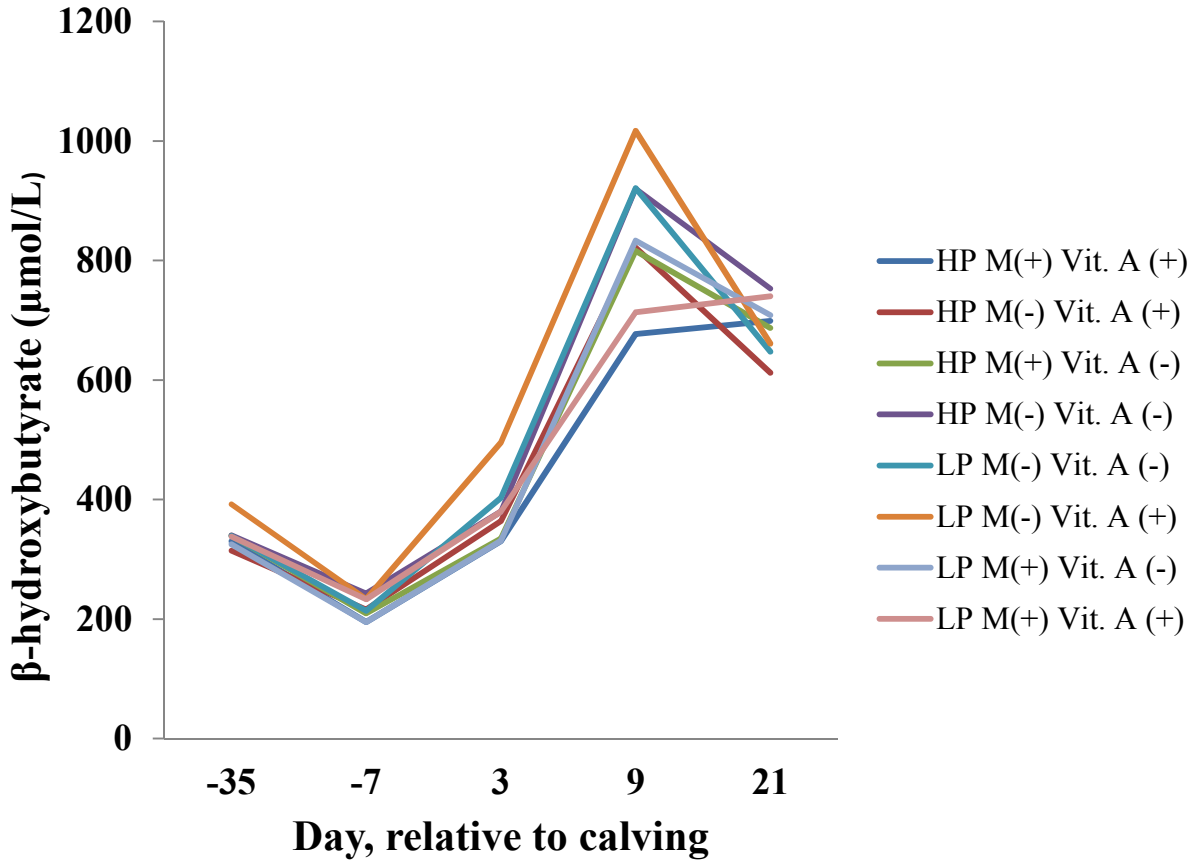

Figure 31. Least square means of serum malondialdehyde (MDA) concentration of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Diet × time interaction ($P = 0.7$)

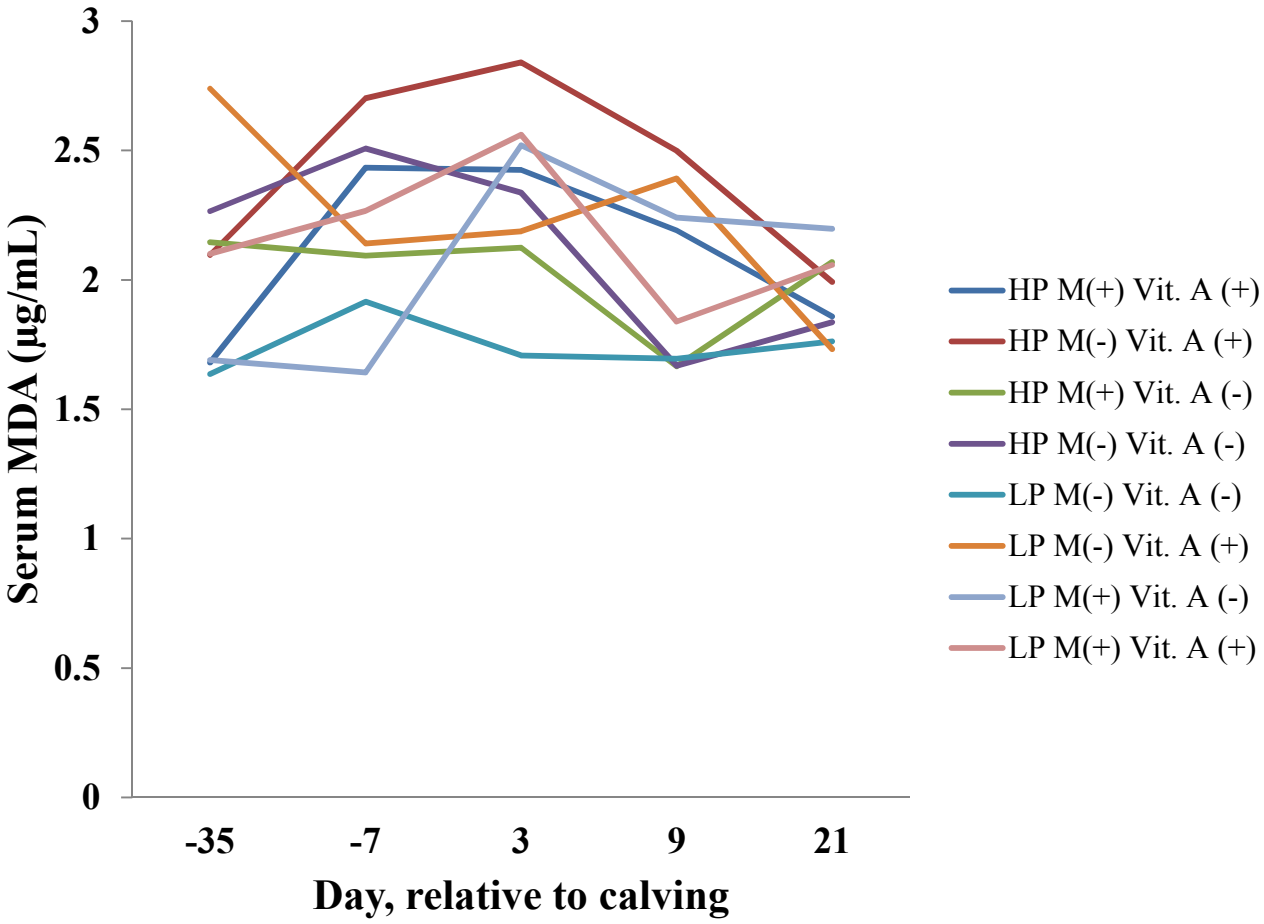

Figure 32. Least square means of serum haptoglobin concentration of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Dietary monensin × time interaction ($P = 0.01$); cows received monensin (black line), or no monensin (dashed line). Asterisks show significant difference at time point specified

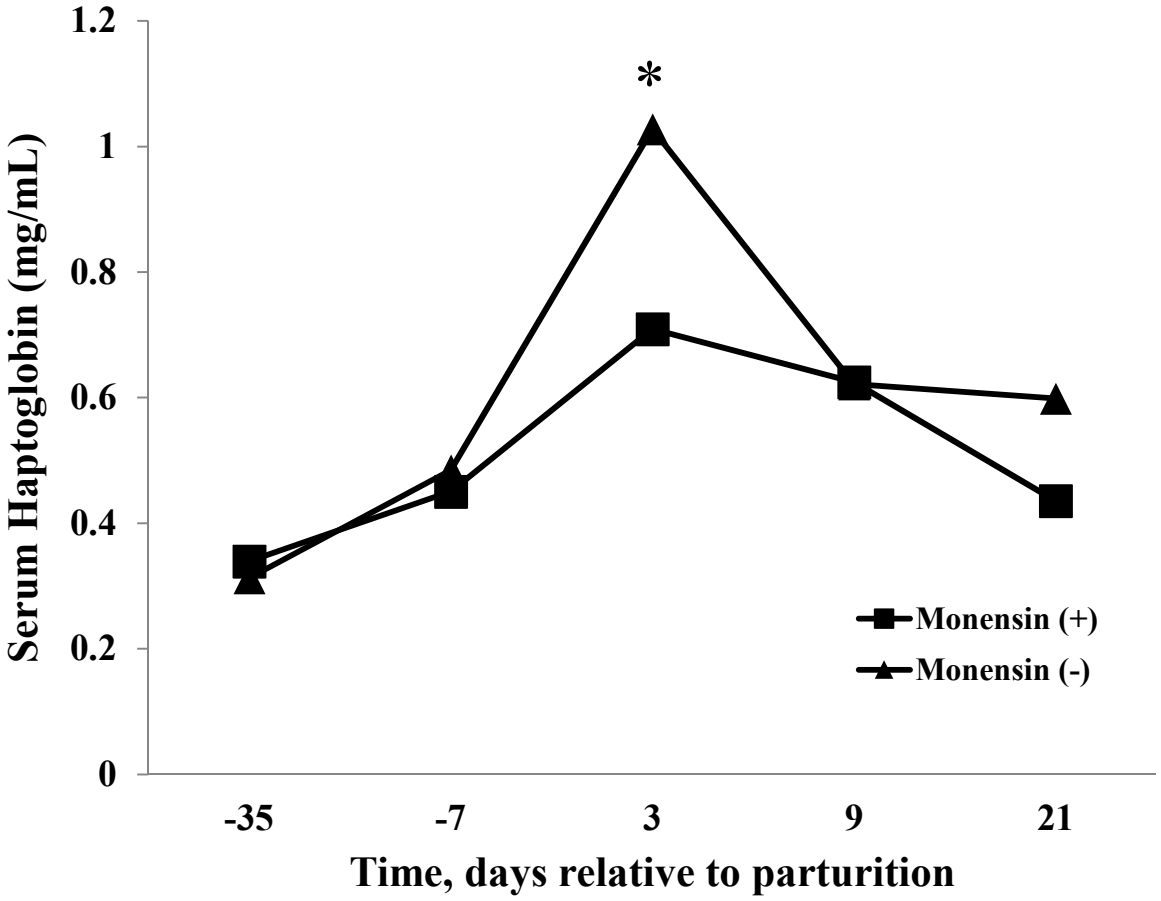

Figure 33. Least square means of serum haptoglobin (mg/mL) of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Dietary crude protein interaction ($P = 0.01$); cows received high crude protein (closed bar), low crude protein (open bar).

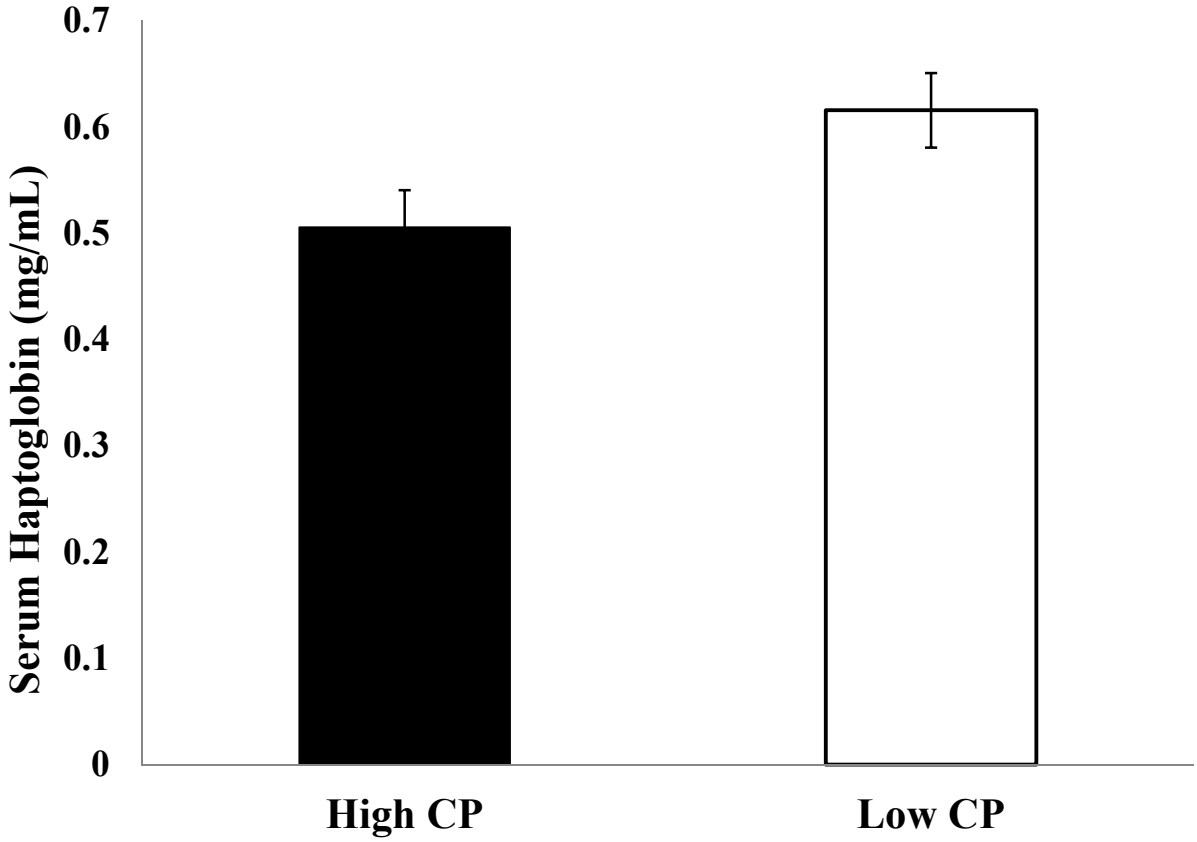

Figure 34. Least square means of serum β-hydroxybuterate concentration of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Dietary monensin × time interaction ($P = 0.02$); cows received monensin (black line), or no monensin (dashed line). Asterisks show significant difference at time point specified

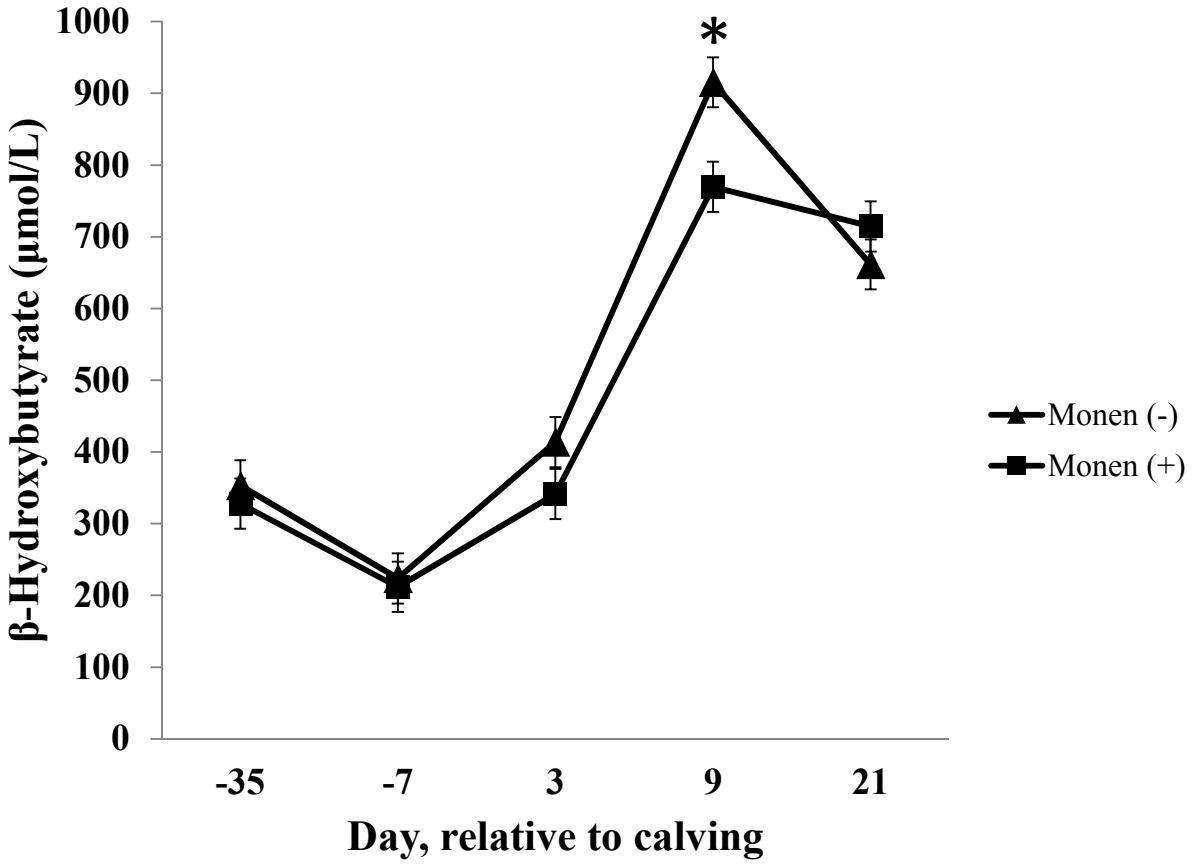

Figure 35. Least square means of Malondialdehyde (MDA) detected of dairy cows (n=80) throughout the transition period in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Dietary vitamin A interaction ($P = 0.06$); cows received vitamin A (black bar), no vitamin A (open bar).

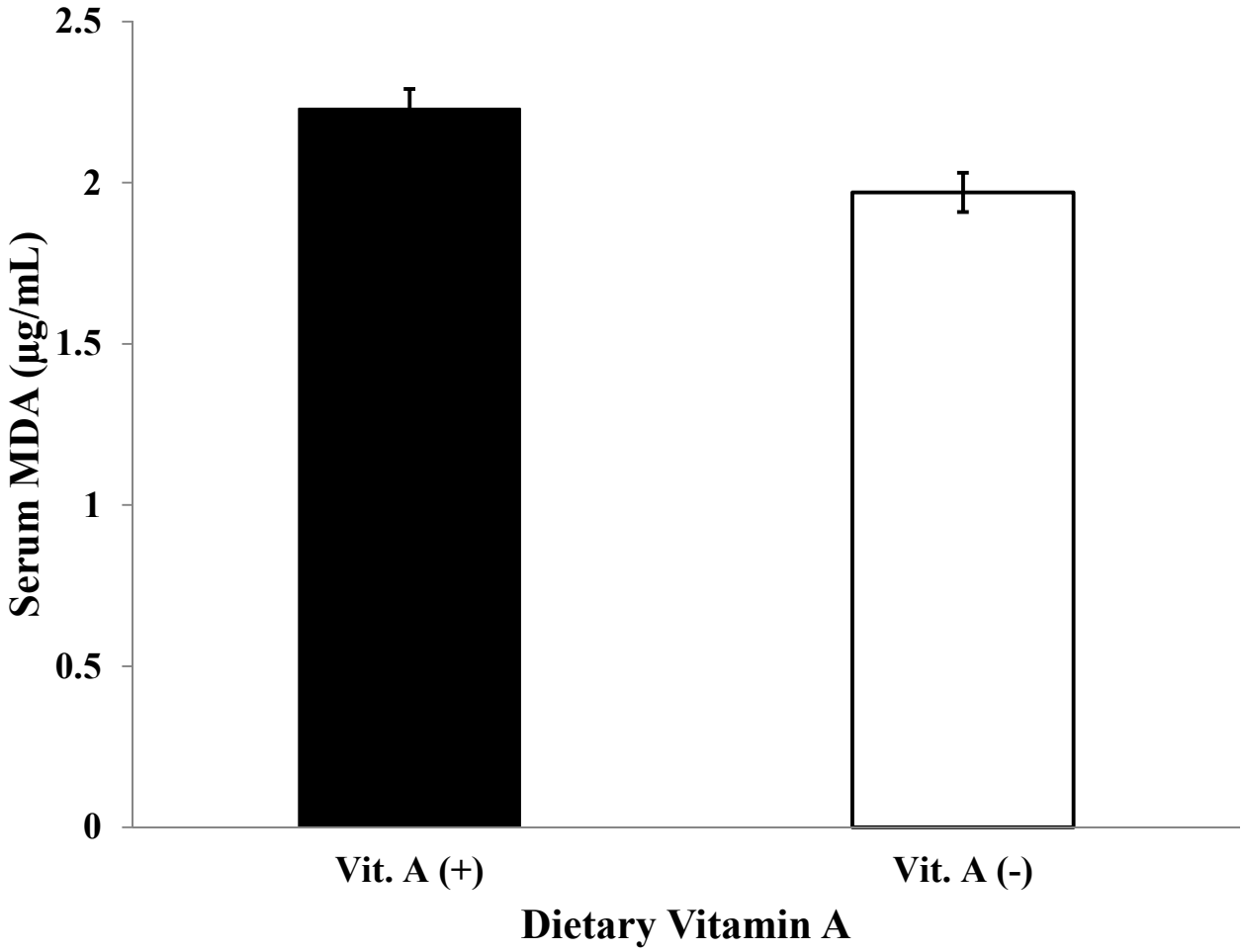

Figure 36. Least square means of serum retinol concentration of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Diet × time interaction ($P = 0.89$)

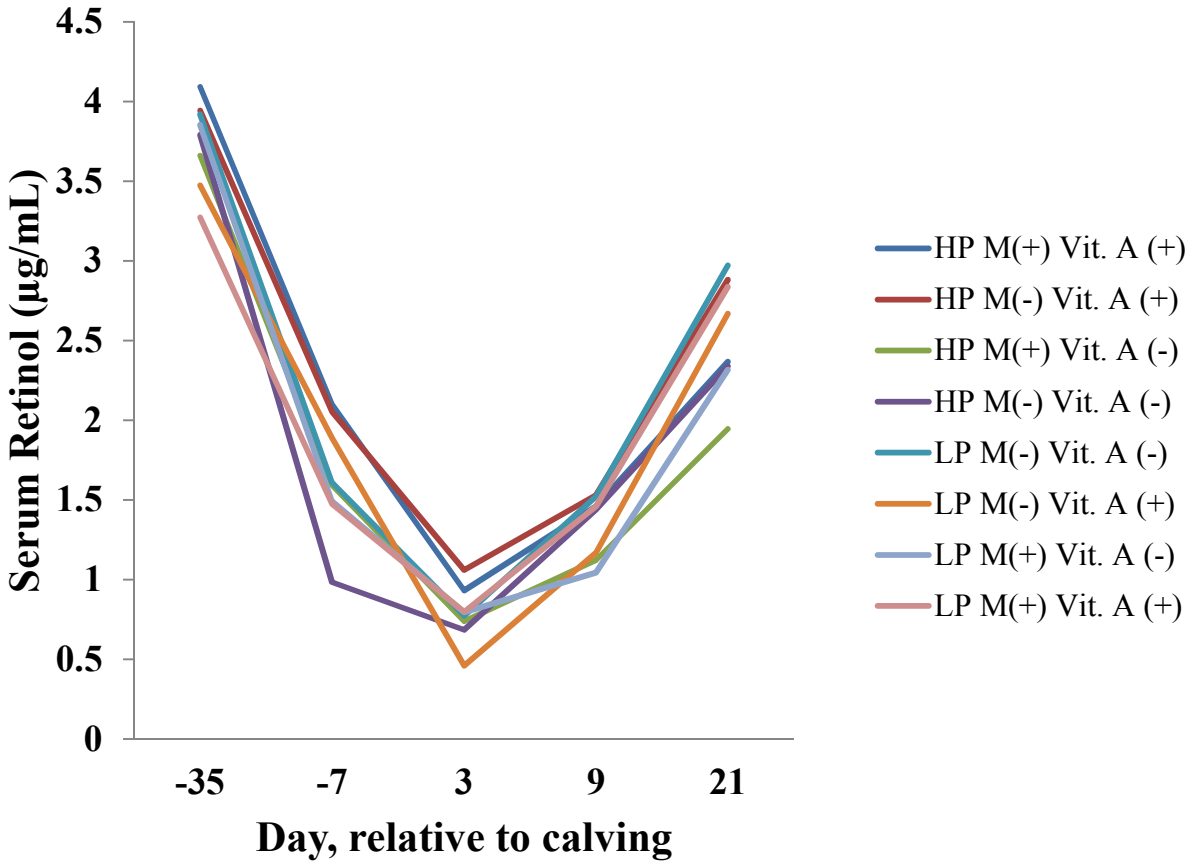

Figure 37. Least square means of serum α-tocopherol concentration of dairy cows (n=80) in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Diet × time interaction ($P = 0.96$)

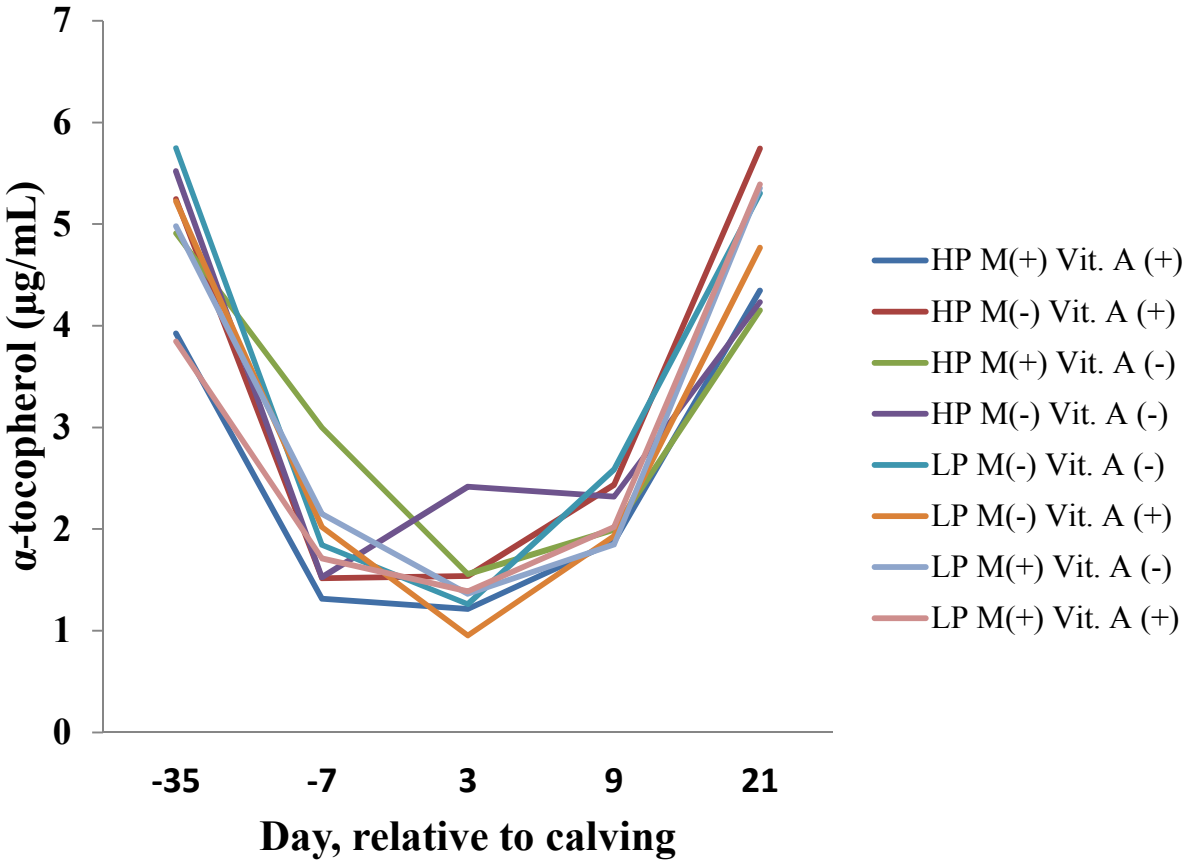

Figure 38. Least square means of serum *13 cis* retinoic acid concentration of dairy cows (n=80) throughout the transition period in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Diet × time interaction ($P = 0.98$)

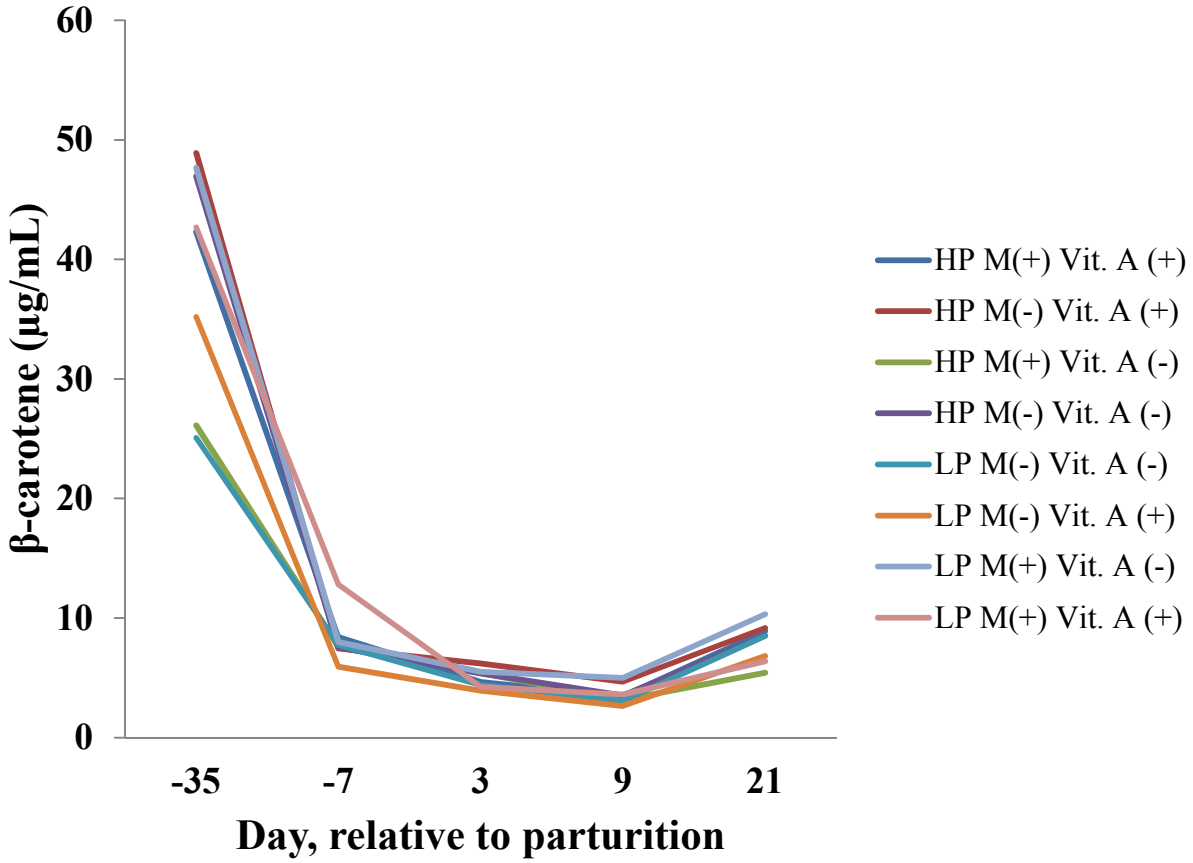

Figure 39. Least square means of serum *13 cis* retinoic acid concentration of dairy cows (n=80) throughout the transition period in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Diet × time interaction ($P = 0.3$)

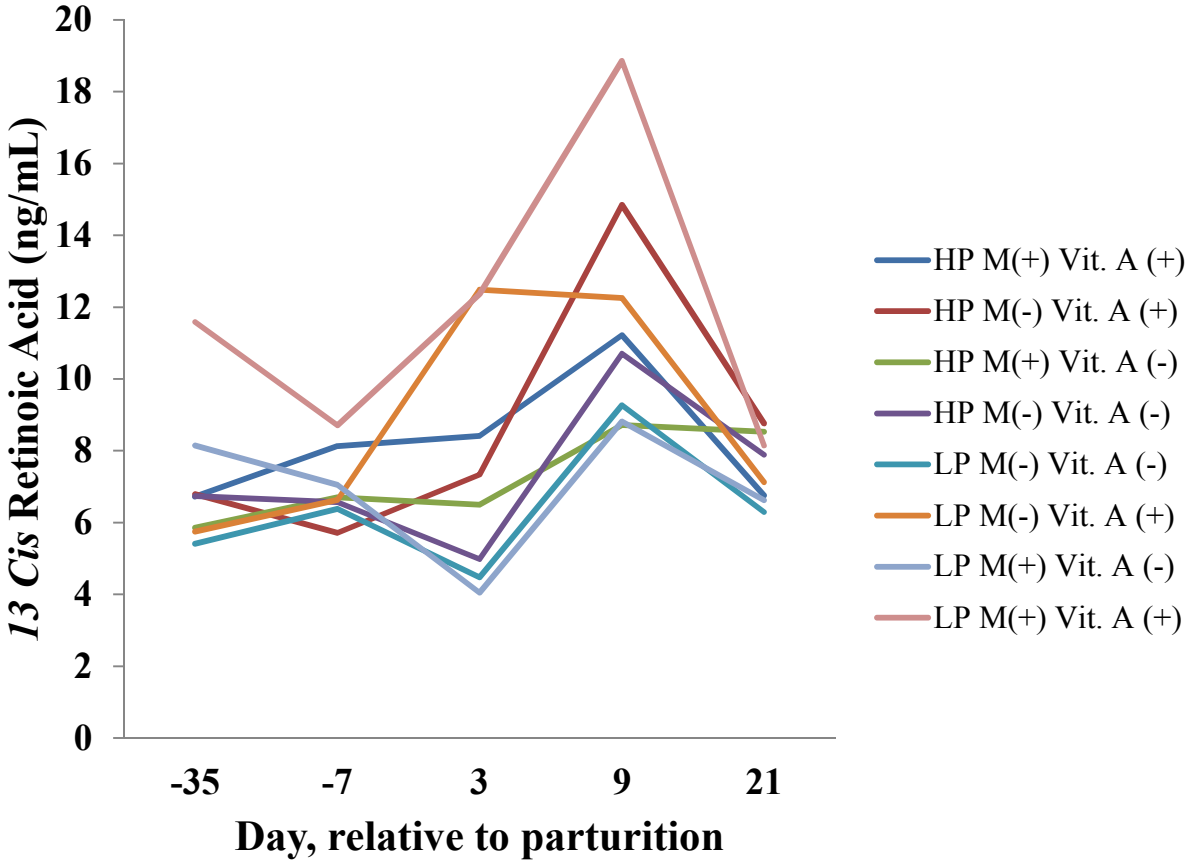

Figure 40. Least square means of serum *13 cis* retinoic acid concentration of dairy cows (n=80) throughout the transition period in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Diet × time interaction ($P = 0.8$)

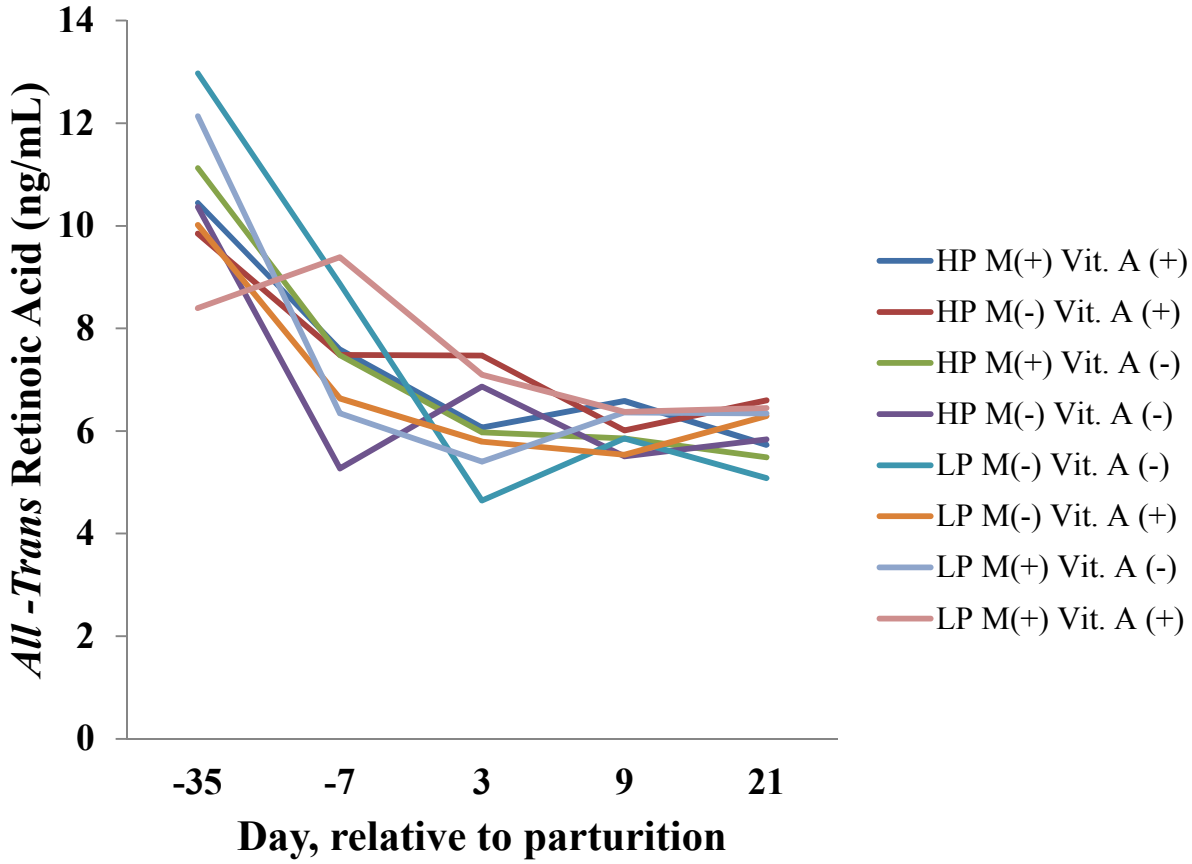

Figure 41. Least square means of serum *13 cis* retinoic acid concentration (µg/mL) of dairy cows (n=80) throughout the transition period in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Dietary protein × vitamin A interaction ($P = 0.02$); cows that did not receive vitamin A but received high crude protein (closed bar), cows that did not receive vitamin A and low crude protein (open bar), cows that received vitamin A and high crude protein (grey bar), and cows that received vitamin A and low crude protein (slashed bar).

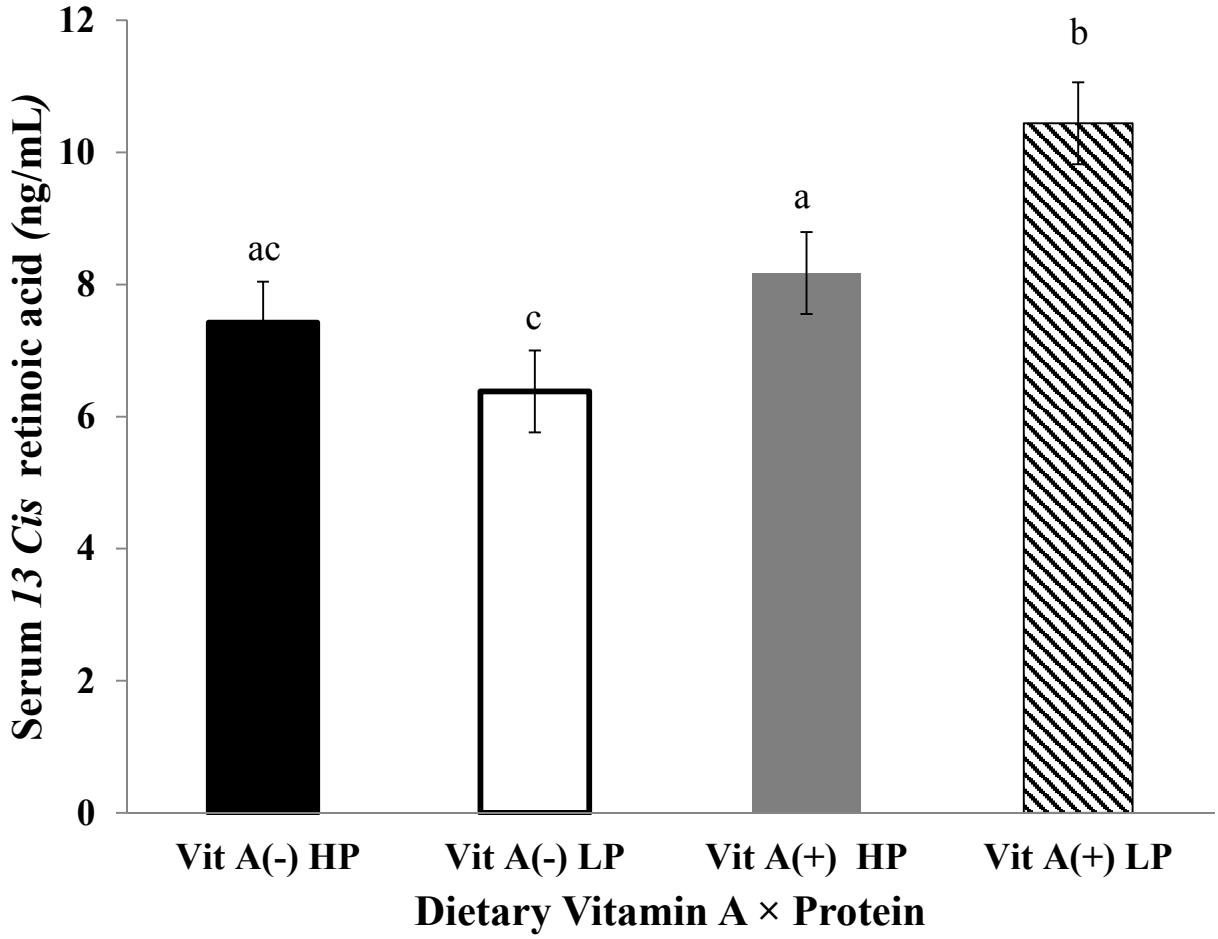

Figure 42. Least square means of serum *13 cis* retinoic acid concentration of dairy cows (n=80) throughout the transition period in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Dietary vitamin A × time interaction (P = 0.004); cows received vitamin A (black line), no vitamin A (dashed line). Asterisks show significant difference at time points specified.

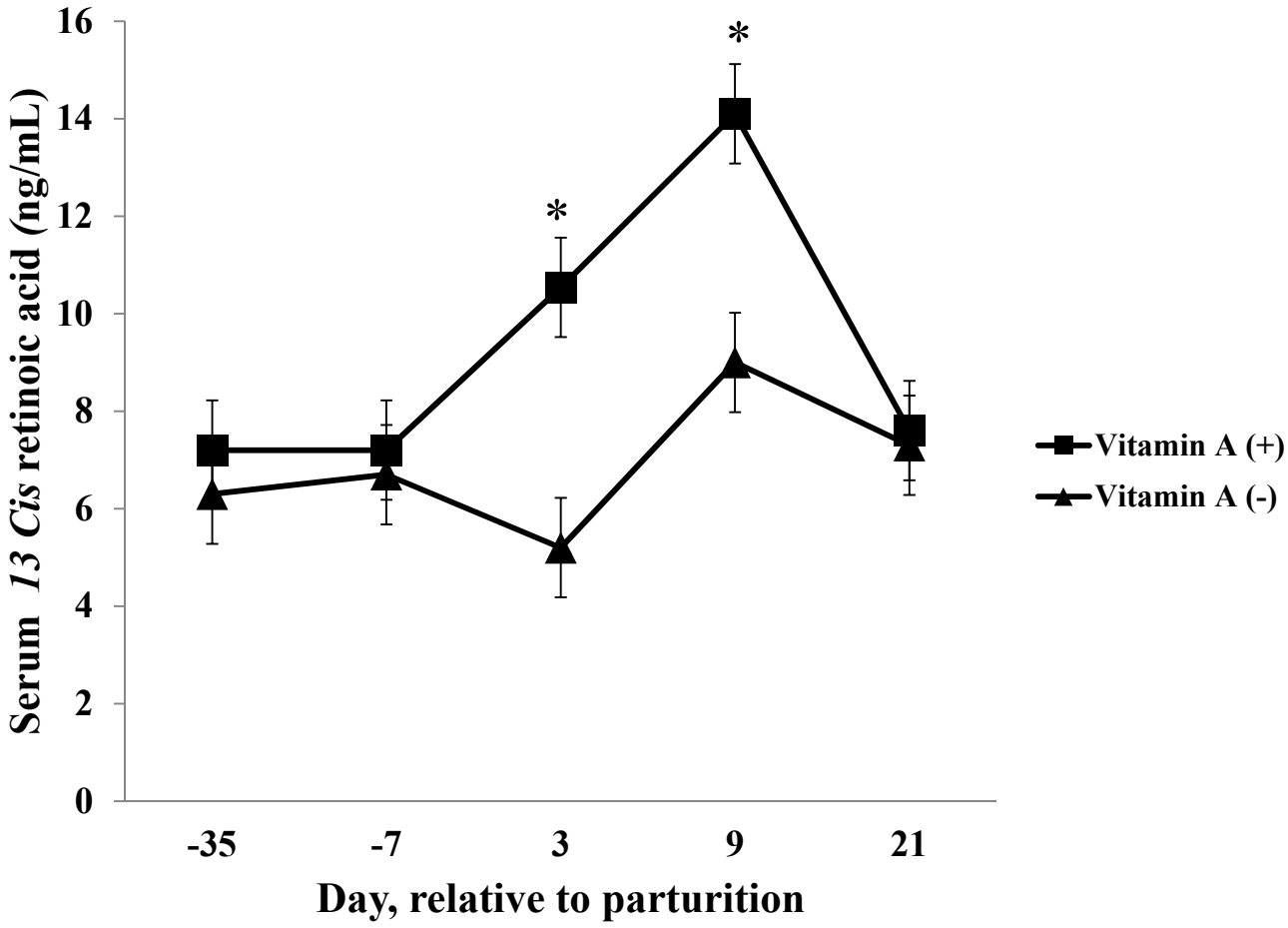

Figure 43. Least square means of serum *all-trans* retinoic acid concentration of dairy cows (n=80) throughout the transition period in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Dietary vitamin A × time interaction (P = 0.005); cows received vitamin A (black line with square), no vitamin A (dashed line with triangle). Asterisks show significant difference at time point specified.

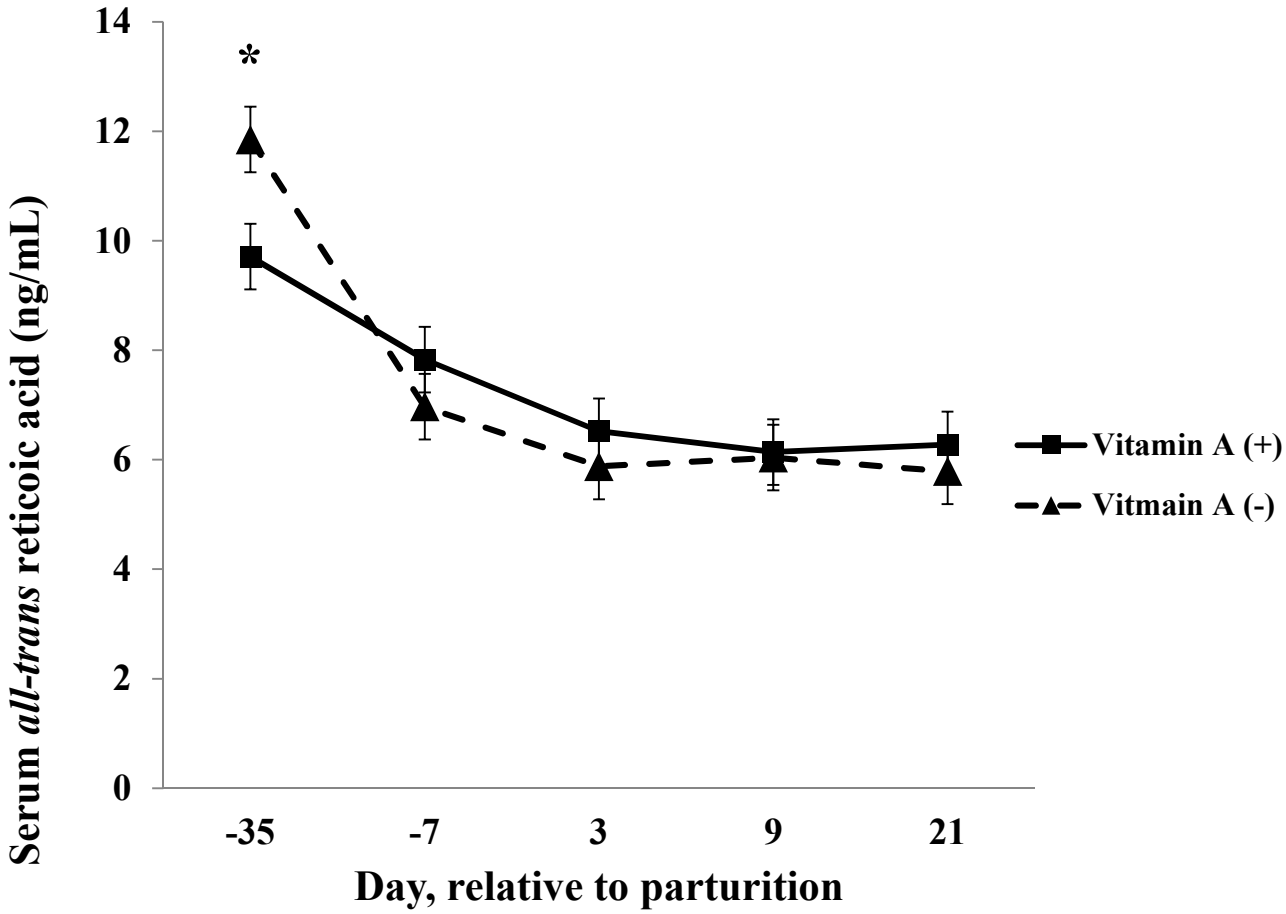

Figure 44. Least square means represented as fold change of adipose tissue gene expression of tumor necrosis factor alpha (TNF-α) of dairy cows (n=80) throughout the transition period in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Dietary monensin × vitamin A interaction ($P = 0.04$); cows that received monensin but no vitamin A (closed bar), cows that did not receive monensin nor received vitamin A (open bar), cows that received monensin and vitamin A (grey bar), and cows that did not receive monensin but received vitamin A (slashed bar).

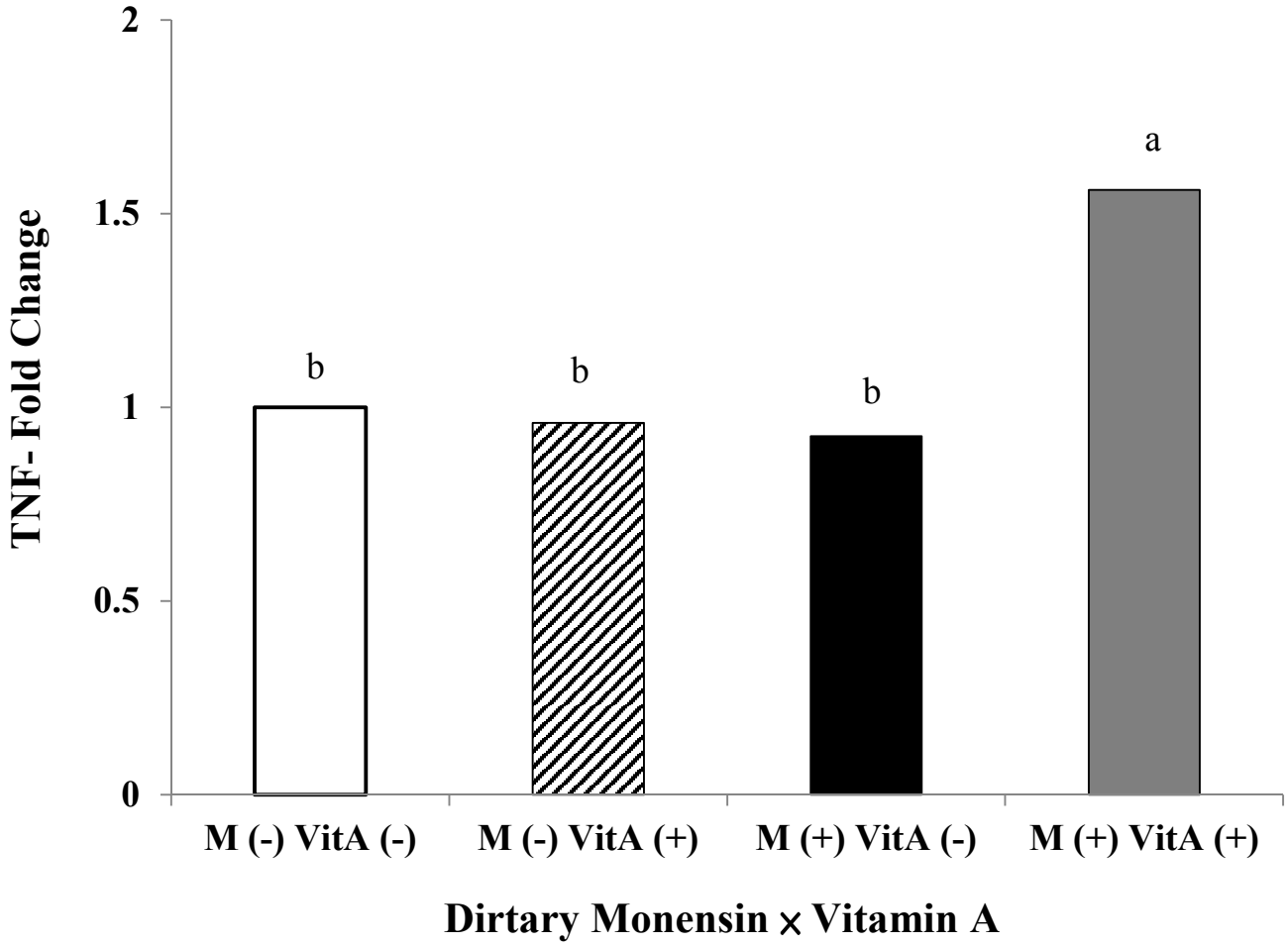

Figure 45. Least square means represented as fold change of adipose tissue retinol binding protein (RBP) gene expression of dairy cows (n=80) throughout the transition period in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Dietary monensin × vitamin A interaction ($P = 0.06$); cows that received monensin but no vitamin A (closed bar), cows that did not receive monensin nor received vitamin A (open bar), cows that received monensin and vitamin A (grey bar), and cows that did not receive monensin but received vitamin A (slashed bar).

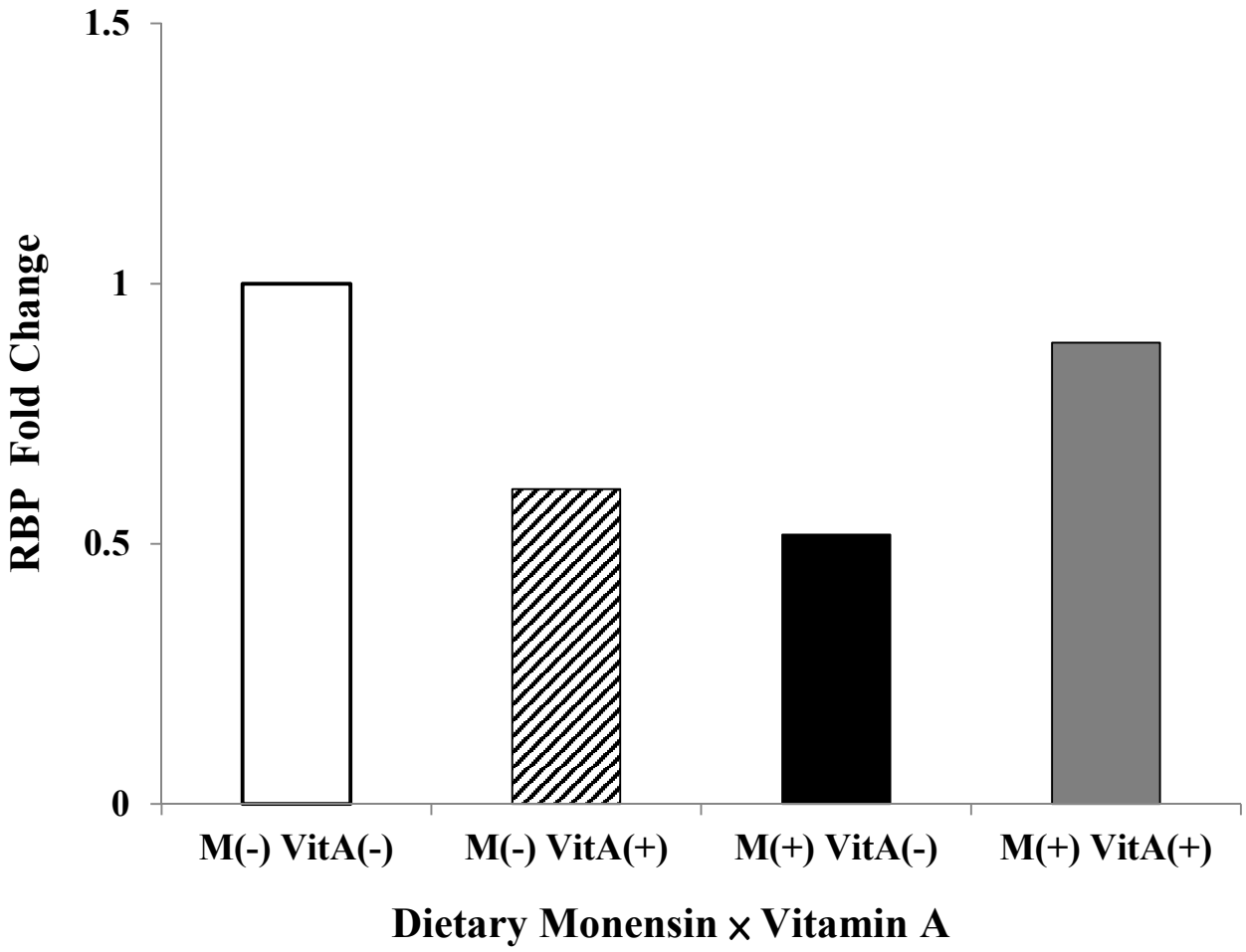

Figure 46. Least square means represented as fold change of adipose tissue gene expression of retinol binding protein (RBP) of dairy cows (n=80) throughout the transition period in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Dietary protein × vitamin A interaction ($P = 0.06$); cows that did not receive vitamin A but received high crude protein (closed bar), cows that did not receive vitamin A but received low crude protein (open bar), cows that received monensin and vitamin A (grey bar), and cows that received vitamin A and received low crude protein (slashed bar).

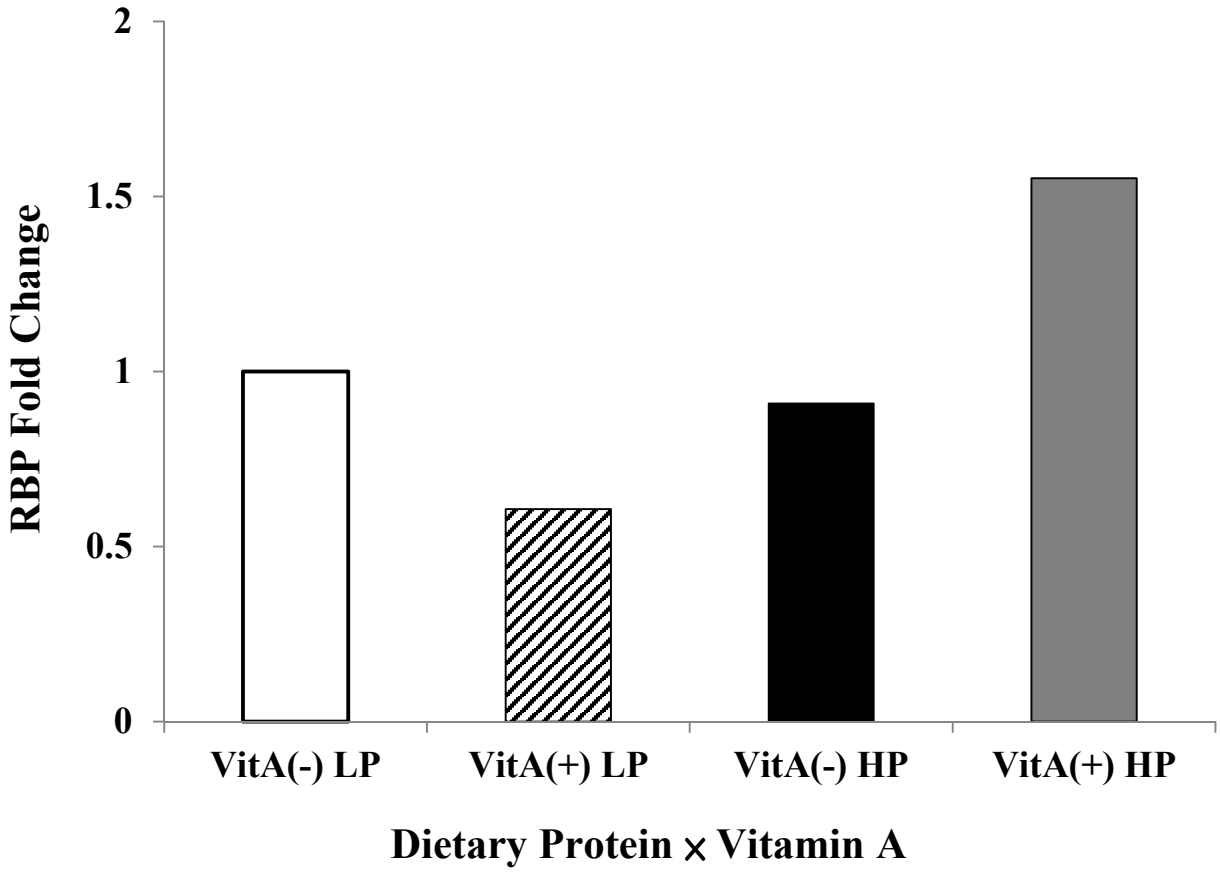

Figure 47. Least square means represented as fold change of PBMC gene expression of tumor necrosis factor-α (TNF-α) of dairy cows (n=80) throughout the transition period in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Dietary crude protein interaction ($P = 0.04$); cows received high crude protein (closed bar), low crude protein (open bar).

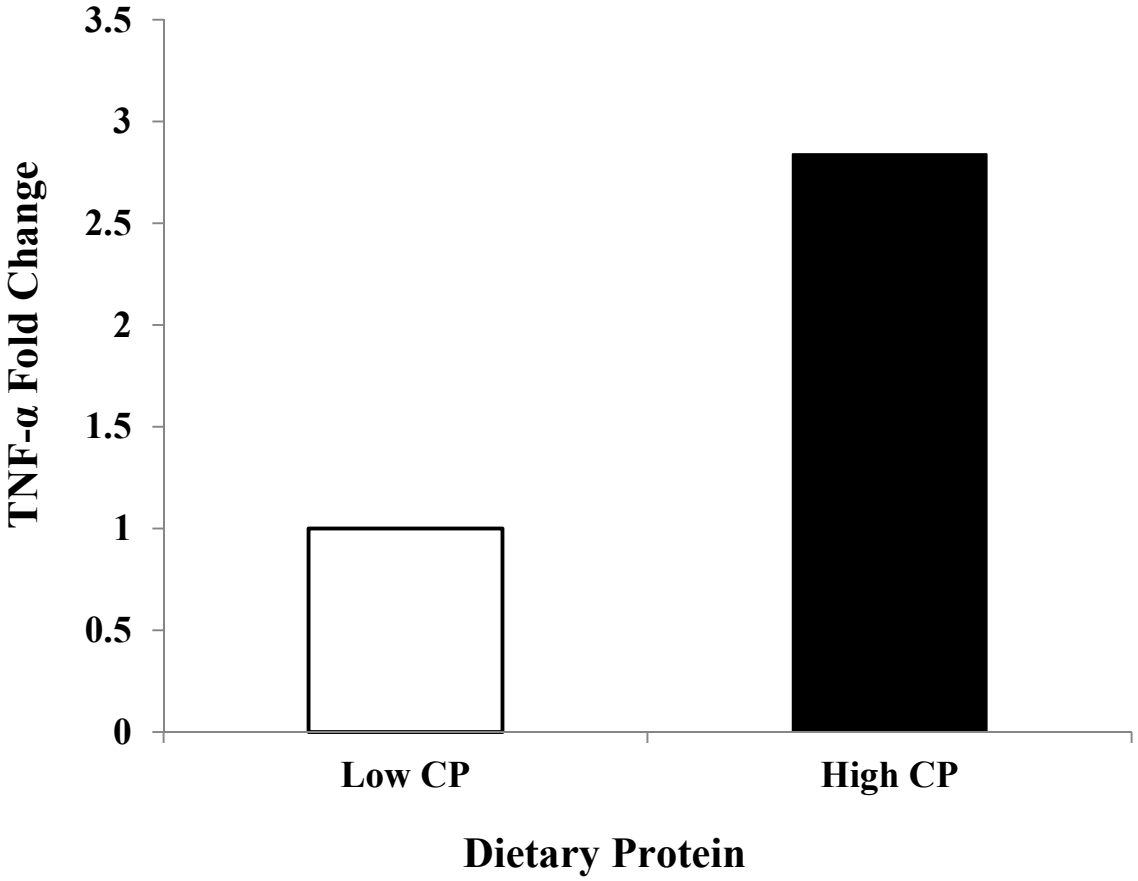

Figure 48. Least square means represented as fold change of PBMC gene expression of intercellular adhesion molecule-1 (ICAM) of dairy cows (n=80) throughout the transition period in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Dietary protein × vitamin A interaction ($P = 0.05$); cows that did not receive vitamin A but received high crude protein (closed bar), cows that did not receive vitamin A but received low crude protein (open bar), cows that received monensin and vitamin A (grey bar), and cows that received vitamin A and received low crude protein (slashed bar).

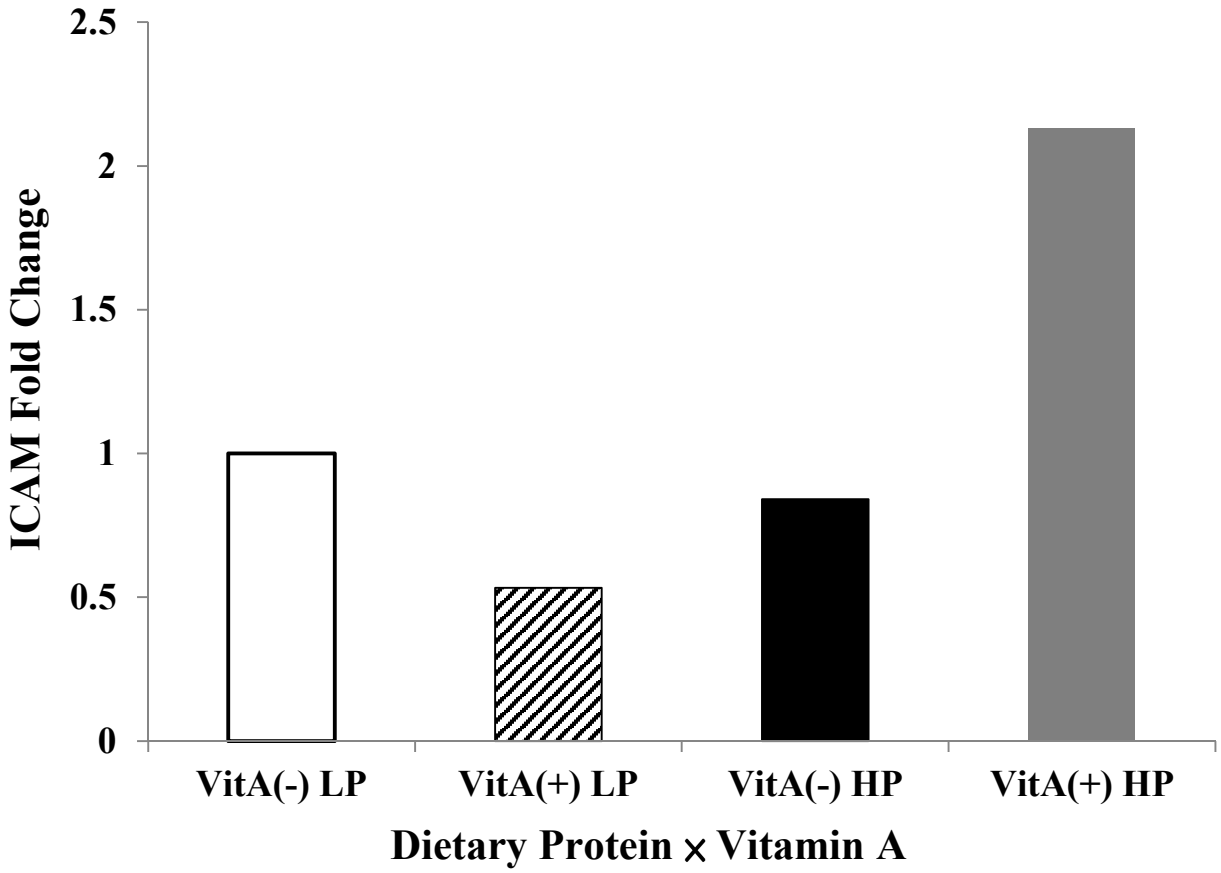

Figure 49. Means of percent killed bacteria on day +7 by neutrophil (killing assay) obtained from dairy cows (n=80) throughout the transition period in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Dietary protein interaction ($P = 0.05$); cows received high crude protein (closed bar), or low crude protein (open bar)

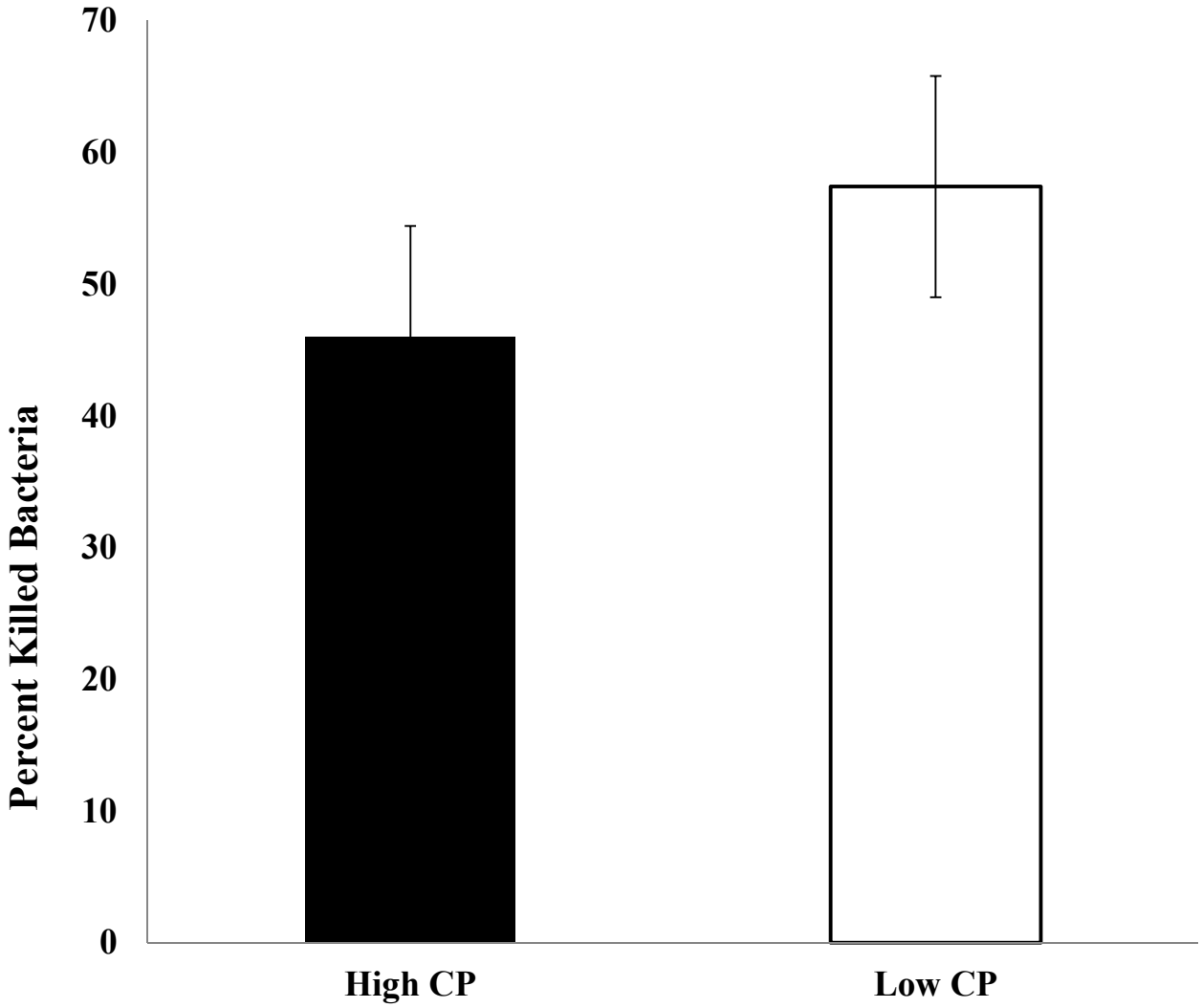

Figure 50. Least square means of serum retinol binding protein (RBP) concentration in dairy cows (n=80) throughout the transition period in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. All cows received a common lactation ration postpartum through d 21. Dietary protein × monensin × time interaction ($P = 0.004$); cows received monensin and high crude protein (solid line with closed square), no monensin and high crude protein (solid line closed circle), received monensin and low crude protein (solid line with open square), and no monensin and low crude protein (solid line and open circle).

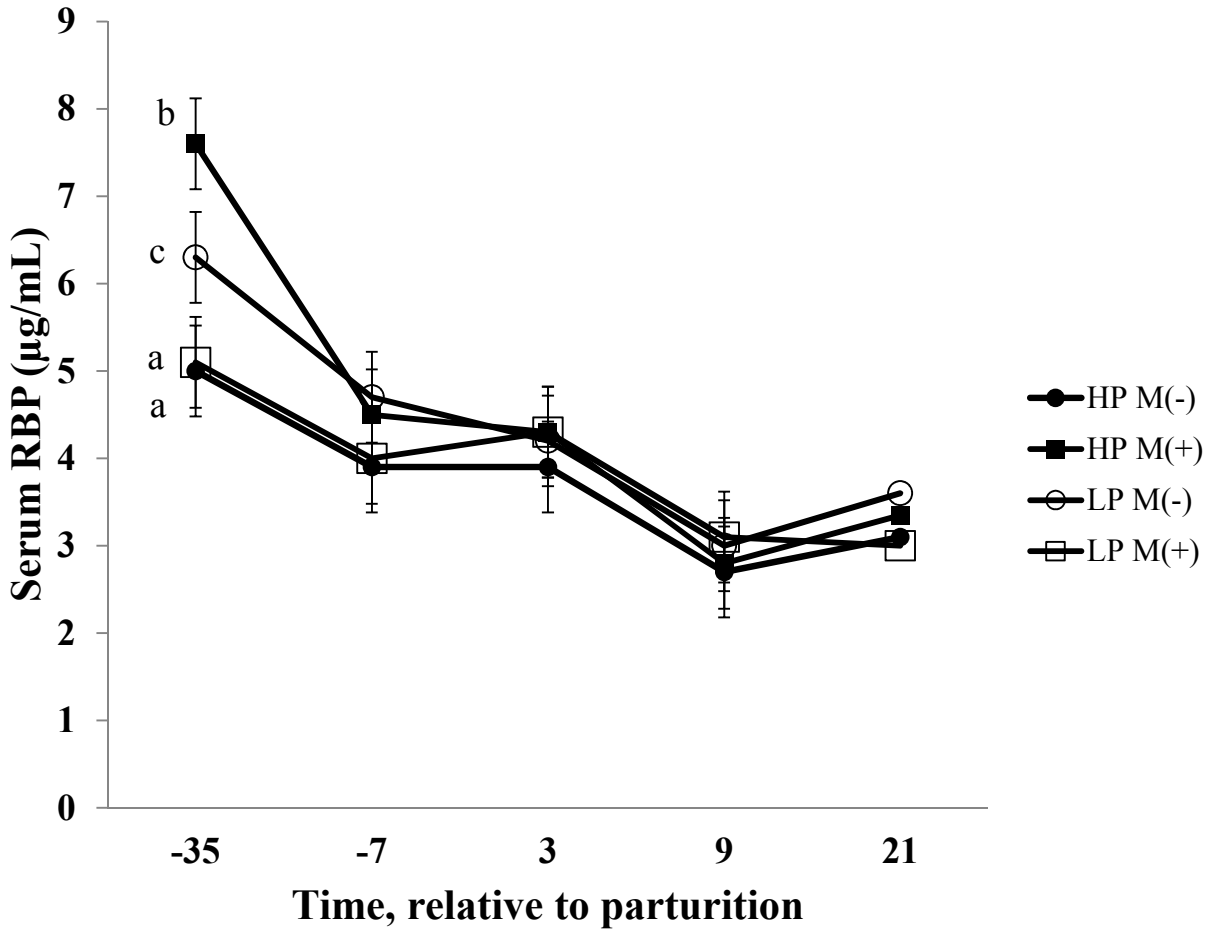

Figure 51. Least square means of postpartum energy balance for dairy cows (n=80) throughout the transition period in which cows either received monensin (M; 0 or 400 mg/hd per day), vitamin A (Vit. A; 0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from d -35 through parturition. Dietary protein × time interaction ($P = 0.03$); cows receiving high crude protein prepartum (solid line), and cows receiving low crude protein prepartum (dashed line). Asterisk shows significant difference at time points specified.

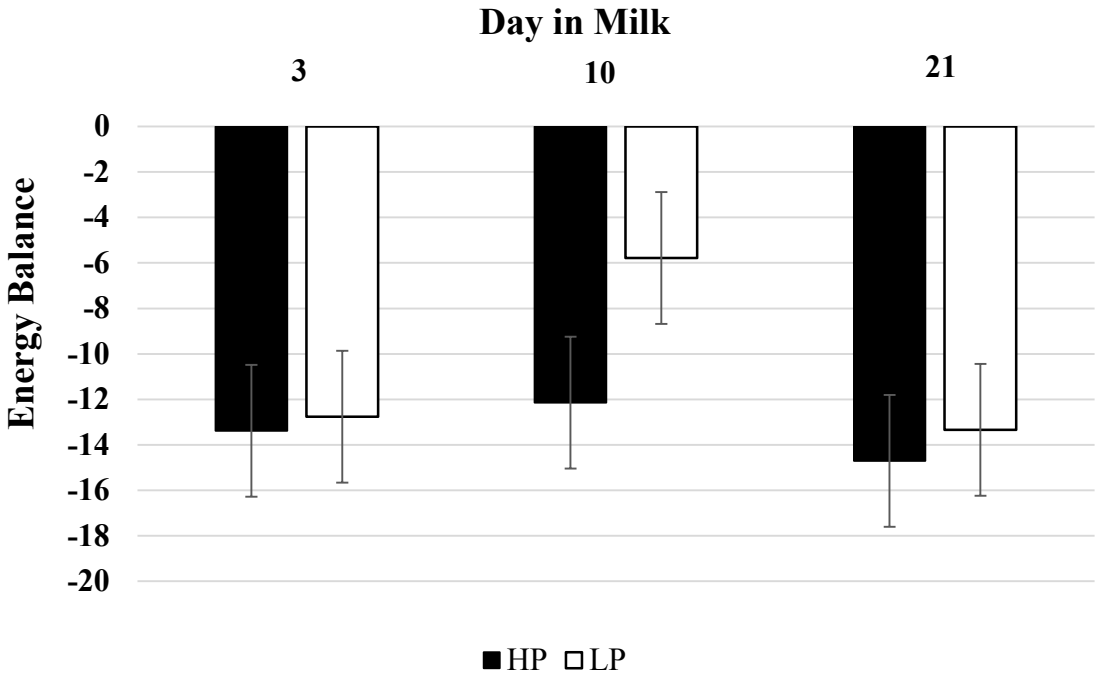

Table 1. Least square means of milk composition and yield for dairy cows (n=80) in which cows either received monensin (0 or 400 mg/hd per day), vitamin A (0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from day -35 to the day of calving. All cows received a common lactation ration postpartum through d 21.

	Main Effect												
	Vitamin A		Protein		Monensin			P-value (main effect)			P-value (interactions)		
	(+)	(-)	High	Low	(+)	(-)	SEM[1]	Pro	Mon	Vit. A	Pro × Vit. A	Mon × Vit. A	Pro × Mon
Milk Comp. (%)													
Fat	4.24	4.19	4.28	4.15	4.18	4.24	0.17	0.59	0.76	0.83	0.25	0.15	0.50
Protein	4.55	4.72	4.66	4.61	4.58	4.69	0.90	0.66	0.38	0.15	0.59	0.65	0.003
Lactose	4.18	4.26	4.22	4.21	4.20	4.24	0.05	0.92	0.54	0.23	0.05	0.76	0.77
Milk Yield (kg/day)													
Yield	26.0	28.4	28.4	26.0	27.6	26.8	1.39	0.20	0.69	0.21	0.12	0.83	0.89
Fat	1.03	1.15	1.18	1.00	1.08	1.10	0.08	0.10	0.86	0.24	0.67	0.06	0.75
Protein	0.98	1.10	1.01	0.99	1.02	1.05	0.05	0.19	0.66	0.07	0.10	0.45	0.53
Lactose	1.17	1.30	1.29	1.18	1.25	1.22	0.07	0.27	0.75	0.14	0.10	0.99	0.97
ECM[3]	28.9	32.1	32.3	28.7	30.4	30.6	1.60	0.11	0.93	0.14	0.28	0.17	0.96
Solid Non-Fat	9.56	9.85	9.73	9.68	9.63	9.80	0.10	0.70	0.26	0.04	0.50	0.79	0.02
SCC[2]	706.9	1246.3	1003.8	949.5	1025.	927.5	198.1	0.84	0.71	0.04	0.99	0.35	0.48
SCLs[4]	2.77	3.09	2.94	2.91	2.99	2.86	0.15	0.86	0.52	0.11	0.99	0.34	0.50

[1]Largest SEM reported

[2]SCC: somatic cell count (× 1000/mL)

[3]ECM: 0.327 × milk yield kg + 12.95 × fat yield kg + 7.21 × protein yield kg

[4]SCLs: somatic cell linear score

Table 2. Least square means of milk composition and yield for dairy cows (n=80) not including samples from the 1st, 2nd, and 3rd milking's, in which cows either received monensin (0 or 400 mg/hd per day), vitamin A (0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from day -35 to the day of calving. All cows received a common lactation ration postpartum through d 21.

	Main Effect							P-value (main effect)			P-value (interactions)		
	Vitamin A		Protein		Monensin								
	(+)	(-)	High	Low	(+)	(-)	SEM[1]	Pro	Mon	Vit. A	Pro × Vit. A	Mon × Vit. A	Pro × Mon
Milk Comp. (%)													
Fat	3.91	3.92	3.94	3.90	3.96	3.88	0.19	0.88	0.74	0.97	0.13	0.08	0.50
Protein	3.42	3.54	3.51	3.45	3.45	3.51	0.05	0.30	0.33	0.05	0.17	0.13	0.31
Lactose	4.52	4.53	4.51	4.54	4.52	4.53	0.05	0.62	0.85	0.97	0.23	0.35	0.39
Milk Yield (kg/day)													
Yield	33.2	36.6	36.2	33.5	35.4	34.4	1.81	0.26	0.69	0.17	0.16	0.90	0.96
Fat	1.27	1.44	1.46	1.25	1.36	1.35	0.10	0.13	0.91	0.22	0.81	0.04	0.83
Protein	1.13	1.27	1.24	1.16	1.20	1.20	0.06	0.27	0.98	0.08	0.55	0.53	0.55
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-
ECM[3]	35.61	39.8	39.7	35.7	38.0	37.4	2.1	0.16	0.83	0.15	0.37	0.15	0.93
Solid Non-Fat	8.81	8.95	8.88	8.87	8.84	8.91	0.07	0.92	0.42	0.12	0.05	0.76	0.10
SCC[2]	441.2	1008.6	786.4	663.6	637.8	812.1	129.2	0.47	0.31	0.001	0.82	0.05	0.17
SCLs[4]	2.40	2.73	2.62	2.51	2.59	2.54	0.15	0.56	0.82	0.10	0.79	0.22	0.21

[1]Largest SEM reported

[2]SCC: somatic cell count (× 1000/mL)

[3]ECM: 0.327 × milk yield kg + 12.95 × fat yield kg + 7.21 × protein yield kg

[4]SCLs: somatic cell linear score

Table 3. Least square means of milk vitamin concentrations (µg/mL) for dairy cows (n=80) throughout the transition period in which cows either received monensin (0 or 400 mg/hd per day), vitamin A (0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from day -35 to the day of calving. All cows received a common lactation ration postpartum through d 21.

	Main Effect							*P*-value (main effect)			*P*-value (interactions)		
	Vitamin A		Protein		Monensin								
	(+)	(-)	High	Low	(+)	(-)	SEM[1]	Pro	Mon	Vit. A	Pro×Vit.A	Mon×Vit.A	Pro×Mon
Retinol	2.23	1.96	2.23	1.96	2.16	2.03	0.12	0.07	0.45	0.09	0.68	0.35	0.01
α-TOC	0.40	0.45	0.43	0.43	0.42	0.44	0.02	0.99	0.47	0.10	0.51	0.20	0.04
β-CAR	1.03	1.03	1.14	0.93	0.98	1.08	0.11	0.13	0.46	0.97	0.81	0.80	0.23

[1]Largest SEM reported

Table 4. Least square means of serum vitamin concentrations for dairy cows (n=80) throughout the transition period in which cows either received monensin (0 or 400 mg/hd per day), vitamin A (0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from day -35 to the day of calving. All cows received a common lactation ration postpartum through d 21.

	Main Effect												
	Vitamin A		Protein		Monensin			*P*-value (main effect)			*P*-value (interactions)		
	(+)	(-)	High	Low	(+)	(-)	SEM[1]	Pro	Mon	Vit. A	Pro×Vit.A	Mon×Vit.A	Pro×Mon
Retinol (µg/mL)	2.10	1.92	2.03	2.00	1.96	2.06	0.12	0.82	0.52	0.27	0.12	0.69	0.94
13 cis (ng/mL)	9.42	7.0	7.90	8.5	8.60	7.80	0.41	0.24	0.19	0.0001	0.02	0.36	0.06
All-trans (ng/mL)	7.30	7.2	7.2	7.30	7.30	7.2	0.28	0.83	0.76	0.78	0.38	0.98	0.86
α-Tocopherol (µg/mL)	2.92	3.21	3.03	3.09	2.91	3.22	0.20	0.83	0.29	0.32	0.89	0.62	0.63
β-carotene (µg/mL)	12.9	12.3	13.3	11.9	12.6	12.7	1.66	0.75	0.82	0.60	0.56	0.78	0.09

[1]Largest SEM reported

Table 5. Least square means of prepartum and postpartum dry matter intake and energy balance (EB) for dairy cows (n=80) throughout the transition period in which cows either received monensin (0 or 400 mg/hd per day), vitamin A (0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from day -35 to the day of calving. All cows received a common lactation ration postpartum through d 21.

	Main Effect							*P*-value (main effect)			*P*-value (interactions)		
	Vitamin A		Protein		Monensin								
	(+)	(-)	High	Low	(+)	(-)	SEM[1]	Protein	Monensin	Vitamin A	Pro×Vit.A	Mon×Vit.A	Pro×Mon
Prepartum	26.3	26.7	26.9	26.0	26.1	26.8	0.9	0.38	0.50	0.72	0.71	0.86	0.90
Postpartum	34.4	38.4	37.3	35.5	36.7	36.2	1.8	0.38	0.83	0.11	0.39	0.62	0.17
Postpartum EB	-11.3	-12.8	-13.4	-10.6	-10.6	-13.4	2.1	0.34	0.33	0.61	0.35	0.86	0.67

[1]Largest SEM reported

Table 6. Least square means of serum concentration of various metabolites in dairy cows (n=80) throughout the transition period in which cows either received monensin (0 or 400 mg/hd per day), vitamin A (0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from day -35 to the day of calving. All cows received a common lactation ration postpartum through d 21.

	Main Effect												
	Vitamin A		Protein		Monensin			*P*-value (main effect)			*P*-value (interactions)		
	(+)	(-)	High	Low	(+)	(-)	SEM[1]	Pro	Mon	Vit. A	Pro×Vit.A	Mon×Vit.A	Pro×Mon
NEFA	0.62	0.62	0.62	0.62	0.60	0.65	0.03	0.95	0.13	0.92	0.86	0.51	0.17
Hpt	0.57	0.54	0.50	0.61	0.54	0.57	0.03	0.01	0.54	0.44	0.76	0.77	0.13
BHBA	490.0	496.6	481.3	505.3	513.6	473.0	16.1	0.28	0.07	0.77	0.10	0.83	0.64
TBARS	2.22	2.01	2.15	2.08	2.07	2.16	0.08	0.53	0.43	0.06	0.77	0.24	0.14
RBP	4.16	4.09	4.12	4.13	4.21	4.05	0.21	0.98	0.58	0.81	0.93	0.65	0.04

[1]Largest SEM reported

Table 7. Least square means of PBMC gene expression (delta Ct) of interleukin 1 beta (IL1 β), interleukin 6 (IL6), tumor necrosis factor alpha (TNF-α), and intercellular adhesion molecule 1 (ICAM) for dairy cows (n=80) throughout the transition period in which cows either received monensin (0 or 400 mg/hd per day), vitamin A (0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from day -35 to the day of calving. All cows received a common lactation ration postpartum through d 21.

	Main Effect												
	Vitamin A		Protein		Monensin			*P*-value (main effect)			*P*-value (interactions)		
	(+)	(-)	High	Low	(+)	(-)	SEM[1]	Pro	Mon	Vit A	Pro×Vit.A	Mon×Vit.A	Pro×Mon
IL1 β	4.84	5.28	4.81	5.31	4.91	5.21	0.71	0.54	0.71	0.59	0.32	0.74	0.65
IL6	13.2	12.5	13.0	12.7	12.8	12.9	0.86	0.77	0.84	0.45	0.73	0.67	0.32
TNF α	4.62	5.06	4.09	5.6	4.34	5.34	0.62	0.04	0.17	0.54	0.07	0.15	0.23
ICAM	7.42	7.63	7.09	7.96	7.22	7.82	0.49	0.13	0.29	0.71	0.05	0.61	0.34

[1]Largest SEM reported

[2] Gene expression data were analyzed using Ct values normalized to the average of the endogenous control genes, GAPDH and RPS9, and are presented graphically as fold change ($2^{-\Delta\Delta Ct}$) relative to the control treatment.

Table 8. Least square means of adipose tissue gene expression[2] of retinol binding protein (RBP), peroxisome proliferator-activated receptor gamma (PPAR-γ), and tumor necrosis factor alpha (TNF-α) for dairy cows (n=80) throughout the transition period in which cows either received monensin (0 or 400 mg/hd per day), vitamin A (0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from day -35 to the day of calving. All cows received a common lactation ration postpartum through d 21.

	Main Effect												
	Vitamin A		Protein		Monensin			*P*-value (main effect)			*P*-value (interactions)		
	(+)	(-)	High	Low	(+)	(-)	SEM[1]	Protein	Monensin	Vitamin A	Pro×Vit.A	Mon×Vit.A	Pro×Monen
RBP	-0.59	-0.56	-0.88	-0.27	-0.47	-0.67	0.32	0.11	0.58	0.95	0.06	0.06	0.08
PPAR-γ	2.24	2.18	2.08	2.34	2.20	2.12	0.15	0.15	0.33	0.77	0.06	0.24	0.39
TNF α	8.47	8.15	0.04	8.79	8.49	8.79	0.16	0.11	0.10	0.10	0.38	0.04	0.30

[1]Largest SEM reported

[2] Gene expression data were analyzed using Ct values normalized to the average of the endogenous control genes, GAPDH and RPS9, and are presented graphically as fold change ($2^{-\Delta\Delta Ct}$) relative to the control treatment.

Table 9. Least square means of hepatic gene expression (delta Ct) of phosphoenol pyruvate carboxykinase (PEPCK), pyruvate carboxylase (PC), tumor necrosis factor alpha (TNF-α), and retinol binding protein (RBP) for dairy cows (n=80) throughout the transition period in which cows either received monensin (0 or 400 mg/h per day), vitamin A (0 or 110 IU /kg BW per day), and crude protein (10% or 12%) during prepartum period from day -35 to the day of calving. All cows received a common lactation ration postpartum through d 21.

	Main Effect												
	Vitamin A		Protein		Monensin			*P*-value (main effect)			*P*-value (interactions)		
	(+)	(-)	High	Low	(+)	(-)	SEM[1]	Pro	Mon	Vit. A	Pro×Vit.A	Mon×Vit.A	Pro×Mon
PEPCK	-1.69	-1.75	-1.77	-1.67	-1.72	-1.72	0.10	0.42	0.98	0.68	0.22	0.29	0.46
PC	0.69	0.84	0.77	0.76	0.78	0.75	0.10	0.91	0.83	0.22	0.79	0.73	0.29
TNF-α	8.62	8.68	8.56	8.74	8.70	8.60	0.11	0.20	0.47	0.70	0.44	0.45	0.73
RBP	-3.27	-3.25	-3.25	-3.26	-3.23	-3.29	0.10	0.94	0.62	0.87	0.19	0.44	0.51

[1]Largest SEM reported

[2] Gene expression data were analyzed using Ct values normalized to the average of the endogenous control genes, GAPDH and RPS9, and are presented graphically as fold change ($2^{-\Delta\Delta Ct}$) relative to the control treatment.

Table 10. Ingredient composition (% of DM) of prepartum and postpartum rations. Prepartum rations designated by protein concentration (HP or LP).

	Ration		
Item	Prepartum(HP)	Prepartum(LP)	Postpartum
Alfalfa hay	18.11	18.06	15.9
Grass hay	23.50	22.06	5.0
Wheat Straw	11.01	10.15	-
Soybean, Hulls	12.8	12.8	-
Rolled barley	4.68	9.42	17.0
Dry distiller corn grain with solubles	7.96	11.03	12.82
Corn, dry	3.12	11.84	15.32
Sodium bicarbonate	-	-	0.41
*Liquid mineral/vitamin pre-mix[3]	4.53	4.64	4.50
Calcium soaps of fatty acids[4]	-		1.35
Triticale Silage[1]	-	-	13.01
Canola Meal	-	-	14.99
Chemical Analysis			
DM	59.10	57.28	63.83
NDF	49.43	46.94	40.83
ADF	32.87	30.98	26.68
CP	12.22	10.26	16.82
Soluble (as % of CP)			
Ether extract	1.40	1.46	3.22
NFC	28.03	32.42	30.03
Ash			
Ca	0.7	0.7	0.7
P	0.3	0.3	0.5
Mg	0.2	0.2	0.2
K	1.92	1.74	1.48
NE_L, Mcal/kg of DM	1.34	1.38	1.47

[1]Triticale silage 37% DM (as fed).

[2]50:50 Oat and pea silage 32% DM (as fed).

[3]Performix, Caldwell, ID.

* Vitamin A excluded in prepartum

[4]EnerGII; Virtus Nutrition, Corcoran, CA.

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