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EFFECTS OF AN EXTRUDED FLAXSEED SUPPLEMENT ON TRANSITION COW IMMUNE FUNCTION AND EFFECTS OF A METHANE INHIBITOR ON TRANSITION COW OVARIAN ACTIVITY

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Abstract

In cattle, the transition period, which encompasses three weeks prior to three weeks after calving, is a critical time in the cow's life with increased incidence of metabolic and infectious diseases. During this time cows suffer from immunosuppression and negative energy balance. Negative energy balance (NEB) and immunosuppression are related in transition dairy cows and occur due to extreme metabolic stress and changing hormones as the cow transitions from a pregnant to energy-demanding lactating state coupled with decreased dry matter intake. Incidences of periparturient diseases are not only detrimental to the overall health and well-being of the cow, but they can also decrease milk output and reproductive efficiency, causing economic loss to the producer. For these reasons, improved management during the transition period that improves the health and well-being of the cow are paramount for setting the stage for successful reproduction and high milk production.

Two approaches were taken to address the challenges of the transition period. The first approach was to feed an omega-3 fatty acid extruded flaxseed supplement and measure effects on transition dairy cow immune function, plasma metabolites, and fatty acid composition of milk fat, plasma, and red blood cells. Polyunsaturated fatty acids (PUFA) decrease expression of pro-inflammatory cytokines promoting an anti-inflammatory state. Further benefits of feeding fats include improved milk production and composition and improved fertility.

The second approach was to supplement transition dairy cows with a small molecule methane inhibitor and observe effects on return of ovarian activity. In a recent study, cows that received a methane inhibitor had increased body weight gain compared to cows that received a placebo. It is hypothesized that reducing methane production could make more energy available to the cow for other productive functions. To test this hypothesis, progesterone was measured in

defatted milk via enzyme-linked immunoassay (ELISA). Parameters measured were days to evidence of a first corpus luteum (CL), length of first luteal phase, days to evidence of second CL, and interval between luteal phases. Evidence suggests that ovarian activity cannot resume until negative energy balance ceases and cows enter a positive energy balance. Cows that enter positive energy balance and cycle more before first service are more likely to have higher conception and pregnancy rates.

These two approaches were taken because NEB and immunosuppression are the primary causes of negative health events in transition dairy cows. Overall, the first approach showed cows receiving the flaxseed supplement had greater ALA in plasma and milk fat, reduced mRNA abundance of TNF and IL6, and reduced neutrophil phagocytosis and ROS. Results indicate that the omega-3 supplement altered the activation status of the immune system and reduced inflammatory responses. This reduction in neutrophil activity may increase energy available to the cow for productive functions, although this speculation needs to be confirmed with further research. The milk fat and plasma exhibited greater ALA the longer the cows were on the diet indicating this supplement can be used to produce milk with higher omega-3 fatty acids, which is an area of increased consumer interest. In the methane inhibitor study, there were no differences observed between treatment groups for any of the reproductive parameters. Results from both studies provide further support for the importance of maintaining proper energy balance during the transition period to support immune function and productive functions in dairy cattle.

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Earning this degree and completing my Master's work was a journey. Just like my favorite book trilogy, *The Lord of the Rings*, I couldn't have finished this work without help. Similar to how the Fellowship and various characters helped Frodo reach Mount Doom and return home safely to the Shire, the help of my colleagues, friends, and family, was essential for completing this journey. My adviser, Dr. Troy Ott, provided me with exceptional guidance. Dr. Ott taught me to expand my way of thinking and to think like an animal scientist, to think critically, and to never stop asking questions. Dr. Ott has helped me to grow and he never stopped pushing me to be the best that I can be. Although he will never quite break me of my grammatically incorrect country way of talking, he has inspired me to never stop learning. I need to extend my sincerest gratitude to Dr. Joy Pate. Dr. Pate was a constant source of knowledge and an irreplaceable member of my committee. My other committee member, Dr. Harvatine, was always patient with my questions and his expertise in dairy cattle nutrition was essential to completing my work. I cannot thank each member of my committee enough for the roles they played in helping me to complete this journey.

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Chapter 1: Review of Literature

Introduction

Last year in the United States greater than 200 billion pounds of dairy products were produced and sold (USDA ERS). The majority of Americans include dairy in their daily diet. The Food and Drug Administration (FDA) Dietary Guidelines for 2015-2020 recommends 24 ounces (or equivalent) of dairy products for a healthy 2000 calorie diet (FDA Dietary Guidelines, 2015). Consumption of milk and other dairy products is linked to essential growth and development for children. Children who avoided drinking milk were found to be shorter, have smaller skeletons, and lower total-body bone mineral content compared to children of the same age and sex in the same community who consumed dairy on a daily basis (Black et al., 2002). For adults, the evidence is strong that a high calcium diet promotes bone density and strength and reduces incidence of osteoporosis, and dairy products are naturally good sources of calcium and other essential nutrients (Heaney, 1999).

With several sectors of the dairy industry experiencing rapid growth (cheese and yogurt), and an increasing emphasis on reducing the environmental impact dairy production, dairy producers must constantly work to improve efficiency of production to keep products affordable for consumers and to keep up with the demand of a growing population. According to the United Nations, the world population is projected to be 9.7 billion by 2050 (United Nations Department of Economic and Social Affairs).

The main ways dairies have attempted to improve efficiency is through selective breeding, improved housing and environmental conditions, milking protocols, and nutrition. Thus far research has allowed dairies to keep up with demand while reducing the total number of cows and the environmental impact of dairy production. To keep dairy products an affordable source of nutrition, while continuing to reduce the environmental impact of dairy production, will be a challenge for animal scientists (Capper et al., 2009).

Current research is not only focused on efficiency, but making dairy products more nutritious and beneficial to human health. Typically, milk is high in saturated and omega-6 polyunsaturated fatty acids, but by selective feeding, milk can be enriched in omega-3 fats, which some research suggests are more healthful (Welter et al., 2016). One product that is enriched in omega-3 fatty acids and is readily available to consumers is Eggland's Best® Eggs. Eggland's Best® markets their eggs as being superior to ordinary eggs in nutrition with reduced saturated fatty acids, increased polyunsaturated fatty acids (PUFA), specifically omega-3 fatty acids, and increased protein (Nutritional Benefits of Eggland's Best® Eggs). Eggland's Best ® Eggs contain 115 mg of omega-3 fatty acids compared to 49 mg in traditional eggs (Eggland's Best® Superior Nutrition). One study showed that egg type (eg free-range, omega-3 enriched) was just as important to consumers as price when choosing which eggs to buy (Tamini et al., 2014). This suggests that consumers may be willing to pay more for a product that is perceived to have greater health benefits. Taking this into account, although the dairy industry has changed immensely in the United States over the last 200 years, there is still room for improvement. Using Eggland's Best® as a model, there is evidence that consumers want and are willing to pay for products that are enriched in omega-3 fatty acids (Tamini et al., 2014). This provides the dairy industry with an avenue for additional expansion.

Poor fertility is a source of substantial inefficiency and economic loss to the dairy industry (Lucy, 2001; DeVries, 2006; Thatcher et al., 2006). For example, conception rates decreased from 65% in 1950 to 40% in 1996 (Butler, 1998; Norman et al., 2009). Reduced reproductive efficiency is due, at least in part, to a large emphasis on genetic selection of cows for milk production and milk components and neglecting other important traits (Pritchard et al., 2012). Only recently have fertility traits been emphasized in genetic selection indexes (Pritchard et al., 2012). For example, in the most recent Net Merit $ index, direct fertility traits were weighted at 10% and another 19% weight was given to productive life, an indirect measure of fertility (USDA AIP Research Report). Genetically selecting cattle for improved milk production and fertility simultaneously, along with strategic management, can greatly increase reproductive performance in dairy cattle (Berry et al., 2016).

Another area of cow management which requires improvement is the transition period. During this time leading up to and after parturition, cows suffer from metabolic disorders, reduced immunity, and are at high risk for periparturient diseases. The health of a cow after parturition is pivotal. Cows that suffer from retained placenta, metritis, or endometritis have decreased reproductive performance (Han and Kim, 2005). Cows that do not suffer from severe metabolic disorders or health events after parturition are more likely to cycle sooner and become pregnant sooner with fewer services than cows that struggle with health events (Wathes et al., 2007). The national pregnancy rate is estimated to be 18% in the United States, if this average was improved to 22% it would carry an economic benefit of greater than $480 million per year for the dairy industry alone (DeVries, 2006). Improving cow health during the transition period is likely the most critical limitation to improved reproductive efficiency.

Background

European settlers first arrived in North America in the early 1600s and brought with them a variety of breeds of cows to supply their families with meat, hides, milk products, and to serve as draft animals. As small towns and cities began to develop in America there was an increasing need for milk production as the population grew. Since the 1800s there have been widespread technological advancements in the dairy industry such as improved nutrition, milking parlors, and pasteurization and homogenization to ensure the safety and marketability of milk products (USDA, Natural Agricultural Library). Recently, perhaps the biggest advancements in the dairy industry are the cows themselves, which are now capable of producing much more milk per cow. For example, the number of milk cows in the United States decreased from 12 million in 1970 to 9.3 million in 2016 but during this time milk production increased (USDA, ERS Dairy Report 2016). The average yearly milk production per cow in 1970 was 9,751 lbs compared to 22,000 lbs in 2017 (USDA ERS, Dairy Report 2016). This represents an approximate 225% increase or an average 5% year-on-year improvement in milk production per cow.

The projected milk production in the United States for 2016 is 212.5 billion pounds (USDA ERS, Dairy Forecast). This rapid improvement in milk yield is not only occurring in America, but in other countries as well. In Denmark, for example, average milk production per cow per year increased from 14,755 pounds in 1989 to 19,804 pounds in 2009 (Ingvartsen and Moyes, 2012). During this same period the number of dairy farms in the U.S. has decreased and the size of farms has increased. From 1970 to 2006 the number of dairy farms decreased by 88% from 648,000 to 75,000 (MacDonald et al., 2007). By 2015 the number of dairy operations decreased further to 43,584 (Progressive Dairyman, 2015 U.S. Dairy Stats). In 1992 more than two-thirds of dairies in the US had herd sizes of 200 or fewer cows and farms of 1,000 cows or

more only accounted for 10% of dairies. By 2006 large dairies accounted for more than one-third of farms (MacDonald et al., 2007). While there are fewer dairies, farms with more than 2000 cows now account for over 20% of dairy farms (USDA, ERS). The gradual move to larger farms was driven by the need for farms to become more efficient to be profitable. This has also served to keep dairy products affordable for the consumer (MacDonald et al., 2007). Similar changes are occurring around the world. Increased milk production per cow is attributed to wide adoption of artificial insemination, intense genetic selection, better management, and better nutrition (Lucy, 2001). Despite improvements in milk production, other areas of the dairy industry still need improvement. Although increasing the size of dairies and selectively breeding cows for milk yield led to increased milk production per cow over the past twenty years, this was accompanied by a reduction in measures of cow health and reproductive performance (Adamczyk et al., 2016).

Fertility in the Dairy Industry

Perhaps the trait which has been most affected by the changes in the dairy industry is fertility. Fertility and milk production are negatively correlated in dairy and beef cattle (Berry et al., 2016). However, the effect of milk production on fertility is small and well-managed dairies can achieve high milk production and high fertility (Thatcher et al., 2006). Nonetheless, the decline in fertility has been profound. First service conception rates in 1951 were 65% compared to only 40% in 1996 (Butler, 2003). Conception rates have changed little since 1996 and remain about 40% on well-managed herds (De Vries, 2006). In a study of Michigan dairy herds, first service conception rates were 43% and pregnancy rates ranged from 11.4% to 22.6% (Linderoth, 2011). Conception rate is calculated by total number of conceptions divided by total number of

inseminations on a farm, and pregnancy rate refers to a cow that conceives and carries a pregnancy to term. The reason for large variation between conception and pregnancy rate is partly due to the adoption of timed artificial insemination (TAI) programs, rather than breeding to natural estrus (Galvao et al., 2013). It can be difficult to visually observe signs of estrus in large dairies, which is why utilization of TAI programs has increased. The submission rate on large dairies has increased on large dairies due to TAI which has increased pregnancy rates. Because poor reproductive management results in large losses to dairy producers, strategies to improve dairy cattle health and reproduction are a key focus for dairy producers and researchers. Costs for reproductive management include cost of hormones for synchronization programs, frozen semen, labor, heat detection aids, hormone injections, and artificial insemination. It is estimated the average cost of breeding per cow per year is $33 (DeVries, 2006). In another study, three breeding programs were evaluated and total cost for a first service ranged from $13.10 - $29.20 per cow with subsequent services costing an extra $17.80 - $33.90 per cow per service (Giordano et al., 2011). The cost per farm for artificial insemination programs in conjunction with a synchronizing program can vary greatly depending on number of cows, labor cost, and management practices (Olynk, 2008). For producers to maximize efficiency and profitability, an optimal calving interval of approximately 13.3 months is desirable. This interval allows for maximum milk production per cow and optimal timing for introducing replacement heifers to the lactation cycle (Arbel et al., 2001).

To achieve a 13 month calving interval cows must conceive in the third month following calving. Most dairies have a voluntary waiting period of about 60-70 days after calving before they will attempt insemination (Arbel et al., 2001). The optimal time after calving for a cow to become pregnant is between 110-130 days in milk (DIM) which can save producers

approximately $6.96-$7.07 per cow/per day (Giordano et al., 2011). The cost per day of cows that are still open after 130 DIM accelerates rapidly and, on a yearly basis, can cost in excess $343 per cow in the herd (Giordano et al., 2011). On average >2.5 inseminations are required to establish a pregnancy in Holstein dairy cows and >2.3 inseminations for Jerseys (Norman et al., 2009). Furthermore, 20-30% of cows are not cycling by 60 days in milk, thus increasing the calving interval and reducing milk production on dairy farms (USDA, 2009). A successful pregnancy returns an average of $278, whereas an aborted pregnancy costs producers an average of $555 (De Vries, 2006). Efficiency on a dairy farm is driven by optimal calving interval. Cows that cannot become pregnant or lose a pregnancy interrupt the ideal calving interval and become a financial burden to producers.

Because of the challenges of low fertility in high producing dairy cows, culling rates have also increased. In 2007 reproductive problems accounted for 30% of culled cows, in 2012 they accounted for 55.1% of culls (Adamczyk et al., 2016). The adoption of sexed semen and the ability to have more replacement heifers readily available has also increased the rate at which cows are culled (Adamczyk et al., 2016). Fewer cows are being culled for low milk yield than for reproductive disorders. In a recent study in Poland, out of 135,496 Holstein-Friesian cows, only 3,053 of those cows were culled for low milk yield, compared to 53,593 that were culled for infertility and reproductive problems (Adamczyk et al., 2016). For dairy farms to be efficient and profitable, management strategies need to balance high milk production with high fertility, and animal welfare.

The Transition Period

An important time in the cow's life where management can improve reproductive function is the transition period. The transition period encompasses approximately three weeks prior to and three weeks after parturition in dairy cattle (Sordillo and Raphael, 2013). The cow is transitioning from a pregnant state to a non-pregnant, energy demanding lactating state. During this time the cow exhibits reduced immune cell numbers and function and elevated markers of inflammation (Silvestre et al., 2011). The increased energy demands of lactation, coupled with decreased dry matter intake before parturition, as the cow transitions between diets, causes negative energy balance (NEB). This is thought to contribute to compromised immune function (Hammon et al., 2006). The combination of metabolic stress, compromised immune function and pathogen exposure at calving and during lactation makes the cow more susceptible to diseases including metritis, endometritis, mastitis, ketosis, retained placenta, displaced abomasum, and other detrimental health events (LeBlanc, 2012). These disorders are not only detrimental to the overall health and well-being of the cow, but they also decrease milk output and reproductive efficiency, amplifying the negative economic impact beyond the cost of disease treatment (Ingvartsen and Moyes, 2012). In fact, mortality during the transition period accounts for 57% of deaths within a herd; this is compared to less than 20% of deaths that occur after 120 days in milk (Kabara et al., 2014). Cows that fail to return to a normal cycling state within 60 days after parturition express decreased fertility and are more likely to be culled from the herd (Thatcher et al., 2006). For these reasons, improved management during the transition period that improves the health and well-being of the cow is paramount for improving reproduction in high producing dairy cows.

THE IMMUNE SYSTEM

The immune system of a cow is comprised of two components: innate and adaptive immunity (Sordillo et al., 2009). The innate immune system provides the initial, rapid defense against infections and is responsible for controlling most infections. Innate immunity includes skin barriers such as epithelial and endothelial cells, as well as immune cells including macrophages, neutrophils, and natural killer cells (Sordillo et al., 2009). Polymorphonuclear neutrophils (PMN or neutrophils) are a key component of innate immunity and one of the immune cell types that act as a first line of defense against pathogens. Neutrophils are alerted to invading pathogens by the release of cytokines including tumor necrosis factor (TNF) and interlukin-6 (IL6) from infected tissues (Silvestre et al., 2011). Neutrophils extravagate from blood vessels following these cytokine signals and engulf invading pathogens through a process known as phagocytosis. When neutrophils phagocytose they generate reactive oxygen species (ROS) causing an oxidative burst (Silvestre et al., 2011). The oxidative burst from the ROS damages pathogens through the production of H_2O_2 in an NADH-dependent reaction (Slauch, 2011). Reactive oxygen species are also involved in signal transduction and gene activation in inflammatory responses (Asehnoune, et al., 2004). Although ROS are necessary for pathogen removal, excess ROS can lead to cellular damage in the host (Sordillo et al., 2009, Asehnoune et al., 2004). Excess production of ROS was shown to cause activation of the pro-inflammatory nuclear factor kappa B or NF-κB pathway (Asehnoune et al., 2004). Measurement of phagocytosis and ROS are ways to assess neutrophil function (Silvestre et al., 2011; Hammon et al., 2006).

During the transition period total immune cell numbers decrease. This is particularly the case for neutrophils. Neutrophil numbers not only decline, but so does their ability to function.

Neutrophils in transition cows have decreased myeloperoxidase activity, which is necessary for phagocytosis, as well as reduced ROS formation and chemotaxis (Hammon et al., 2006; Ingvartsen and Moyes, 2015). Neutrophils are also activated by macrophages, another innate immune cell, which communicates with the adaptive immune system through antigen presentation (Ingvartsen and Moyes, 2015). Macrophages play a sentinel role in the immune system, constantly patrolling the body for invading pathogens (Elhelu, 1983). When macrophages encounter pathogens they become activated and up-regulate expression of class II major histocompatibility complex (MHC) molecule, allowing them to function as antigen presenting cells to alert other immune cells to the presence of a pathogen (Elhelu, 1983; Chattergoon et al., 1998). This is important because macrophages are capable of stopping small pathogenic invasions through phagocytosis, but in cases of large infections they require help from other immune cells like neutrophils and T cells (Chattergoon et al., 1998).

As a direct result of the changes outlined above, inadequate response by the adaptive immune system is a problem experienced by transition cows (Silvestre et al., 2011). However, the adaptive immune system complements the innate immune system in the fight against pathogens. Adaptive immunity requires assistance from the innate immune system to develop strong, pathogen-specific responses. This assistance comes primarily from macrophages and dendritic cells, which activate the adaptive immune system to produce antibodies against the invading pathogen and to activate T cells to help kill pathogens (Ingvartsen and Moyes, 2015). This one-two punch of innate and adaptive immunity is able to clear most infections. Furthermore, T cells and B cells develop a memory response to specific pathogens from previous infections (Sordillo et al., 1997). B cells are responsible for producing antibodies which are important for fighting infection (Ingvartsen and Moyes, 2015). The adaptive immune response

takes longer to develop, but after it has encountered a pathogen once and developed memory toward that specific pathogen, the second response will be much faster and more effective at eliminating the invading pathogen (Sordillo et al., 2009). Memory allows the adaptive immune system to respond more quickly in subsequent infections by the same pathogen (Sordillo, 2005; Sordillo et al., 2009). The principle of immune memory is the foundation for vaccination programs.

T cells play critical roles in the memory response of adaptive immunity. T cells are classified as T-helper and T-cytotoxic cells. T-helper cells are essential for a successful cell-mediated immune response as they produce interlukin-2 (IL-2) and interferon gamma (IFNγ) which are inflammatory cytokines necessary for recruiting more immune cells for pathogen clearance (Palm and Medzhitov, 2009). T-helper cells recruit macrophages to the site of an infection to promote intracellular killing of pathogen-infected cells (Fearon and Locksley, 1996). T helper cells also promote antibody production of B cells and clonal expansion of cytotoxic T cells (Fearon and Locksley, 1996). Cytotoxic T cells identify infected or damaged cells by antigen presentation. Antigen presentation occurs when innate immune cells, like macrophages and natural killer cells, break down proteins of pathogens in the endosomal compartment. The breakdown of proteins causes a release of peptides that bind MHC II to activate T cells (Fearon and Locksley, 1996). This complex of antigen presentation is the main way the innate and adaptive immune cells work together to clear pathogens. These intricate and specific actions are what make the immune system so effective at fighting off a wide array of infections

INFLAMMATION

Although responses by the immune system are critical for clearing pathogens, a balanced immune response, which maintains tissue homeostasis, is essential. Inflammation is not directly caused by the invading pathogen, but rather indirectly by the response of the immune system to the invading pathogen. Inflammation is a natural response to kill pathogens and to begin the healing process in infected tissues. But if not controlled, excess inflammation can cause more damage to the tissues than the pathogen (Sordillo et al., 2009). Inflammation involves increased blood supply to the infected tissue, increased vascular wall permeability, migration of immune cells from the blood to the tissue, and release of cytokines (Calder, 2012).

Inflammation occurs in a continuum, but the two extremes of inflammation are acute and chronic. Acute inflammation is caused by the initial response of the immune cells traveling from the blood to affected tissues and is typically resolved in a short period of time (Calder, 2012). When acute inflammation fails to be resolved in a timely manner it becomes known as chronic inflammation. Chronic inflammation can damage the host's tissues, due to the uncontrolled, lengthy inflammatory response (Calder et al., 2012). Inflammatory responses are activated when the toll-like receptor 4 (TLR4) recognizes lipopolysaccharide (LPS), which is a component of the cell wall of gram-negative bacteria. Specifically, TLR4 recognizes and binds the lipid A moiety of LPS (Sordillo et al., 2009). Binding of the TLR4 causes activation of NF-κB and increases expression of pro-inflammatory cytokines (Sordillo et al., 2009). The main problem during the transition period is trying to balance the immune response so that there is neither too little nor too much immune response to pathogens. To do this and avoid the negative effects of chronic inflammation, the immune system must switch to an anti-inflammatory state.

Resolution of Inflammation

Acute inflammation is a self-limiting process that generally resolves in a short period of time (Willoughby et al., 2000). Resolution of inflammation is accomplished by loss of immune cells from the site of inflammation to allow the tissue to return to homeostasis (Serhan et al., 2008). Lipid mediators, including eicosanoids, lysophospholipids, phosphoinositides, diacylglycerol, and phosphatidic acid, are known to regulate acute and chronic inflammation (Serhan et al., 2008; Sordillo et al., 2009). The eicosanoid family includes prostaglandins, prostacyclins, leukotrienes, lipoxins, and thromboxanes (Sordillo et al., 2009). Cyclooxygenase-2 (COX2) is an enzyme that has previously been thought to only induce inflammation via biosynthesis of prostaglandins (PG) including PGE_2, $PGF_{2\alpha}$, and thromboxane A_2 (Sordillo et al., 2009). Cyclooxygenase-1 and 2 has been the target of non-steroidal anti-inflammatory drugs (NSAID) to inhibit PG synthesis (Sales and Jabbour, 2003). Recent research suggests that COX2 can reduce and resolve inflammation by selectively inhibiting prostaglandin synthesis (Rajakariar et al., 2006; Wallace, 2006). Arachidonic acids, DHA, and EPA are precursors for eicosanoids and eicosanoids can enhance or resolve inflammation (Calder, 2006; Sordillo et al., 2009). Resolution of inflammation can be initiated when DHA and EPA are metabolized by the lipoxygenase (LOX) or COX2 pathways. This increases prostaglandin D2 synthase (PGD_2) and 15-deoxy-delta-12,14-prostaglandin J_2 ($15d\text{-}PGJ_2$) which inhibits leukocyte adhesion to endothelial cells and blocks the NF-κB pathway to decrease pro-inflammatory cytokine expression (Rajakariar et al., 2006; Prasad et al., 2008; Sordillo et al., 2009). Lipoxins are important mediators in the anti-inflammatory response. Lipoxins are derived from arachidonic acid and have anti-inflammatory and pro-resolution actions (Serhan, 2005). Specifically, lipoxins signal to keep neutrophils and eosinophils from entering the inflamed tissue, activate

macrophage phagocytosis of apoptotic cells, and increase expression of antimicrobial defense molecules (Serhan, 2002; Serhan, 2007; Campbell et al., 2007). Similar actions are also carried out by specialized pro-resolving mediators (SPM) including resolvins and protectins, which are derived from EPA and DHA via the LOX pathways (Basil and Levy, 2015). Activation of NF-κB is regulated by the IKK complex, specifically IKKα and IKKβ (Tak and Firestein, 2001; Lawrence et al., 2005). Phosphorylation of IKKβ allows for prolonged expression of NF-κB, and thus prolonged inflammation (Tak and Firestein, 2001). A new model suggests that IKKα plays a role in resolution of inflammation by inhibiting macrophage activation and increasing the rate at which NF-κB subunits RelA and c-Rel are turned over, reducing their ability to activate pro-inflammatory genes (Lawrence et al., 2005). Resolution of inflammation is imperative for keeping the immune system in balance during an immune response to avoid host damage.

The transition period results in suppression of both innate and adaptive immunity, which causes decreased immune cell numbers and a decrease in lymphocyte proliferation, as well as decreased ability of the immune system to self-regulate (Ingvartsen and Moyes, 2015). Although the exact causes of immunosuppression are still being investigated, it is thought to be due, in part, to the negative energy balance experienced by cattle during early lactation (Kimuara et al., 2014).

Metabolic Challenges

Glucose is a primary energy source for lactation and glucose concentrations are low in transition dairy cows (Sordillo and Rapheal, 2013). Glucose also happens to be the primary energy source for immune cells (Ingvartsen and Moyes, 2015). After parturition, fatty acids stored in adipose tissue are mobilized to meet the energy demands of lactation. This elevates blood concentrations of non-esterified fatty acids (NEFA) and beta-hyrdoxybutyrate (BHBA)

(Graugnard et al., 2012). In vitro treatment of cells with BHBA, at concentrations that mimic those in a postpartum cow, impaired the phagocytic ability of PMNs (Suriyasathaporn et al., 1999). A similar study showed increased NEFA concentrations *in vitro* decreased bovine PMN viability (Scalia et al., 2006). During the late dry period, cows are fed with the intent of avoiding the negative health events associated with NEB postpartum. However, overfeeding cows did not reduce the incidences of periparturient diseases; it decreased fertility, and increased body condition loss (Graugnard et al., 2012; Beever, 2006). Overfeeding energy in transition cows can actually increase fat deposition in the viscera, which can lead to impaired liver metabolism after parturition and increase NEFA and BHBA in the blood as well as excessive body condition loss and increased inflammation and periparturient disease (Graugnard et al., 2012, Beever, 2006, Dann et al., 2006). Overfed cows exhibited decreased neutrophil phagocytic capacity compared with cows that were fed to meet requirements. Overfed cows also exhibited higher blood insulin concentrations, which could impair PMN phagocytic capabilities by reducing plasma glucose (Graugnard et al., 2012). Antibodies that help fight infections, including IgG and IgM, were also reduced after parturition, potentially contributing to immunosuppression (Esposito et al., 2014). It is clear that over-conditioned cows have more health problems compared to cows with a lower body condition score during the transition period and that this leads to reduced fertility (Kim and Suh, 2003).

Clearly, proper feeding and management of transition cows is critical to reduce diseases postpartum. These diseases are costly to treat, result in premature culling, reduced milk income, and reduced fertility. Cows need to be fed enough energy to support a rapidly growing calf in late gestation and to produce large quantities of milk after parturition. One strategy that is of

growing interest to overcome this challenge is to feed fats as an energy source during the transition period.

Fatty Acids

Fatty acids can play a role in immune regulation by inhibiting or promoting receptor binding, second messenger signaling, and transcription factor activation, and by inhibiting arachidonic acid metabolism (Sordillo et al., 2009; Gandra et al., 2016). There are two main types of fats, saturated and unsaturated fatty acids. Each type affects the immune system differently. Saturated fatty acids such as palmitic, oleic, and lauric, which are found in common ingredients in TMRs, can bind TLR4, and activate NF-κB and pro-inflammatory responses (Lee at al., 2001, Sordillo et al., 2009). Increased saturated fatty acids in blood from adipose tissue due to negative energy balance may be linked to reduced PMN viability, although the mechanism is not completely understood (Ingvartsen and Moyes, 2012; Scalia, 2006). Feeding saturated fatty acids was associated with increased milk yield, increased negative energy balance, and decreased fertility (Leroy et al., 2014). Saturated fatty acids induced insulin resistance, which increased the amount of glucose available for lactation, but at the cost of increasing adipose lipid mobilization. This caused increased NEFA concentrations and thus elevated NEB (Leroy et al., 2014). Conversely, polyunsaturated fatty acids (PUFA) including alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), inhibit LPS-induced NF-κB activation. The lipid A moiety of LPS is inhibited from binding TLR4 by PUFAs (Ingvartsen and Moyes, 2012, Sordillo et al., 2009). Unsaturated fatty acids were associated with decreased milk fat percentage and yield, which can improve energy balance (Leroy et al., 2014). Alpha linolenic acid (ALA; 18:3n3), an essential omega-3 fatty acid, reduced production of inflammatory cytokines including interleukin-1, interleukin-6, and tumor necrosis factor

(Lessard et al., 2003, Elis et al., 2016). In tissues, ALA is converted to stearidonic acid (18:4n3) by delta-6 desaturase. Elongases convert stearidonic acid to eicosatetraenoic acid (20:4n3), which is then desaturated by delta-5 desaturase to EPA (20:5n3) and the final conversion in the synthesis pathway is to DHA (22:6n3) through delta-2 desaturase, elongases, and beta-oxidation (Figure 1.1; Calder, 2012). Eicosapentaenoic acid (EPA) and DHA both inhibit inflammatory responses (Lee et al., 2003). When bovine mammary epithelial cells were cultured with PUFA, mRNA abundance of IL6 and IL8 were reduced, both are pro-inflammatory cytokines (Rezamand and McGuire, 2011). There is growing evidence that, although feeding fats can increase NEFA, the type of fat influences the type of NEFA produced and the type of NEFA can modulate immune cell response (Ingvartsen and Moyes, 2012). A thorough understating of how different fatty acids affect immune responses may lead to advancements in feeding strategies for dairy cattle during the transition period.

Various types of supplements are fed to influence dry matter intake, milk production, and health of dairy cows. Antioxidants and minerals are common feed supplements. Vitamin E, selenium, copper, chromium, zinc, and beta-carotene improve health and immunity in transition dairy cows (Spears and Weiss, 2008). Fats are fed to increase energy intake in the postpartum cow; around parturition dry matter intake decreases so it is important to increase energy density of the diet (Leroy et al., 2014; Sordillo and Raphael, 2013). The benefits of feeding fats includes reduced calving interval and increased first service pregnancy rates (Rodney et al., 2015). It is hypothesized that feeding fats leading up to parturition decreases fat mobilization and accumulation in the liver post-partum, which reduces metabolic diseases (LeRoy et al., 2014). Traditionally, omega-6 fatty acids, a family of polyunsaturated fatty acids that includes alpha-linoleic acid (LA), are fed around parturition to stimulate prostaglandin production to improve

uterine health (Elis et al., 2016). Omega-6 fatty acids also induce a pro-inflammatory state, which is thought to help with clearance of pathogens from the uterus after parturition by stimulating production of prostaglandin F2α (Lessard et al., 2004; Leroy et al., 2014). Cows that were fed a diet containing soybeans (high in omega-6 fatty acids) after parturition exhibited a higher omega-6 to omega-3 ratio (Lessard et al., 2004). Closer to breeding, producers often switch from diets enriched in omega-6 fat to diets enriched in omega-3 fats to decrease inflammation and prostaglandin production, making the uterus a more favorable environment for conception and pregnancy (Silvestre et al., 2011; Leroy et al., 2014). Whether or not this feeding strategy is effective in maintaining optimal health for transition dairy cows is a hypothesis which needs to be tested further.

FLAXSEED

Flaxseed is a good source of α-linolenic acid (ALA), which is the shortest member of the omega-3 PUFA family. Feeding flaxseed to transition dairy cows did not impair lymphocyte proliferative responses compared to an omega-6 soybean supplemented diet (Lessard et al., 2004). Improved milk production, composition and reproductive health were also demonstrated in cows fed a flaxseed supplemented diet (Zachut et al., 2010; Petit, 2002). Feeding a dry-extruded or heat-treated source of flaxseed increased protein and PUFA concentrations in the milk and specifically increased ALA (Petit, 2002). Untreated flaxseed undergoes bio-hydrogenation by rumen microbes. Under normal feeding conditions, more than 80% unsaturated fatty acids are bio-hydrogenated in the rumen (Palmquist, 1993; Syadiati et al., 2012). During this process, microorganisms in the rumen hydrogenate double bonds from the unsaturated fatty acids to form saturated fatty acids (Syadati et al., 2012). This greatly reduces the amount of

unsaturated fatty acids available for absorption in the small intestine and delivery to the mammary gland.

Several studies showed that feeding flaxseed diets resulted in increased conception rates, pregnancy rates and reduced embryo mortality (Petit and Twagiramungu, 2006; Ambrose et al., 2006, Dirandeh et al., 2013). Furthermore, the size of pre-ovulatory follicles was increased while numbers of small follicles was decreased in animals fed diets containing flaxseed (Ambrose et al., 2006, Zachut et al., 2010). It was theorized that omega-3 fats caused the generation of a follicle and corpus luteum by inducing proliferation of granulosa cells (Petit and Twagiramungu, 2006). However, this speculation has not been confirmed. There are several hypotheses on how supplementing flaxseed improves fertility. The most common hypothesis is that omega-3 fats decrease uterine production of prostaglandin F2 alpha (PGF2α), thereby reducing early embryo mortality (Elis et al., 2016).

Interestingly, a recent study showed that flaxseed supplemented cows expelled their placentas sooner than cows on a control diet and that flaxseed supplemented cows experienced lower disease incidence postpartum (Ulfina et al., 2015). However, another study found no difference in placenta expulsion rate with an omega-3 supplemented diet, therefore this area needs to be studied further (Kemp et al., 1998). Retained placentas contribute to increased inflammatory responses and uterine infections, which can progress from metritis to endometritis, and cause decreased milk yield (Rajala and Grohn, 1998; Han and Kim, 2005; Ulfina et al., 2015). Another study reported that feeding omega-3 fats enhanced oocyte quality and improved fertilization and embryo development in lactating dairy cows (Elis et al., 2016). Polyunsaturated fatty acids reduce uterine PGF2α secretions which may extend the life of the corpus luteum (CL) by making it less sensitive to PGF2α, allowing for increased embryo survival rates (Staples et al.,

1998). In one study, when cows were supplemented with calcium-soaps of long chain unsaturated fatty acids, the number of large follicles was increased and the number of small follicles decreased (Lucy et al., 1991). The mechanism by which dietary fats improves reproduction is not entirely understood, but proposed hypotheses include: reducing NEB allowing cattle to return to cyclicity sooner postpartum, increased steroidogenesis favorable for improved fertility, reduced insulin resistance to stimulate follicle development, and actions on production and release of PGF2α which may influence the persistence of a CL (Staples et al., 1998).

Omega-3 Fatty Acids and Regulation of Inflammation

Another key source of omega-3 fatty acids is from fish, especially cold water fishes (Harper and Jacobson, 2001; Hooper et al., 2006; Simopoulos, 2000). Both animal and human studies showed that omega-3 fats regulate inflammation. Humans supplemented with 4 g/day of a marine-sourced omega-3 FA for either six weeks or nine months showed reduced neutrophil and monocyte chemotaxis, and the decrease in neutrophil migration was greater in the group supplemented for nine months (Schmidt et al., 1992). Other studies demonstrated that EPA and DHA differentially regulate adhesion molecule expression, including intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule-1(VCAM-1), and endothelial-leukocyte adhesion molecule-1 (ELAM-1, E-Selectin; Collie-Duguid and Wahle, 1996). Adhesion molecules are surface proteins on endothelial cells and leukocytes that promote cell-cell interaction and cause immune cells to travel out of the bloodstream through the blood vessel wall (extravagate) to sites of inflammation (Calder, 2012). Experiments in mice revealed that EPA inhibited LPS-induced monocyte and endothelial adhesion molecule expression (Yamada et al., 2008). Furthermore, EPA and DHA supplementation in the diet reduced pro-inflammatory

cytokines in mice and humans (Caterina et al., 1994; Yaqoob and Calder, 1995; Khalfoun et al., 1997). Incorporation of omega-3 fatty acids into the phospholipids of cell membranes decreased inflammation because cells involved in inflammation typically have more omega-6 FA (eg arachidonic acid) than omega-3 FA (eg EPA or DHA; Calder, 2012). There is increasing interest in the effects of omega-3 fats on cancer and autoimmune diseases based on results in humans and animals (Simopoulos, 2002; Hooper et al., 2006). The effects of omega-3 fatty acids, especially EPA and DHA, on reducing pro-inflammatory cytokines and inhibiting LPS-induced inflammatory reactions supports the concept that omega-3 fatty acids can regulate inflammatory processes in both humans and animals (Caterina et al., 1994; Khalfoun et al., 1997; Yaqoob and Calder, 1995, Yamada et al., 2008). Despite these promising findings, further research is still needed for a full understanding of the mechanisms behind the effects of omega-3 fatty acids.

A recent study demonstrated that reducing the ratio of omega-6 to omega-3 fats in the diet increased dry matter intake (Greco et al., 2015). Decreased DMI by cows fed an omega-6 fatty acid supplement was associated with lower blood glucose concentrations and increased blood concentrations of BHBA (Gandra et al., 2016). Cows supplemented with soybeans (high in omega-6 FA) exhibited reduced proliferation of PBMC in response to concanavalin A (ConA) stimulation when compared to cows supplemented with flaxseed (Lessard et al., 2004). Furthermore, PBMC from soybean-supplemented cows produced more IFN-γ, a pro-inflammatory cytokine, than those fed flaxseed (Lessard et al., 2004). Progesterone concentrations were higher in cows fed flaxseed compared to those fed soybeans and there was 12.4% greater embryonic loss after 28 days in cows fed soybeans (Petit and Twagiramungu, 2006). The effects of omega-3 fatty acids on dairy cows are still being studied, but evidence

suggests that feed sources high in omega-3 fatty acids increases DMI and decreases inflammation and embryonic loss.

Fatty Acids and Prostaglandin Synthesis

The observed differences between omega-3 and omega-6 fats on immune cell function and reproductive performance may be attributed to how each family of FA affects synthesis of prostaglandins. Linoleic acid (18:2n6) can be converted to arachidonic acid and linolenic acid (18:3n3) can be converted to EPA through a series of desaturase and elongase steps (Petit and Twagiramungu, 2006; Lessard et al., 2004). Eicosapentaenoic acid and arachidonic acid are precursors for prostaglandin biosynthesis, but the two lead to prostaglandins with different biological activity (Petit and Twagiramungu, 2006). Omega-6 FA lead to the production of PGE_2 and PGF, and omega-3 FA inhibit the production of PGE_2 and PGF and promote prostacyclin production leading to anti-thrombin and anti-inflammatory processes (Simoloulos, 2002). Furthermore, omega-3 family FA cause a reduction in ovarian and uterine prostaglandin F2α production which can be detrimental to a developing embryo at high concentrations (Petit and Twagiramungu, 2006). Many studies support that the type and amount of fat fed is important because of the downstream effects of each type of fat on inflammatory pathways and prostaglandin synthesis (Simopoulos, 2009; Gandra et al., 2016).

Effects of Fatty Acids on Milk Production and Composition

Of further interest in feeding fats are effects on milk production and composition. Some studies have shown cows supplemented with omega-3 fats tended to produce more milk, but these cows also peaked earlier in their lactation than cows on a diet high in saturated and omega-6 fatty acids (Megalac; Petit, 2002; Petit et al., 2007; Zachuet et al., 2010). In one study that

reported increased milk production in cows supplemented with flaxseed, the flaxseed-supplemented diet contained more fat than the Megalac diet which may explain the increased milk yield (Petit, 2002). There is inconsistency in the literature on the effects of omega-3 fats on milk yield because some studies reported no effect on milk production (Drackley et al., 1992; Christensen et al., 1994; Petit et al., 2002; Chillard et al., 2010). The study by Petit (2002) also showed daily lactose production was not negatively affected by a diet containing formaldehyde-treated whole linseed (omega-3 FA) at 10.4% DM. Milk protein was higher in the linseed-supplemented cows compared to cows supplemented with similar amounts of FA derived from soybeans (Petit, 2002). Results from several studies showed that diets supplemented with unsaturated fatty acids led to depression of milk fat and protein concentrations; however this effect was inconsistent throughout the literature (Romo et al., 1996; Petit, 2002; Mustafa et al., 2003; Petit et al., 2007; Zachuet et al., 2010). Discrepancies in milk fat depression may be due to the varying concentrations at which the fat was supplemented and the source of the fat. It was shown that feeding seed sources of omega-3 fats, like flaxseed, was more beneficial to milk production and components than fish oil. Cows that received fish oil had decreased milk production and milk fat and protein, compared to cows on a flaxseed diet (Petit et al., 2002). The difference was thought to be caused by high levels of trans-fatty acid generation in the rumen (Petit et al., 2002; Baumgard et al., 2000).

Including heat-treated sources of omega-3 FA in the diet increased their amount in milk by protecting the fat from undergoing bio-hydrogenation in the rumen (Petit at al., 2002). Effectively feeding FA so that they end up in the milk is a challenge for dairy producers, but it is possible to increase the amount of omega-3 PUFAs in milk. Consumer's interest in consuming omega-3 FA has driven efforts to increase their concentrations in foods including meat, eggs and

milk. One study reported that omega-3 fatty acid content was almost four times higher in flaxseed supplemented cows than control cows (Zachut et al., 2010). In the same study the omega-6 to omega-3 ratio was reduced to 8.3 from 2.3 (Zachut et al., 2010). Despite the challenges involved with feeding cows to increase omega-3 PUFAs in milk, this study revealed it is possible.

Health Benefits of Omega-3 Fatty Acids for Humans

Producers are interested in finding ways to increase omega-3 fats in milk because of consumer demand. A healthy ratio of omega-6 to omega-3 fats in a human diet is 1:1 or 2:1 (Simopoulos, 2009). The average American diet has a ratio of omega-6 to omega-3 fats of 15:1 (Simopoulos, 2002). High amounts of omega-6 fats in the human diet have been linked to obesity, type 2 diabetes, and cardiovascular disease (Liu et al., 2013). Various studies have shown that supplementing human diets with sources of omega-3 fats reduced inflammation in cases of inflammatory bowel disease and rheumatoid arthritis (Kremer et al., 1987; Stenson et al., 1992; Trebble, 2005; Cleland et al., 2006; Calder, 2012;). Like cows, humans cannot synthesize omega-3 fatty acids because they lack the delta-15 desaturase enzyme necessary for synthesis of alpha linolenic acid (Calder, 2012.) External sources of omega-3 fats must be incorporated in human diets (Arterburn et al., 2006). Omega-3 fats were found to be beneficial to human health and studies have linked omega-3 fats in the diet to decreased incidences of cardiovascular diseases, hypertension, and arthritis (Zachut et al., 2010; Stark et al. 2016; Lemerle et al., 2016; Colussi et al., 2016). Consumers are becoming more health conscious and want to consume healthier foods. Therefore demand for omega-3 supplemented food, including milk, has recently increased (Zachut et al., 2010; Bainbridge et al., 2017).

SUMMARY

For dairy producers to be profitable they need to be efficient. This means they need healthy cows that maintain high milk production and an acceptable calving interval. For this to happen, cows need proper nutrition and reproductive management. Failure to provide cows with the necessary nutrition and management results in loss to the producer. Evidence in the literature supports that omega-3 FA could be used to improve dairy cow health and production, especially during the critical transition period. Specifically, omega-3 fats are shown to possess anti-inflammatory properties by inhibiting the pro-inflammatory NF-κB pathway which reduces production of pro-inflammatory cytokines including TNF and IL6 (Caterina et al., 1994; Yaqoob and Calder, 1995; Khalfoun et al., 1997; Asehnoune et al., 2004). The ability of omega-3 fats to suppress neutrophil chemotaxis and phagocytosis is also of interest as it may help to calm the immune response during the transition period and switch the immune system from an inflammatory response to a resolving, healing state (Scalia et al., 2006, Serhan et al., 2008). Supplementing dairy cows with omega-3 fats was not shown to be detrimental to overall milk production, but in some cases caused milk fat depression, and milk tended to contain less omega-6 FA and more omega-3 FA, potentially making the milk more marketable to the general public (Zachut et al., 2010).

The objective of this study was to examine the effects of feeding an omega-3 fatty acid supplement on the immune response of postpartum Holstein dairy cows. Our working hypothesis is that omega-3 supplementation would alter immune cell function in transition dairy cows by inducing anti-inflammatory immune responses. Furthermore, this would increase the amount of

omega-3 fatty acids in the milk, thus reducing the ratio of omega-6 to omega-3. To address these hypotheses we had the following objectives:

1. To measure the fatty acid profile in milk, plasma, and red blood cells to determine if the concentration of omega-3 fatty acids can be altered.
2. To measure proliferative response and mRNA abundance of PBMCs as a measure of immune function.
3. To measure neutrophil phagocytosis and reactive oxygen species to determine if there is any effect of omega-3 fats on neutrophil function.

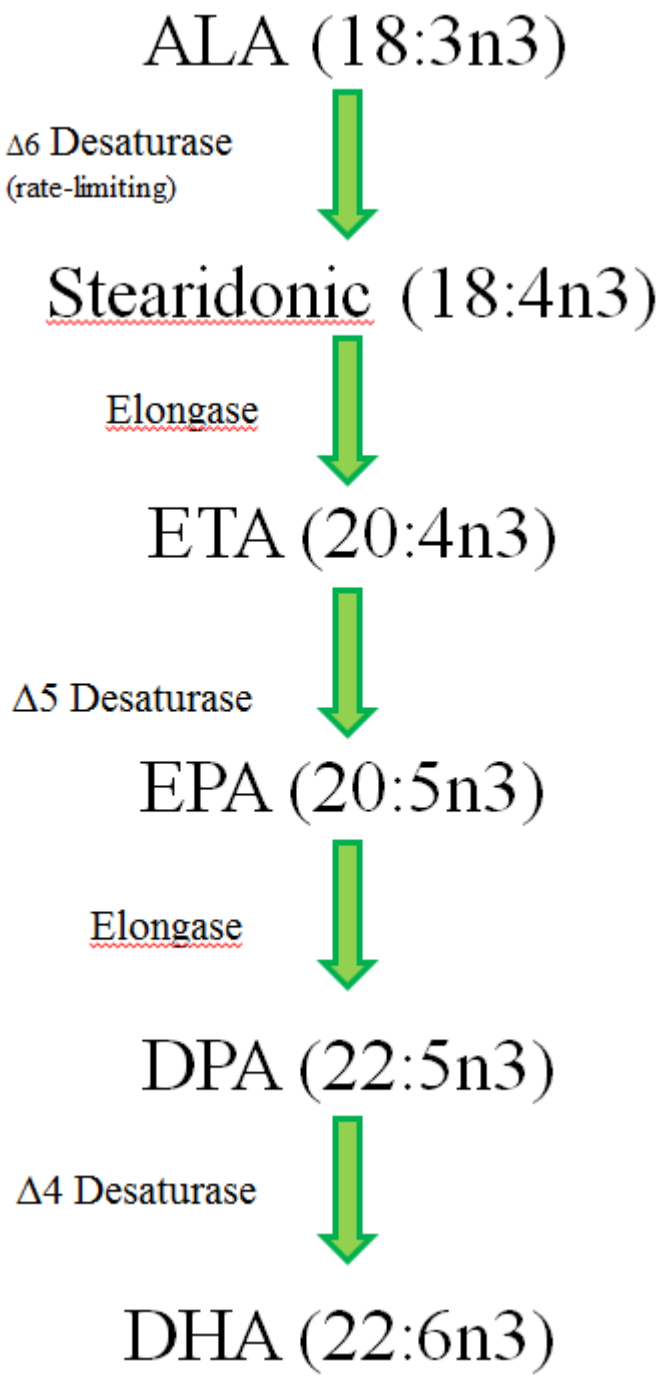

Figure 1.1: Conversion Pathway of ALA to DHA
In tissues, ALA is converted to DHA through a series of desaturase and elongase enzymes. Both ALA and DHA are essential fatty acids and cannot by synthesized in humans or ruminants.

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Chapter 2: Effects of feeding an extruded flaxseed supplement on fatty acids in milk and plasma and immune function in transition dairy cows

Abstract

The transition period, which encompasses three weeks prior to three weeks after calving, is a critical time the life of a cow. During this time cows exhibit reduced immune cell numbers (particularly neutrophils) and function and elevated markers of inflammation. Compromised immune function is thought to be caused, in part, by extreme metabolic stress and changing hormones in response to the sudden transition from a pregnant to energy-demanding lactating state coupled with decreased dry matter intake (Sordillo et al., 2009). These changes in the cow can increase the risk for metritis, endometritis, mastitis, ketosis, and other detrimental health events (LeBlanc, 2012). These health disorders are not only detrimental to the overall health and well-being of the cow, but they can also decrease milk output and reproductive efficiency (Ingvartsen and Moyes, 2012). For these reasons, improved management during the transition period that improves the health and well-being of the cow are paramount for setting the stage for successful reproduction and high milk production.

The objective of this study was to determine the effects of feeding an omega-3 fatty acid flaxseed supplement on transition dairy cow immune function, energy balance, and fatty acid composition of milk, plasma, and red blood cells. Multiparous Holstein dairy cows

(n=15) were randomly assigned to two treatments: control cows (n=8) received whole roasted soybeans at 4.8% dry matter (DM) and flaxseed cows received LinProR® at 3.5% DM (n=7). Cows were fed these diets for the first 21 days of lactation. Blood was sampled on days 1, 7, 14, and 21. Milk was sampled on days 7, 14, and 21. Dry matter intake and milk production did not differ between groups and no differences were observed in plasma non-esterified fatty acids, glucose or tumor necrosis factor (TNF). Total milk fat percentage tended to be higher (P=0.067) in the flaxseed group (4.5%) compared to the control group (3.9%), with no differences in milk protein, lactose, MUN, or somatic cell count. Flaxseed-supplemented cows tended to have more ALA in milk fat (0.7%; P=0.059) and plasma (4.99%; P=0.097) than control cows. Production of reactive oxygen species (ROS) by neutrophils was reduced in cows fed flaxseed (P<0.01) and the amount of neutrophil phagocytosis also tended to be reduced (P=0.087). There were no effects on the proliferation of peripheral blood mononuclear cells (PBMCs), either in the presence or absence of concanavalin A stimulation. The mRNA abundance in PBMCs of TNF (P=0.090), interleukin-6 (IL6, P=0.1), and IL10 (P=0.094) tended to be reduced in flaxseed-supplemented cows.

Overall, cows receiving the flaxseed supplement had greater ALA in plasma and milk fat and reduced neutrophil phagocytosis and ROS. Results indicate that the omega-3 supplement altered the activation status of the immune system and reduced inflammatory responses. This reduction in neutrophil activity may increase energy available to the cow for productive functions, although this speculation needs to be confirmed with further research. The milk fat and plasma exhibited greater ALA the longer the cows were on the diet indicating this supplement can be used to produce milk with high omega-3 fatty acids, which

is an area of increased consumer interest. Furthermore, results provide support for the hypothesis that fat feeding can alter immune function.

Introduction

The transition period encompasses three weeks prior to three weeks after calving and is a critical time in the cow's life. During the transition period proper management and nutrition are essential for reducing the incidence of diseases that typically occur during this time. The increased incidence of disease is thought to result from the combination of decreased dry matter intake and the high energy demand of lactation that causes the cow to enter a negative energy balance. Negative energy balance contributes to compromised immune function including reduced immune cell numbers and function and elevated markers of inflammation (Silvestre et al., 2011; Hammon et al., 2006). Neutrophils in transition cows exhibit decreased myeloperoxidase activity, phagocytosis, reactive oxygen species formation and chemotaxis (Hammon et al., 2006; Ingvartsen and Moyes, 2015). The combination of negative energy balance and compromised immune function during the transition period increases the risk of metritis, endometritis, retained placenta, mastitis, displaced abomasum, and other detrimental health events (LeBlanc, 2012). These health events can delay onset of ovarian activity and cows that fail to return to a normal cyclic state within 60 days after parturition exhibit decreased fertility (Thatcher et al., 2006).

Fatty acids play a role in regulating immune function by promoting or inhibiting toll-like receptor 4 binding, second messenger signaling, transcription factor activation, and by inhibiting arachidonic acid metabolism (Sordillo et al., 2009; Gandra et al., 2016). Of the two main types of fats (saturated and unsaturated), polyunsaturated fatty acids (PUFA) have been associated with decreased milk fat production and thereby improved energy balance (Leroy et al, 2014). Omega-3 fatty acids are members of the PUFA family that have been shown to reduce production of inflammatory cytokines (Lessard et al., 2003, Elis et al., 2016). Flaxseed

is a rich source of α-linolenic acid (ALA), the shortest member of the omega-3 PUFA family. In one study, feeding a flaxseed diet to transition dairy cows did not impair lymphocyte proliferative responses compared to a soybean supplemented diet (omega-6 fat) which reduced lymphocyte proliferation, however no mechanism or explanation was provided for why an omega-6 diet may reduce proliferation (Lessard et al., 2004). In a previous study omega-3 fats in the diet either had no effect or decreased lymphocyte proliferation (Lessard et al., 2003). Due to discrepancies in the literature this area needs to be explored further. It was speculated that overall lymphocyte proliferation is decreased around parturition because cell-mediated immunity is downregulated (Lessard et al., 2004). Benefits in milk production and composition have been reported from a flaxseed supplemented diet including increased milk yield and fat (Zachut et al., 2010). Feeding a rumen-protected source of flaxseed increased milk ALA and milk protein compared to a soybean diet (Petit, 2002). Effects of fat on milk protein is dependent on the fat source, but if supplemented correctly, can allow cows to produce greater concentrations of milk protein sooner in lactation and increase the value of milk (Schingoethe et al., 1996; Petit, 2002). Increased PUFA in milk and decreased omega-6 to omega-3 ratio will improve the nutrition value of milk for humans (Petit, 2002).

Further benefits of omega-3 fats include improved reproductive function. It is theorized that omega-3 fats cause the generation of larger follicles and corpora lutea by inducing proliferation of granulosa cells (Petit and Twagiramungu, 2006; Ambrose et al., 2006; Dirandeh et al., 2013). Feeding flaxseed diets to cows resulted in increased conception and pregnancy rates and decreased embryo mortality (Petit and Twagiramungu, 2006; Dirandeh et al., 2013; Rodney et al., 2015; Elis et al., 2016). There are several hypotheses to explain how supplementing flaxseed improves fertility. One hypothesis is that omega-3 fats

decrease uterine concentrations of prostaglandin F2 alpha (PGF2α), reducing early embryo mortality (Elis et al., 2016). Another hypothesis is that omega-3 fatty acids may enhance oocyte quality and health, but mechanisms behind these theories need to be explored further as there is little research in this area (Moallem et al., 2009; Elis et al., 2016).

Producers are interested in feeding omega-3 fats due to enhance milk production and composition (Zachut et al., 2010). There is some evidence that cows supplemented with omega-3 fats produce more milk, but peak earlier in their lactation than cows fed an omega-6 supplemented diet (Petit, 2002). Another driving force for supplementing cows with omega-3 fats is consumer demand. Omega-3 fats have been found to be beneficial to human health; omega-3 fats in the diet have been linked to decreased incidence of cardiovascular diseases, hypertension, and arthritis (Zachut et al., 2010).

The objectives of this study were to examine the effects of feeding an omega-3 fatty acid supplement on milk production, metabolic and immune responses of Holstein dairy cows after calving. The working hypothesis is that omega-3 fatty acid supplementation will alter immune cell function in postpartum cows by inducing anti-inflammatory immune responses. Furthermore, supplementation will increase the amount of omega-3 fatty acids in milk fat, which market research suggests is a characteristic desired by some consumers (Marshall et al., 1993)

Materials and Methods

ANIMALS

All procedures involving animals were reviewed and approved by the Pennsylvania State University Institutional Animal Care and Use Committee (protocol #46326) and complied with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS Ag Guide, 2010). Fifteen multiparous Holstein dairy cows from the Penn State University dairy herd were housed in a tie stall barn and milked twice a day at 0700 and 1900 h. Milk yields were recorded at each milking. At calving cows were assigned randomly to dietary treatment group. Cows were fed a total mixed ration (TMR) once a day in amounts to ensure 10% refusals. Amount of feed offered and refused were recorded daily to calculate daily dry matter intake for each cow. One group of cows received a commercially available flaxseed supplement (LinPro™-R, kindly supplied by O&T Farms Inc., Regina, Saskatchewan, Canada; n=7) at 3.5% of diet dry matter (DM). The flaxseed supplement was mixed with the TMR at each feeding. The control group (n=8) was supplemented with whole roasted soybeans at 4.8% DM to yield similar crude protein, ether extract, and energy concentrations. Diets were formulated to meet the energy requirements for cows with a mean body weight of 580 kg producing 40 kg/day of milk with 3.5% fat (Nutrient Requirements of Dairy Cattle: Seventh Revised Edition, 2001).

Sample Collection

Blood (500 mL) was collected on Days 1, 7, 14, and 21 after parturition. Day 1 was the day after the cow calved with an average of 16.5 hours (range of 9-30 hours) between calving and first sampling, which did not differ between dietary treatment groups. Milk was collected on Days 7, 14, and 21 and sent to Dairy One (Ithaca, NY) for component analysis by FTIR spectroscopy.

Immune Cell isolation

Blood was collected via the jugular vein into a sterile bottle containing 1 mL of 0.5M ethylenediaminetetraacetic acid (EDTA) per 100 mL of blood and stored on ice until processing. To isolate peripheral blood immune cells (PBL) from plasma and red blood cells, blood was transferred to glass tubes (16x125 mm) and centrifuged at 1494 x g for fifteen minutes at 4° C. This separated the blood into layers with red blood cells on the bottom, buffy coat (containing immune cells) in the middle, and plasma on the top. Plasma was removed and stored at -20 C in 1.5 mL aliquots in Eppendorf tubes for later analysis. Red blood cells were stored in 1.5 mL Eppendorf tubes at -80° C for later analysis. The buffy coat was pipetted into a 50 mL conical tube containing 5 mL of ice cold 1x phosphate-buffered saline (PBS containing 2mM EDTA). Peripheral blood mononuclear cells (PBMC) and polymorphonuclear cells (PMN) were isolated using Ficoll-Paque PLUS (17-1440-02; GE Healthcare Bio-Sciences, Pittsburgh, PA) gradient centrifugation (Davis and Pate, 2007). After centrifugation the top layer contained the PBMC and the lower red layer contained the PMN. Each layer was pipetted into a separate 50 mL conical tube. A red blood cell (RBC)

lysis was performed on the PMN to remove unwanted red blood cells. Twice the volume of RBC lysis buffer was added to the PMN and it was gently inverted for 3 minutes and centrifuged at 664 x g for 10 minutes at 4° C. The supernatant from the PMNs was decanted and the RBC lysis step repeated. The PBMC were washed twice at 664 x g for 10 minutes at 4° C. Both sets of cells underwent further washes with 1x PBS/2mM EDTA three times at 295 x g for 10 min at 4° C, once at 150 x g for 10 min at 4° C, and once at 100 x g for 10 min at 4° C. Cell pellets were resuspended in 2 mL of 1x PBS/2mM EDTA. The viability of cells was determined using the Guava ViaCount Flex reagent (4000-0040; EMD Millipore, Billerica, CA) at a final dilution of 1:500. The cells were counted using a Guava EasyCyte Plus (Guava Technologies, Hayward, CA) with the Cytosoft 5.3 software (Guava Technologies, Hayward, CA).

PBMC Proliferation Assay

Proliferation medium was prepared using 100 mL AimV medium (31035-025; Gibco Life Technologies, Grand Island, NY) with added insulin, transferrin and selenium (ITS; 1uL/mL-354351; Corning, Tewksbury, MA) and gentamicin (2 uL/mL, Gibco). Cells were aliquoted to a 15 mL conical tube and centrifuged at 295 x g for 10 minutes at 4° C. The supernatant was discarded and cells were suspended in a total volume of 2 mL of proliferation medium. CellTrace Carboxyfluorescein succinimidyl ester (CFSE, C34554- ThermoFisher Scientific, Waltham, MA) was prepared by reconstituting the CFSE in Dimethyl sulfoxide (DMSO) to generate a 5 mM stock solution. A working dilution of 2mM CFSE was used by adding 16 uL of CFSE stock solution to 24 uL of sterile PBS. To each tube of cells resuspended in 2 mL of proliferation medium, 7 uL of the working dilution of CFSE were

added for a final concentration of 1.75 uM. Tubes were covered in aluminum foil and incubated at 37° C for 15 minutes. Cells remained protected from light and were incubated at room temperature for an additional 10 minutes. To quench any CFSE not taken up by the cells, 2 mL of 10% fetal bovine serum (FBS-16000044; Gibco)-F12 medium (1165-047; Gibco) were added to the tube. The cells were washed three times with 2 mL of 10% FBS-F12 medium at 295 x g for 10 minutes at 4° C. Uptake of CFSE by the cells was confirmed by analyzing green fluorescence intensity using flow cytometry. After the final centrifugation, cells were suspended in 2 mL of proliferation medium and pipetted into a 96 well plate with 0.5 million cells/well and treatments were applied in triplicates. Cells were cultured with or without Concanavalin A (40 ug/mL; ConA; 234567, Calbiochem, La Jolla, CA) for 72 hours at 37° C and analyzed for loss of CFSE as an indicator of proliferation using the Guava ExpressPro program in the Cytosoft 5.3 software and then analyzed using FlowJo V10 (FlowJo, LLC; Ashland, Oregon). The treatments included unlabeled PBMC with 2 uL of a 1mg/mL solution of propridium iodide dye (PI- P3566; Molecular Probes of ThermoFisher Scientific), CFSE-labelled PBMC's and PI dye, CFSE-labelled PBMC with Concanavalin A (ConA) and PI, and CFSE-labelled PBMC.

Neutrophil Phagocytosis and Oxidative Burst Assay

Neutrophil phagocytosis and oxidative burst was measured as previously described (Smits et al., 1997). Propridium Iodide (PI) and dihydrorhodamine 123 (DHR-D23806; ThermoFisher Scientific) were used to determine phagocytic and oxidative burst activity of neutrophils, respectively. *Streptococcus uberis* were grown overnight in Brain Heart Infusion broth (CM1135B; ThermoFisher Scientific) at 37°C. Bacteria were washed three times in

PBS and killed by heating at 70°C for 60 min (Kindly supplied by Dr. A Hristov, Penn State University). The killed bacteria were suspended in 1 mL PBS and labelled with PI (100 ug/mL for $1x10^9$ bacteria/mL) for 60 min at room temperature. The PI-labelled bacteria were washed three times in PBS, suspended in RPMI 1640 (11835-055; Gibco) and counted on a Guava flow cytometer. Adequate labeling of bacteria was confirmed by observing a small amount of the bacteria under the red fluorescence filter of a microscope. The PI-label bacteria were aliquoted and stored at -80°C and thawed immediately before use. The PMN were aliquoted into a 96 well plate at 0.5 million cells per well and treatments were applied in triplicate. The treatments applied were PMN only, PMN with PI dye, DHR-labelled PMN, PMN with PI-labelled bacteria, and DHR-labelled PMN with PI-labelled bacteria.

The PMN were incubated with 5 uM DHR dye (stock 5 mM) for five minutes in a shaking water bath at 37°C. The PI-labeled bacteria were added to the PMN at a ratio of 15:1 in a total volume of 200 uL. The plate was incubated for 20 minutes at 37°C and washed three times with PBS at 664 x g for 2 minutes at 4°C. Phagocytosis and reactive oxygen species were determined on the Guava flow cytometer in the Guava ExpressPro program in the Cytosoft 5.3 software and analyzed using FlowJo V10. Phagocytosis was determined in PI-positive cell measured in the yellow channel. A small aliquot of PI-positive cells was checked under a fluorescence microscope to determine that no free-floating bacteria or bacteria attached to the cell membrane were being considered as positive events by the flow cytometer. Presence of reactive oxygen species was quantified as cells that appeared in the green channel.

RNA Isolation and Quantitative Real Time PCR

Peripheral blood mononuclear cells were frozen in Trizol (15596018; Ambion of Life Technologies, Grand Island, NY) and RNA was extracted according to the manufacturer's protocol with an added step of heating the samples to 60° C for ten minutes to aid in dissolving the pellet and to volatize any remaining ethanol. The quality and quantity of RNA was determined using a Nanodrop 8.2 spectrophotometer (Thermoscientific, Wilmington, DE). An Experion automated electrophoresis system in conjunction with the Experion RNA StdSens kit (BioRad, Hercule, CA) and the Experion Software Version 3.0 (Agilent Technologies, Wilmington, DE) were used to further confirm the quantity and quality of RNA. DNase treatment was performed on the extracted RNA using RQ1 RNase-Free DNase Kit (M6101; Promega Corporation, Madison, WI) according to the manufacturer's protocol. Complementary DNA Synthesis was performed using DyNAmo cDNA Synthesis Kit (F470L; Thermoscientific, Waltham, MA) according to the manufacturer's protocol using random hexamer primers. The samples were loaded into a DNAEngine Peltier Thermal Cycler (BioRad; Hercule, CA) and it was programmed for the following cycling conditions: 25 ˚C for 10mins, 37 ˚C for 30mins, 85˚C for 5 mins, 4 ˚C hold.

To generate standards for quantitative PCR, gene specific amplicons were generated using GoTaq DNA Polymerase Kit (M3005; Promega Corporation, Madison, WI) according to the manufacturer's protocol. The PCR reaction was programmed on the thermal cycler as follows: 95˚C for 3 minutes (denaturation), 95˚C for 30 seconds (denaturation), gene-dependent temperature (Table 1.1) for 45 seconds (annealing), 72˚C for 1 minute (polymerase extension), repeat steps 2-4 for 39 cycles, 72˚C for 5 minutes (final extension), and 4˚C until

removed from thermal cycler. Primers and annealing temperatures used for real time PCR are listed in Table 2.1. Primes were validated by sequencing the amplicons (Genomics Core Facility, Huck Institutes of Life Sciences, Penn State University.)

A 1.5% agarose gel was ran at 90 volts to verify amplicon size for each gene and DNA was extracted from the gel using the QIAquick Gel Extraction Kit Protocol (28706; Qiagen, Hilden, Germany) according to the manufacturer's protocol. The extracted DNA product was used to generate the standard curves consisting of 12-log dilutions to validate the assays for qPCR analysis. Samples were prepared for qPCR analysis using SensiMix SYBR No-ROX Kit (QT650-05; Bioline USA, Taunton, MA) and measured using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies). The qPCR was programmed for the following: 95˚C for 5 minutes, 95˚C for 30 seconds, gene-dependent temperature (Table 1.1) for 1 minute 72˚C for 1 minute, repeat above for 40 cycles, 72˚C for 5 minutes, 4˚C until removed from the machine.

For each gene of interest, a reference gene, ribosomal protein L19 (RPL19) was used to adjust for template input into the reaction mix following confirmation of the absence of treatment effects on RPL19 abundance. Standard curves exhibited a linear range of 5 to 35 cycles. Samples with Ct values beyond the range were excluded and reanalyzed using different template concentrations. The slopes of the standard curves were between -3 and -3.5 with R^2 values above 0.92 and efficiency between 95% and 105%. Melting curve analysis was used to confirm a single amplicon with the expected temperature (Tm).

Plasma NEFA and Glucose Analysis

Plasma non-esterified fatty acid (NEFA) concentrations were measured using a Wako HR Series NEFA-HR (2) kit (Wako Diagnostics, Richmond, VA) according to the manufacturer's protocol. To collect plasma for glucose analysis, blood was collected into a 5.5 mL FX Sodium Fluoride/Potassium Oxalate Vacuette tube (456020; Greiner Bio-One International, Kremsmünster, Austria). The blood sample for glucose concentration was collected at the same times as the other blood samples. The blood was centrifuged at 1494 x g for fifteen minutes at 4° C to collet plasma. Glucose concentrations were measured in the plasma using a Sigma PGO Enzymes Kit (P7119; Sigma Aldrich, St. Louis, MO) according to the manufacturer's protocol. Tumor necrosis factor (TNF) was measured in plasma using the TNF enzyme-linked immunosorbent assay (ELISA) kit from Cusabio (CSBE12020B, Cusabio College Park, MD). Plasma NEFA, glucose, and TNF were all measured on a VICTOR^3 1420 Multilabel Counter (PerkinElmer, Waltham, MA) using the Wallac 1420 Manager software.

Plasma Fatty Acid Profile

Plasma fatty acids were extracted as described by Hara and Radin (1978) and fatty acids were methylated according to the methods previously described by Kramer et al (1997) with modification. Briefly, the samples were extracted by adding 5.4 mL of hexane: isopropanol (3:2 HIP) to 3 mL of plasma. The samples were thoroughly mixed and 3.6 mL of 7% sodium sulfate were added to the samples and they were thoroughly mixed again. The samples were centrifuged at 1400 x g for 5 minutes at 5° C. After centrifugation, the top

organic layer was transferred to a hexane pre-rinsed extraction tube containing 1 gram of sodium sulfate. The tubes were capped and incubated at room temperature for 30 minutes. After incubation the solution was transferred to a new hexane pre-rinsed extraction tube and evaporated under nitrogen at 40° C before methylation.

For methylation, internal standards C13:1 and C19:0 in toluene (0.5mg/mL) were prepared. To each tube, 250 uL of each internal standard and 2 mL of 0.5 M sodium methoxide in methanol solution were added. The samples were vortexed and then incubated in a 50°C water bath for 10 minutes. Next 2 mL of 5% methanolic HCL, prepared from acetyl chloride and methanol, were added and the samples were vortexed and incubated in an 80°C water bath for 10 minutes. After cooling, 2 mL of hexane and 7.5 ml 6% K_2CO_3 were added and the samples were centrifuged at 295 x g for five minutes. The top layer was transferred to a new extraction tube and dried under nitrogen gas. A total volume of 500 uL of hexane were added to the dried samples and they were transferred to GC vials (06-718-975; Thermoscientific, Wilmington, DE) for analysis.

Red Blood Cell Fatty Acid Profile

Red blood cell fatty acid profile was determined using a modification of the above procedure. Briefly, the samples were extracted by adding internal standards C13:1 and C:19:0 in hexane: isopropanol (0.5 mg/mL, 3:2 HIP) to 1 mL of red blood cells. From here, the extraction procedure was carried out the same as for the plasma samples.

Milk Fatty Acid Profile

Milk fatty acid profile was determined according to methods previously described by Choinard et al., 1999. Briefly, approximately 50 mg of fat cake was weighed into 16x150 extraction tubes that were pre-rinsed with hexane. From here the extraction procedure was carried out the same as for the plasma samples.

To methylate, 2 mL of hexane were added to the dried samples and 40 uL of methylacetate were added to that. The samples were thoroughly mixed and 40 uL of methylation reagent were added and samples were mixed again for two minutes. After allowing the samples to stand for 8 minutes, 60 uL of termination reagent were added and samples were vortexed for 30 seconds. Approximately 100 mg of calcium chloride and 2 mL of hexane were then added and the samples were vortexed and let stand for 1 hour. The samples were centrifuged at 1300 x g for five minutes at 5°C. The top layer was transferred to GC vials for analysis by gas chromatography.

The individual fatty acids for milk, plasma, and red blood cells were quantified by gas chromatography using an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a fused-silica capillary column (SP-2560; 100 m x 0.25 mm i.d. with a 0.2-µm film thickness; Supelco Inc., Bellefonte, PA) and a flame-ionization detector with hydrogen as a carrier gas. Fatty acid peaks were identified in gas chromatograph output using pure fatty acid methyl ester standards (GLC 461, GLC 780, and pure *trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 CLA, NuCheck Prep Inc., Elysian MN). An equal weight reference standard (GLC 461, NuCheck Prep Inc.) was used to determine correction factors for individual fatty acids as described by Rico and Harvatine (2013).

Data Analysis

Data were analyzed using MIXED procedures of SAS (SAS v 9.4) with day and treatment as fixed effects and cow as a random effect. For qPCR, all statistical analysis were performed on ΔCt values (Schmittgen and Livak, 2008) using mixed model ANOVA and data are depicted as fold change ($2^{-\Delta\Delta Ct}$). The data collected on Day 1 were used as a covariate for Day 7, 14, and 21 for all analysis. Statistical significance was declared for p-values less than 0.05 and tendencies for p-values between 0.05 and 0.1. In cases where data were not normally distributed the data were log or square root transformed and normal distribution was confirmed. Least squares means with pooled standard errors were plotted on graphs. All graphs were created using GraphPad (Version Prism 5; GraphPad Software Inc., La Jolla, CA).

Results

Dry matter intake and milk yield were not different between the two treatment groups (Figure 2.1A and Figure 2.1B). For both the control and flaxseed groups milk yield increased over time ($P<0.01$). Milk fat percentage tended to be greater in the flaxseed group (0.6 percentage points; $P=0.079$, Figure 2.2A). In both, groups milk fat percentage decreased over time ($P<0.01$). There were no treatment effects observed on the other milk components measured (protein, lactose, milk urea nitrogen, and somatic cell count, Figures 2.2B, 2.3A-C). Protein percent ($P<0.01$, Figure 2.2B) and milk somatic cell count ($P<0.05$, Figure 2.3C) decreased over time whereas milk lactose percentage increased ($P<0.01$, Figure 2.3A). The average milk urea nitrogen in the control group was 9.9 mg/dL and 10.1 mg/dL for the flaxseed group (Figure 2.3B). No treatment effects were observed in plasma glucose (Figure 2.4B), non-esterified fatty acids (Figure 2.4A) or TNF (Figure 2.5A). Plasma NEFA concentrations decreased ($P<0.01$) over time and plasma glucose concentrations increased ($P<0.01$).

The results showed that the percentage of neutrophils producing ROS was lower in flaxseed-supplemented cows compared to the control group ($p<0.01$, Figure 2.7A). It also appeared that the percent of neutrophils phagocytosing was reduced in the flaxseed cows, but due to the large variation among cows there was no statistical difference detected ($P>0.1$, Figure 2.8A). However, there was a tendency for the amount of phagocytosis per neutrophil to be lower in the flaxseed-fed cows ($P=0.09$, Figure 2.8B). The percentage of neutrophils producing reactive oxygen species tended to decrease over the 21-day period ($P=0.07$). The percentage of neutrophils exhibiting phagocytosis showed a tendency to increase ($P=0.097$). There was no effect of treatment on the amount of reactive oxygen species produced (Figure

2.7B). There was no treatment effect observed on proliferation of unstimulated (Figure 2.9A) or ConA stimulated PBMCs (Figure 2.9B), although overall proliferation decreased over time in unstimulated cells ($P<0.01$). There tended to be less mRNA expression for TNF in the PBMC of flaxseed-supplemented cows ($P=0.09$, Figure 2.5B). The mRNA expression of IL6 was reduced in flaxseed supplemented cows ($P=0.1$, Figure 2.6A). Lastly, there was no effect of diet on mRNA expression of IL10 ($P>0.1$, Figure 2.6B).

The fatty acid profiles showed that ALA concentration tended to be greater in milk fat of the flaxseed group ($P=0.06$, Figure 2.10A), and the percentage of ALA (g/100g FA) continued to increase in the flaxseed cows the longer the cows were on the diet and decreased in the control cows, with a tendency for a treatment by day interaction ($P=0.07$). There was no treatment effect on milk fat yield of ALA (g/day, Figure 2.10B). In plasma, there was a tendency for ALA to be greater in the flaxseed cows ($P=0.09$, Figure 2.11A), and the concentration of ALA in the plasma continued to increase to day 21 ($P<0.01$). A treatment by day effect of diet on plasma ALA was observed ($P<0.01$). Of the other omega-3 fatty acids in the synthesis pathway of ALA (ETA, EPA, DPA, and DHA) there were no treatment effects observed except for DHA, the longest chain in the synthesis pathway (Figure 2.11B). There was a tendency for plasma DHA to be increased in the flaxseed cows compared to the control cows ($P=0.09$), but unlike ALA, DHA decreased in both groups over the 21 day trial period ($P<0.01$). There were no differences in g/100g of ALA in the red blood cell fatty acids.

Discussion

The objective of this study was to determine if feeding a flaxseed supplement affects immune function in transition dairy cows. The hypothesis that a flaxseed supplement would shift the immune response to a more anti-inflammatory state was supported by the results.

The percentage of neutrophils producing reactive oxygen species (ROS) was less in flaxseed-fed cows than in control cows. The amount phagocytosis by neutrophils tended to be reduced in flaxseed-fed cows. The reduction of both phagocytosis and ROS in cows supplemented with flaxseed is physiologically linked because when neutrophils phagocytose it causes generation of ROS (Silvestre et al., 2011). The oxidative burst is responsible for damaging pathogens, but in excess causes damage to host cells and tissues (Sordillo et al., 2009, Asehnoune et al., 2004). Reactive oxygen species are also a sign of a proinflammatory state, so the reduced ROS in the treatment group reflects a reduced inflammatory state (Sordillo et al., 2009, Asehnoune et al., 2004). Our results differ from another study that reported increased phagocytosis in cows supplemented with whole soybeans, but this study measured phagocytosis by monocytes, macrophages, and neutrophils, whereas we measured neutrophils alone (Gandra et al., 2016).

There was no treatment effect observed on ex vivo proliferation of PBMC regardless of whether they were stimulated with ConA. In one study, PBMC from cows fed soybeans proliferated less than cows supplemented with flaxseed when stimulated with ConA and supplemented with autologous serum, but not with fetal bovine serum (Lessard et al., 2004). The researchers speculated that the lymphocyte proliferative response in the soybean-supplemented cows was due to factors in the fetal bovine serum (Lessard et al., 2004). This same study reported that the proliferative response of PBMC increased by six weeks

postpartum, which differs from our results (Lessard et al., 2004). Another study reported decreased lymphocyte proliferation in rats supplemented with ALA (Albers et al., 2002; Calder et al., 2002). The discrepancies in the literature may be due to species differences and source and amount of PUFA supplemented in the diet. Proliferation of T cells and B cells is important in the transition period to generate more immune cells (Sordillo et al., 2009; Silvestre et al., 2011). If proliferation does not occur at an adequate rate during an immune challenge, pathogens will not be effectively cleared from the cow's system (Silvestre et al., 2011). The general lack of an increase in proliferation by PBMC stimulated with ConA, regardless of diet, reflects the immunosuppression and inadequate adaptive immune response that often occurs in the transition cow. Although there was no difference observed in proliferation between the treatment groups in this study, there was an overall decrease in proliferation during the 21 day sampling period, indicating there may be inhibitory effects of the diets (omega-3 and omega-6 FA) on PBMC proliferation.

By 21 days postpartum most cows are consuming an adequate amount of feed and NEFA and glucose concentrations are returning to normal (Drackley, 1992). Complete uterine involution is typically completed by 12 days postpartum, with most involution occurring up to three days post-partum (Leslie, 1983). Uterine involution is a proinflammatory process that includes breaking down caruncles and bacterial infection is common with up to 93% of cows exhibiting symptoms of uterine infections up to 15 days post-partum (Leslie, 1983). By day 21 postpartum, cows are recovering from negative energy balance and uterine involution will be complete in healthy cows.

The mRNA expression of TNF and IL6, two proinflammatory cytokines, was reduced in the treated cows, and there was no effect of diet on IL10, an antiinflammatory cytokine.

TNF and IL6 are products of the NF-κB pathway and result in inflammation (Sordillo et al., 2009). Decreased expression of TNF and IL6 in PBMC of treated cows supports our hypothesis that a flaxseed supplement would promote an antiinflammatory state. We postulated that the antiinflammatory IL10 would be greater in treated cows but there was no difference between diets. In another study, there was no effect of diet (flaxseed vs. soybean) on in vitro culture of monocytes activated with LPS on IFN-γ or TNF production, however, production of these proinflammatory cytokines decreased from week one through week six after calving (Lessard et al., 2004). T cells decline and monocytes increase during the transition period, indicating that the innate immune system properties are upregulated and adaptive immune properties are downregulated (Sordillo et al., 1995; Lessard et al., 2004). There was an effect of diet on mRNA abundance of TNF, but not protein concentration, this may be due to posttranslational and posttranscriptional modification of TNF (Taquet et al., 2009). Cells are also able to individually synthesize and degrade proteins and the half-life of proteins is highly variable; therefore mRNA abundance and protein concentration are not always closely coordinated (Taquet et al., 2009).

There were no differences observed for plasma glucose and NEFA between the control and flaxseed groups. Leading up to parturition, plasma NEFA concentrations steadily increase and peak after parturition around 1000 uM (Vazquez-Anon and Luck, 1994). For the next three weeks NEFA concentrations decrease and stabilize around 300 uM (Vazquez-Anon and Luck, 1994). The cows in this study remained within the acceptable range of plasma NEFA concentrations, and as expected, the concentration of plasma NEFA decreased in both groups by day 21. Normal plasma glucose concentrations in fresh cows range from 55-70 mg/dL (Bertics et al., 1992). Both treatment groups in this study remained within the normal

range of plasma glucose concentrations and concentrations increased over time indicating the energy balance for the cows in both treatment groups was improving. Glucose is not only an important energy source for the cow and milk production, but it is also needed for immune cells (Ingvartsen and Moyes, 2015). Although both groups were within the normal range of glucose concentrations for plasma, they were at the low end of the acceptable range. It may be beneficial to increase the concentration of glucose so that there is ample fuel to meet all of the energy demands of the transition dairy cow (Bertics et al., 1992). In one study cows supplemented with whole flaxseed had increased glucose concentrations during the pre- and postpartum period with a range of 65-80 mg/dL compared to a soybean diet with a range of 55-65 mg/dL (Gandra et al., 2016).

Milk yield was the same between both groups, but flaxseed-supplemented cows tended to have higher milk fat percentage. Our results differ from previous studies that reported milk fat depression and decreased milk protein when cows were supplemented with fat early in lactation (Schingoethe and Casper, 1991). In that study cows were supplemented with soybeans and sunflower seed sources of omega-6 fatty acids, at 5.2% DM, whereas our cows were supplemented with omega-3 fats at 3.5% DM, which may account for the differences observed (Schingoethe and Casper, 1991). Cows supplemented with fats were previously reported to reach peak lactation earlier than cows not supplemented, although our study did not examine peak lactation (Zachut et al., 2010; Petti, 2002; Schingoethe and Casper, 1991). The goal of this experiment was to supplement flaxseed at a concentration high enough to increase omega-3 fats in the milk, but not so much as to cause milk fat depression, which we accomplished. It is hypothesized that seed sources of omega-3 fat are better than fish oil as seed sources have been found to maintain and even increase milk fat in

dairy cows (Cant et al., 1997; Petit et al., 2002). Milk fat depression typically occurs when rumen available sources of fats, like fish oil, are fed, whereas seed sources can be treated to be protected from rumen bio-hydrogenation (Cant et al., 1997). Fish oils are not very palatable to cows and up to10% decrease in DMI were observed which could account for decreased milk production and milk fat (Cant et al., 1997; Donovan et al., 2000). The flaxseed-supplemented cows tended to have more ALA in milk fat compared to the control group and the concentration of ALA continued to increase over the 21-day trial in the supplemented cows. This fat formulation (LinPro™-R), which undergoes extrusion processing, was able to bypass the rumen at a rate sufficient to increase omega-3 fatty acids in the milk, although the percentage increase was quite small, partly because the extrusion process is not especially efficient at protecting ALA from rumen bio-hydrogenation. The control group had higher stearic acid (18:0) concentrations, a saturated fatty acid, than the flaxseed group ($P<0.05$). These results are consistent with other studies that showed that supplementing cows with omega-3 fats in the diet increases increased ALA and other PUFAs and decreased saturated fatty acids in the milk (Petit at al., 2002; Zachut et al., 2010). Furthermore, the same trends were observed in plasma ALA in the flaxseed cows. In plasma, DHA was increased in the flaxseed-supplemented cows. There were no differences observed in the red blood cell fatty acid profile for g/100g of ALA. This was not surprising because red blood cells have a half-life of 175 days in dairy cattle (Kurata et al., 1993).

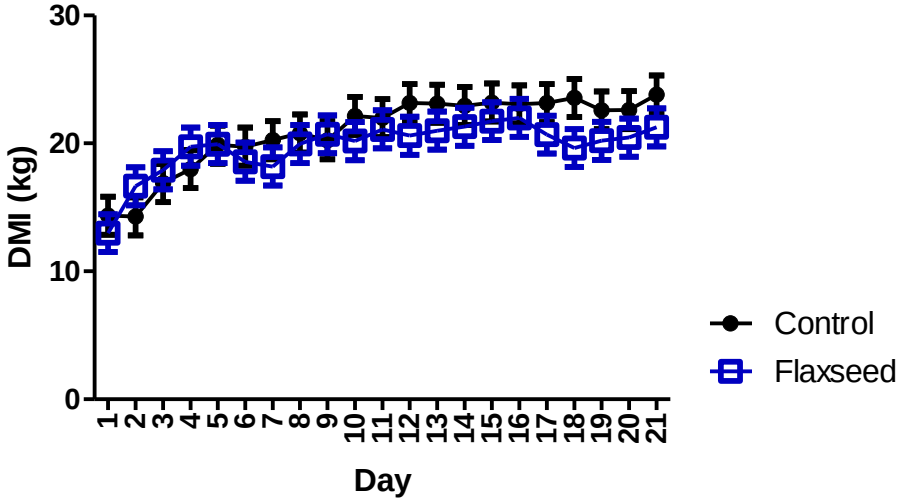
In conclusion, feeding a commercially available flaxseed supplement (LinPro™-R) to transition dairy cows did not reduce feed intake, milk production, and increased the percentage of milk fat produced in the supplemented cows. There was a reduction in neutrophil reactive oxygen species and phagocytosis. This may indicate that the omega-3

supplement reduced the activation status of the immune system and reduced inflammatory responses. We hypothesize this reduction in neutrophil activity could increase energy available to the cow for productive functions, although this speculation needs to be confirmed in future studies. The milk fat fatty acid profile showed that ALA increased in milk fat, and that the longer the cow was on the diet the more ALA accumulated in the milk. It would be interesting to determine the level of ALA enrichment that could be achieved without reduced milk fat percentage in the cow. Cows supplemented with formaldehyde-treated whole linseed at 6.7% DM had ALA making up 13.9% of total fatty acids in the milk without reducing milk total fat percentage (Petit et al., 2002). Alpha-linolenic acid was 18 times greater in cows that were supplemented with extruded flaxseed at 7.9% DM pre-partum and 9.2% post-partum, but milk total milk fat percentage was decreased (Zachut et al., 2010). The plasma fatty acid profile showed that two omega-3 fats, ALA and DHA, increased in plasma in cows supplemented with LinPro™-R. Our results are consistent with previously published data where the ratio of omega-6 to omega-3 in plasma was reduced in cows supplemented with extruded flaxseed and ALA was increased as well (Zachut et al., 2010).

The results provide some support for the hypothesis that fat feeding can alter immune function in transition dairy cows. But these results should be interpreted with caution due to the low number of cows used in this study and the variability exhibited by some of the dependent variables. What is not yet known is how this supplement could affect health events or reproductive rates. It would be valuable to test the hypothesis that when neutrophil function is reduced more energy is available to the cow for productive function. However, it is critical to determine if feeding omega-3 fats affects the incidence of periparturient diseases. Furthermore, if this product decreases health events and decreases metabolic stress it would

likely increase reproductive efficiency and decrease embryonic loss. These questions are being addressed in an on-going study where cows are supplemented during the entire transition period. Incidences of periparturient and metabolic diseases are being recorded and data will be collect on conception rates, pregnancy rates, and embryonic loss. The data from this large study will be combined with the immune cell function data from this study to address the question whether supplementing cows with an omega-3 fatty acid can improve their health, efficiency, and fertility.

A



B

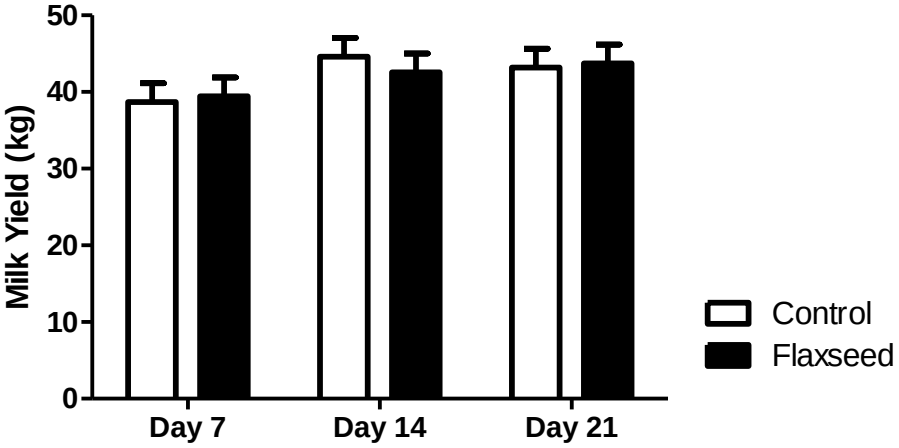


Figure 2.1: Dry Matter Intake (A) and Milk Yield (B)
There was no difference in dry matter intake among groups (P>0.1) and DMI increased over time for both groups (P<0.01). Milk yield did not differ between treatments (P>0.1) and increased over time for both groups (P<0.01)

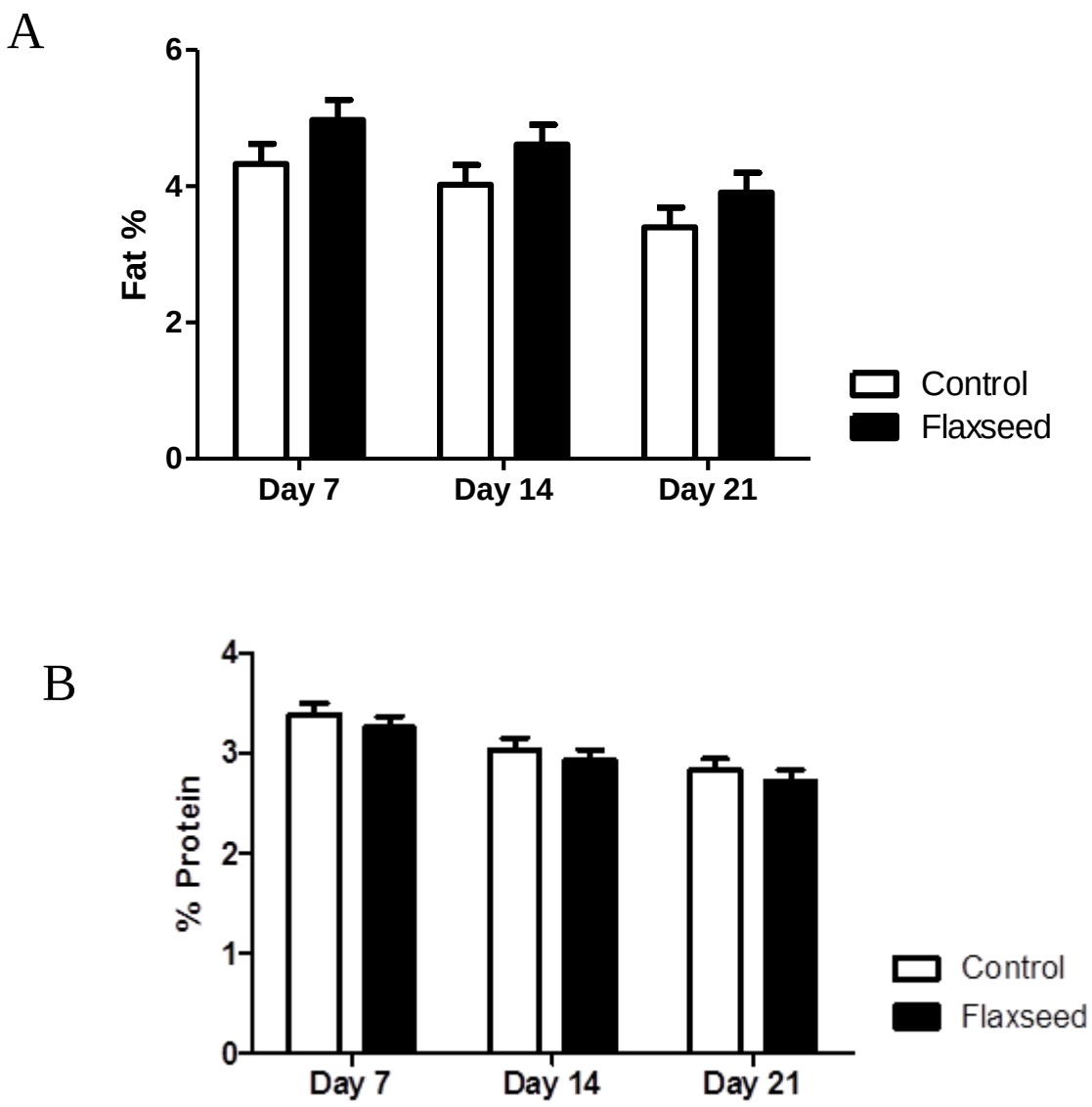


Figure 2.2: Percentage Fat (A) and Protein (B) in Milk
Mean concentration of milk fat was 0.6 percentage points greater in the flaxseed group (Treatment: P=0.067) which represents a 15% increase in milk fat. For both groups milk fat percent decreased over the 21-day period (Day: P<0.01), which coincided with the increase in milk yield over time.There was no difference in percent milk protein between groups (Treatment: P>0.1) and the percentage of protein in milk decreased over time (Day: P<0.01).

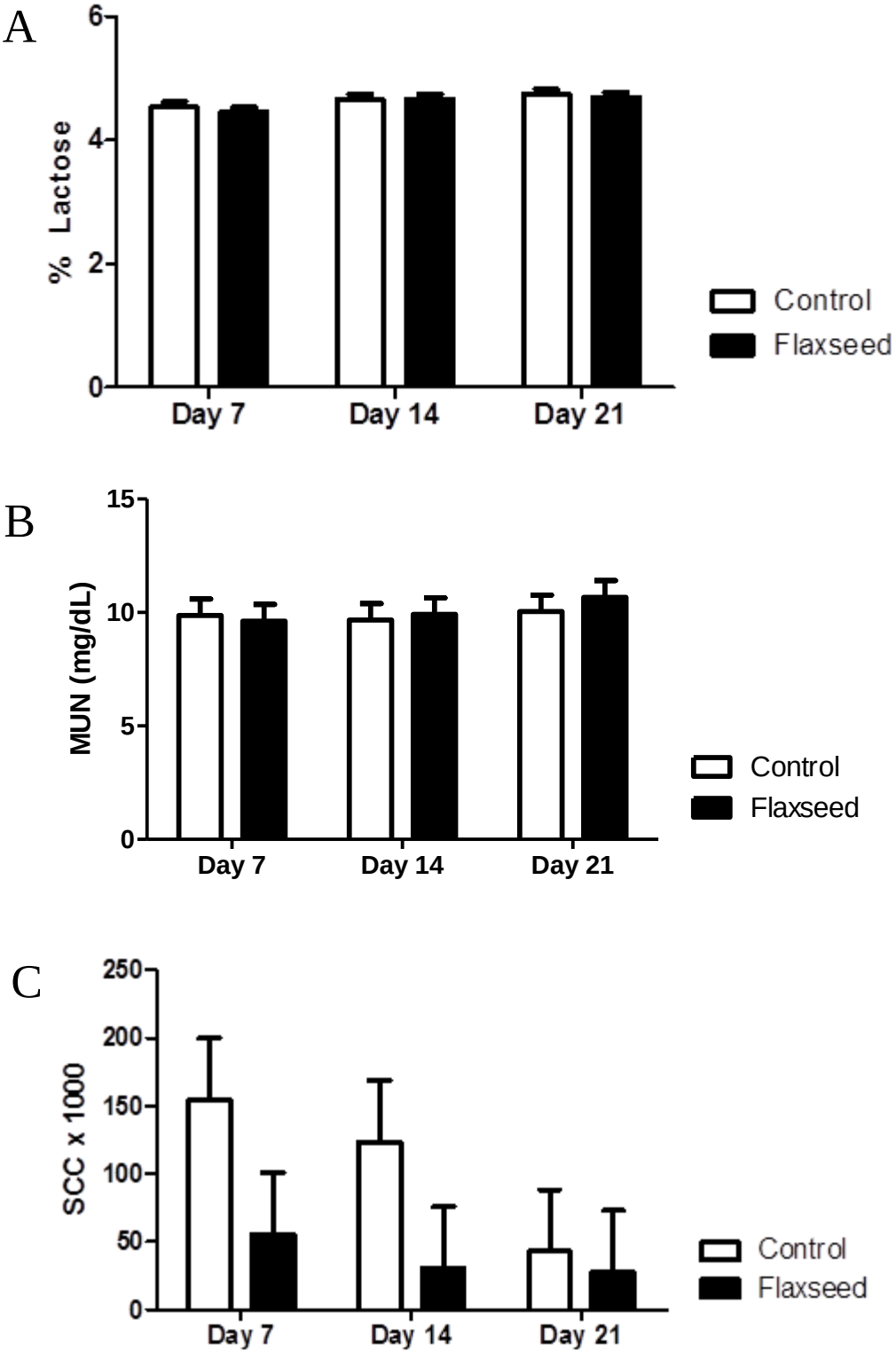
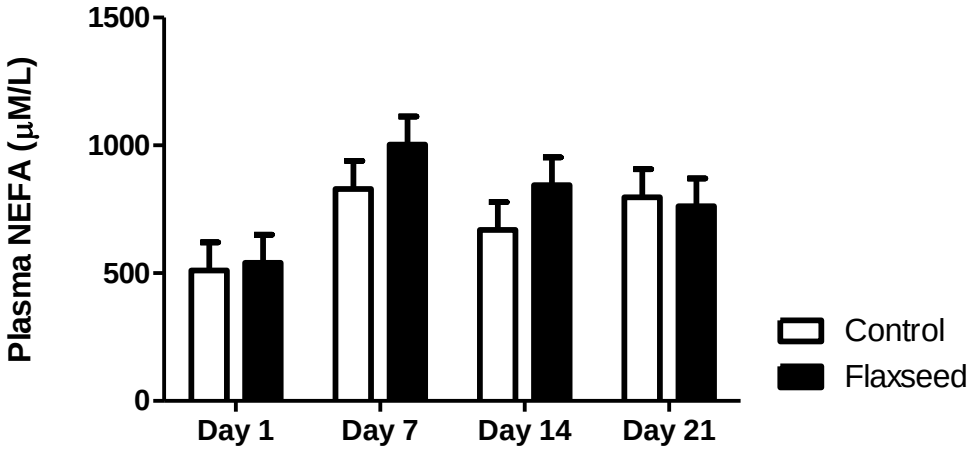


Figure 2.3: Percentage Milk Lactose (A), Milk Urea Nitrogen (MUN; B), and Milk Somatic Cell Count (SCC; C)
There was no difference between groups for milk lactose, MUN, or SCC (Treatment: $P>0.1$). The percentage of lactose in milk increased over time and SCC decreased (Day: $P<0.01$).

A



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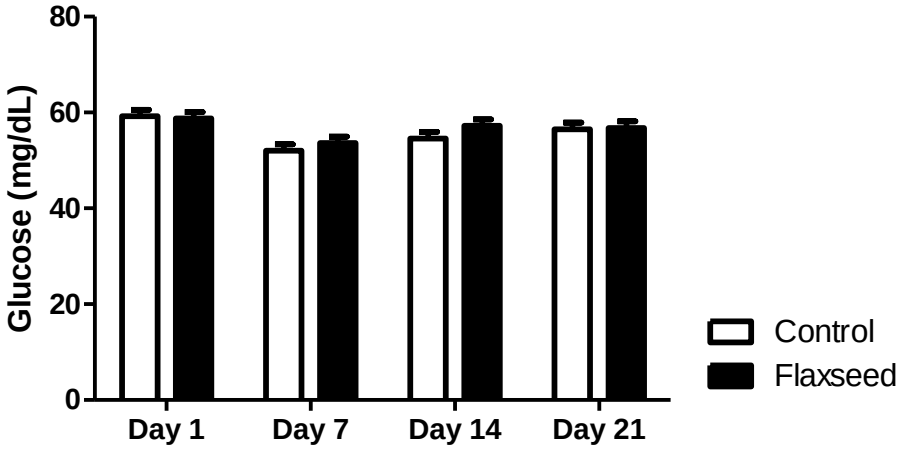
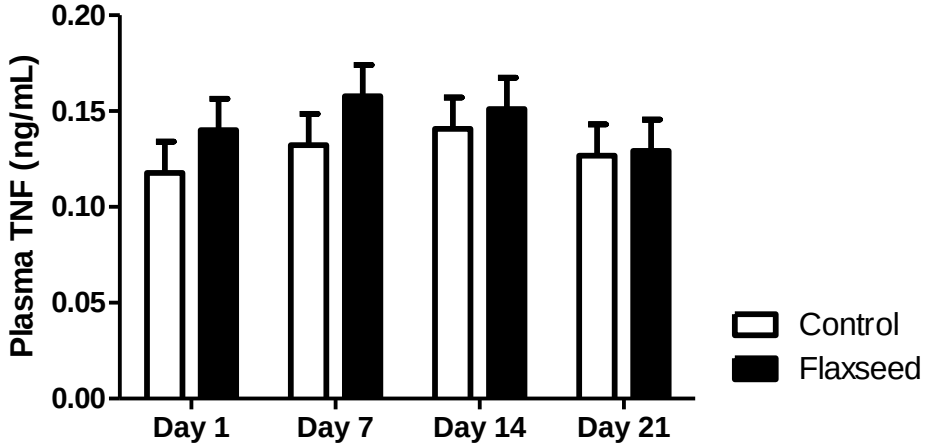


Figure 2.4: Concentration of Plasma Glucose (A) and Non-Esterified Fatty Acids (B) There was no difference between groups for concentration of plasma glucose or NEFA (Treatment: P>0.1).

A



B

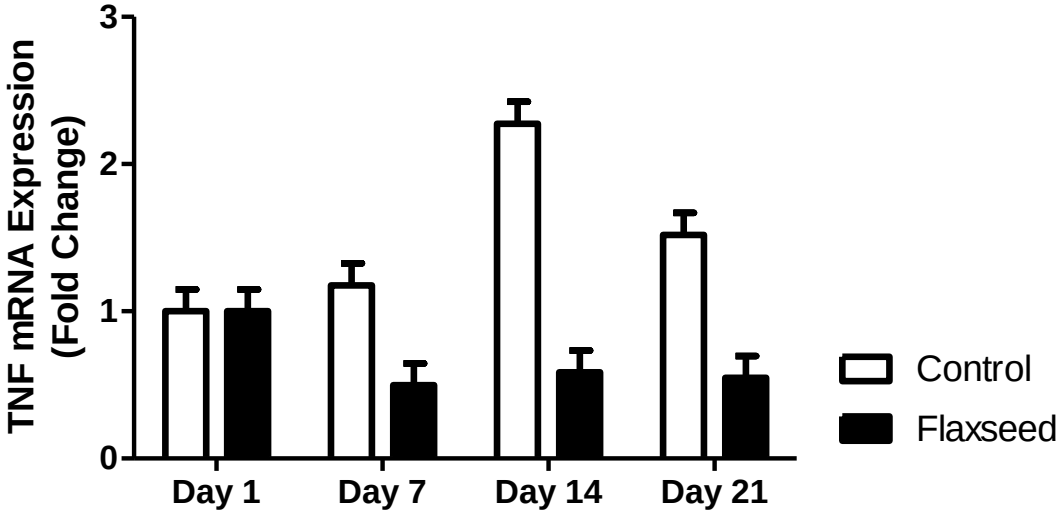


Figure 2.5: Concentration of Tumor Necrosis Factor (TNF) in Plasma (A) and mRNA Expression of TNF (B).
There was no difference between groups for plasma concentration of TNF (Treatment: P>0.1). The mRNA expression of TNF tended to be less in the flaxseed-fed cows compared to controls (P=0.09)

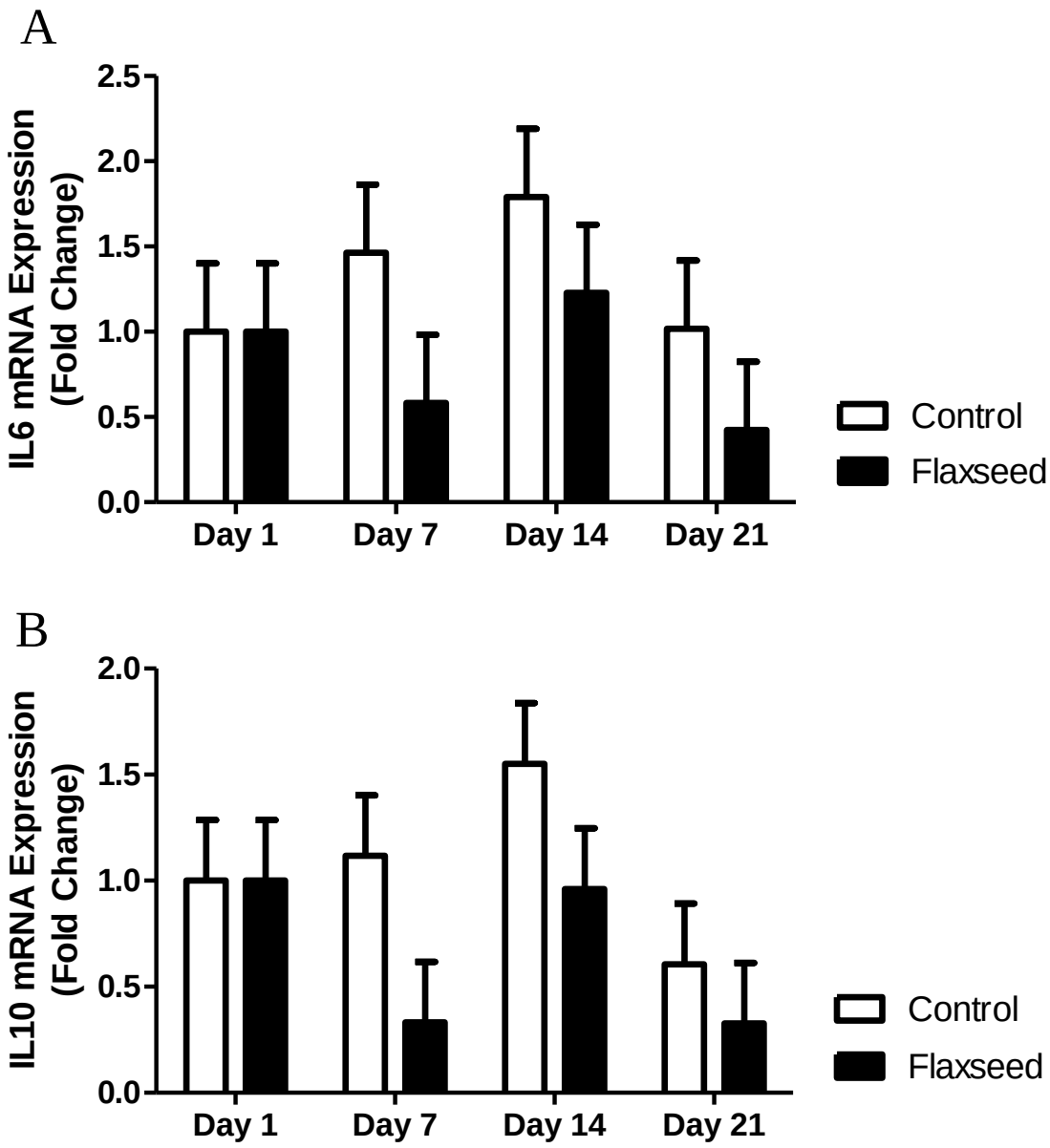


Figure 2.6 Expression of IL6 (A) and IL10 (B) mRNA
The mRNA expression of IL6 was less in flaxseed-fed cows compared to controls (Treatment: $P<0.01$) and there was no effect of diet on mRNA expression of IL10 (Treatment: $P>0.1$).

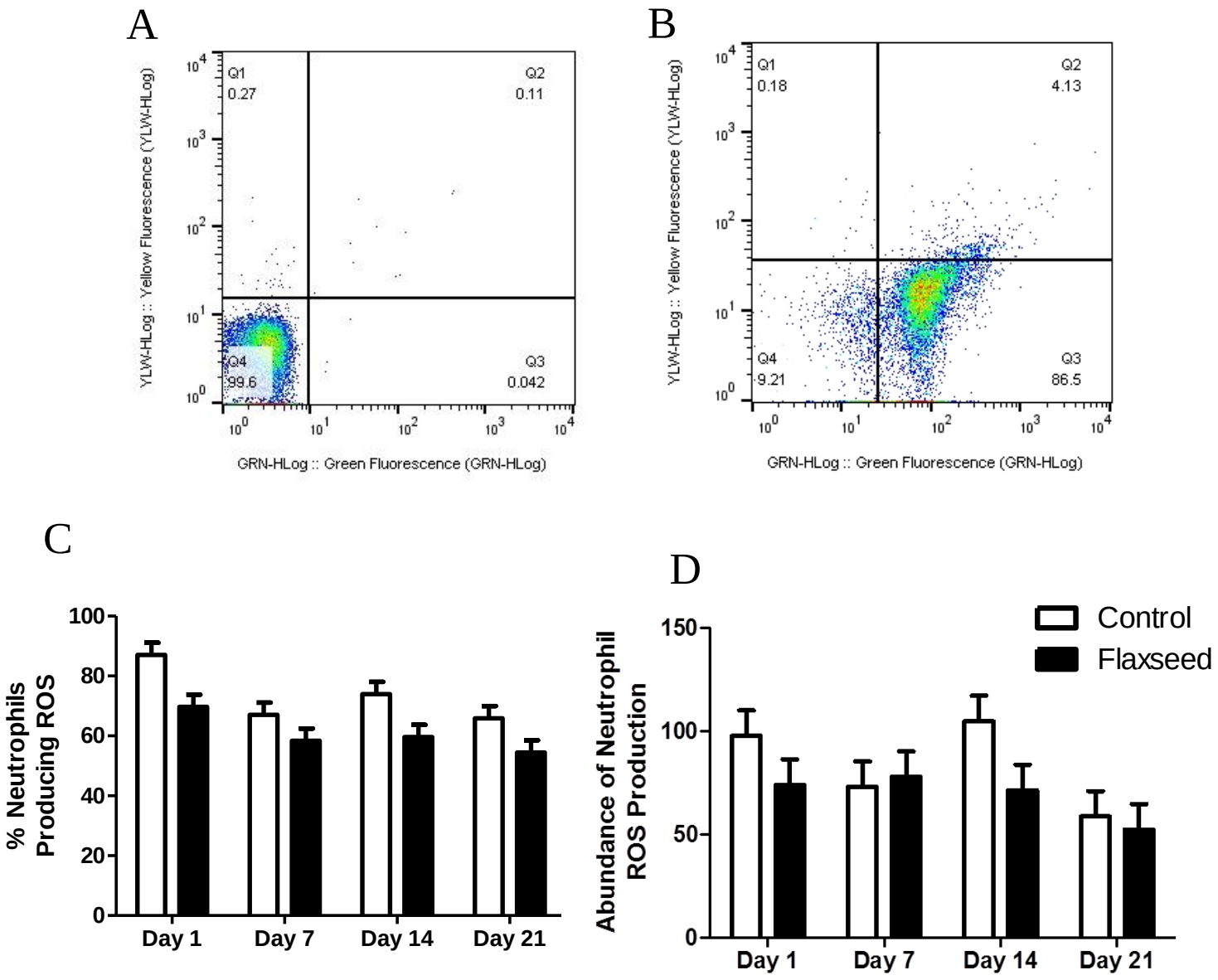


Figure 2.7: Percentage of Neutrophils Producing Reactive Oxygen Species (C) and Abundance of ROS Produced by Neutrophils

Representative dot plots (A & B) and results (C) showing the percentage of neutrophils producing ROS and the amount of ROS produced per neutrophil. Figure 2.14A represents the negative control of neutrophils only and Figure 2.14B depicts neutrophils labeled with DHR which fluoresces green to show ROS production.

The percentage of neutrophils producing ROS was less in flaxseed-fed cows compared to control (Treatment: $P<0.01$) and the percentage of neutrophils producing ROS tended to decrease over time in both groups ($P=0.07$). The amount of ROS produced by neutrophils decreased over time (Day: $P<0.05$) and there was no effect of diet (Treatment: $P>0.1$).

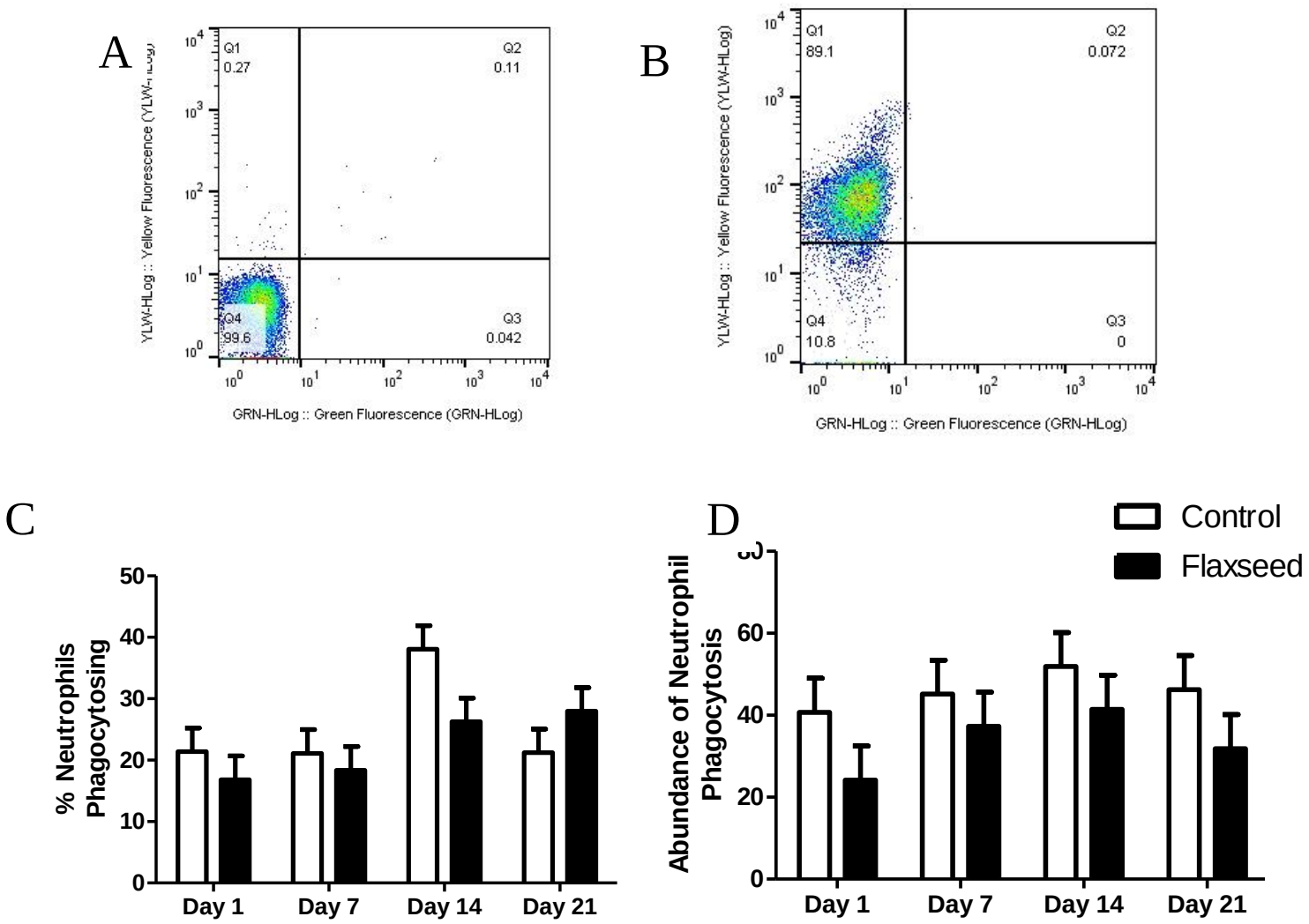


Figure 2.8: Neutrophils Exhibiting Phagocytosis
Representative dot plots (A & B) and results (C) showing the percentage of neutrophils exhibiting phagocytosis. Figure 2.14A represents the negative control of neutrophils only and Figure 2.14B depicts neutrophils incubated with PI labelled bacteria which fluoresces yellow to show the occurrence of phagocytosis. There was no effect of diet on the percentage of neutrophils phagocytosing (Treatment: P>0.1).
There was a tendency for the amount of phagocytosis per neutrophil to be decreased in the flaxseed group compared to the control group (Treatment: P=0.09).

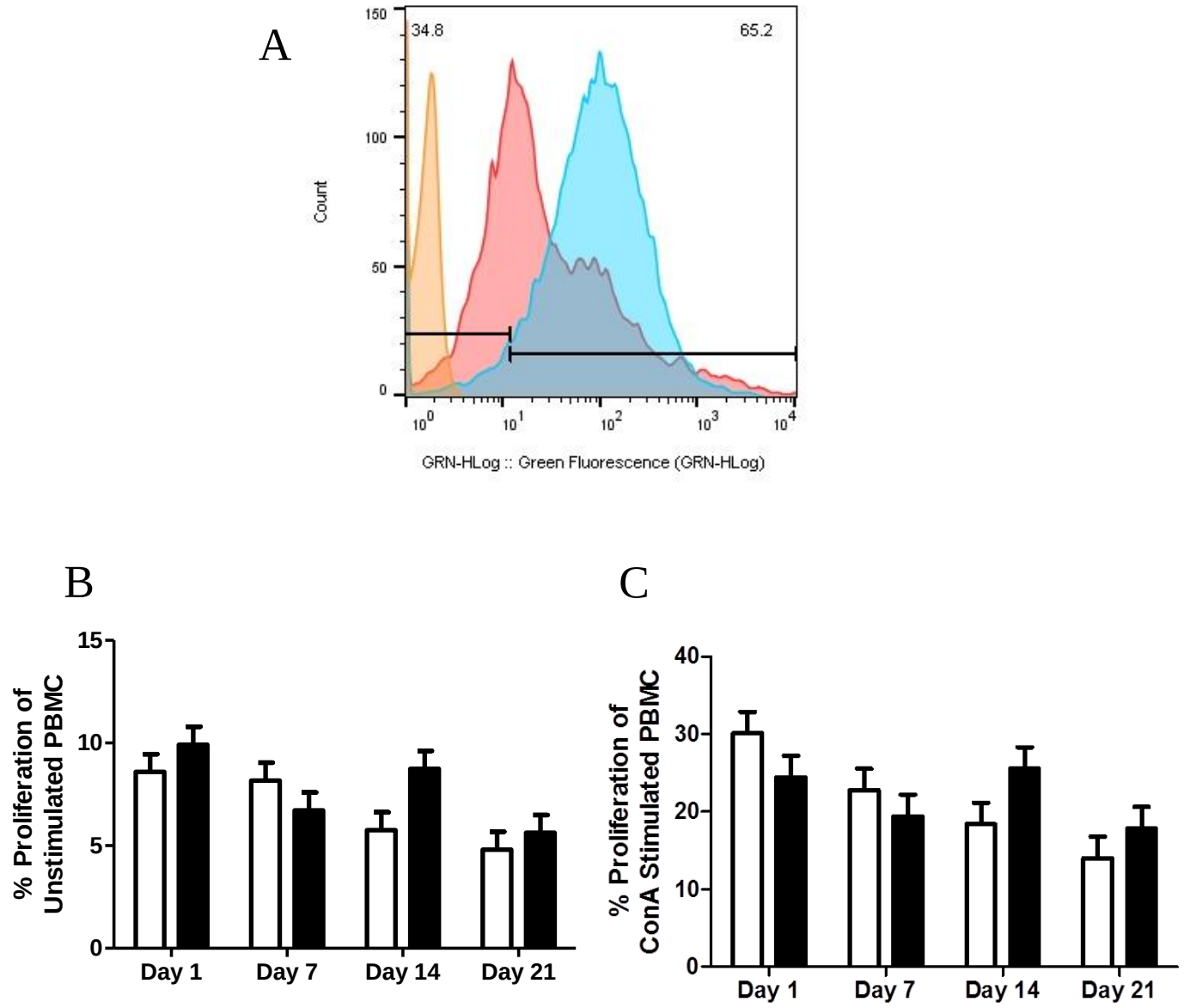


Figure 2.9: Proliferation of Unstimulated (B) and ConA Stimulated PBMC (C) Representative histogram (A) and results (B) of percentage of PBMC that proliferated without ConA stimulation and percentage of PBMC that proliferated with ConA stimation (C). The orange peak of the histogram represents the negative cell population and the pink peak represents ConA stimulated PBMC and the blue peak unstimulated PBMC There was no effect of treatment for both the stimulated and unstimulated cells (Treatment: $P>0.1$). In both groups the percent proliferation decreased over time (Day: $P<0.01$).

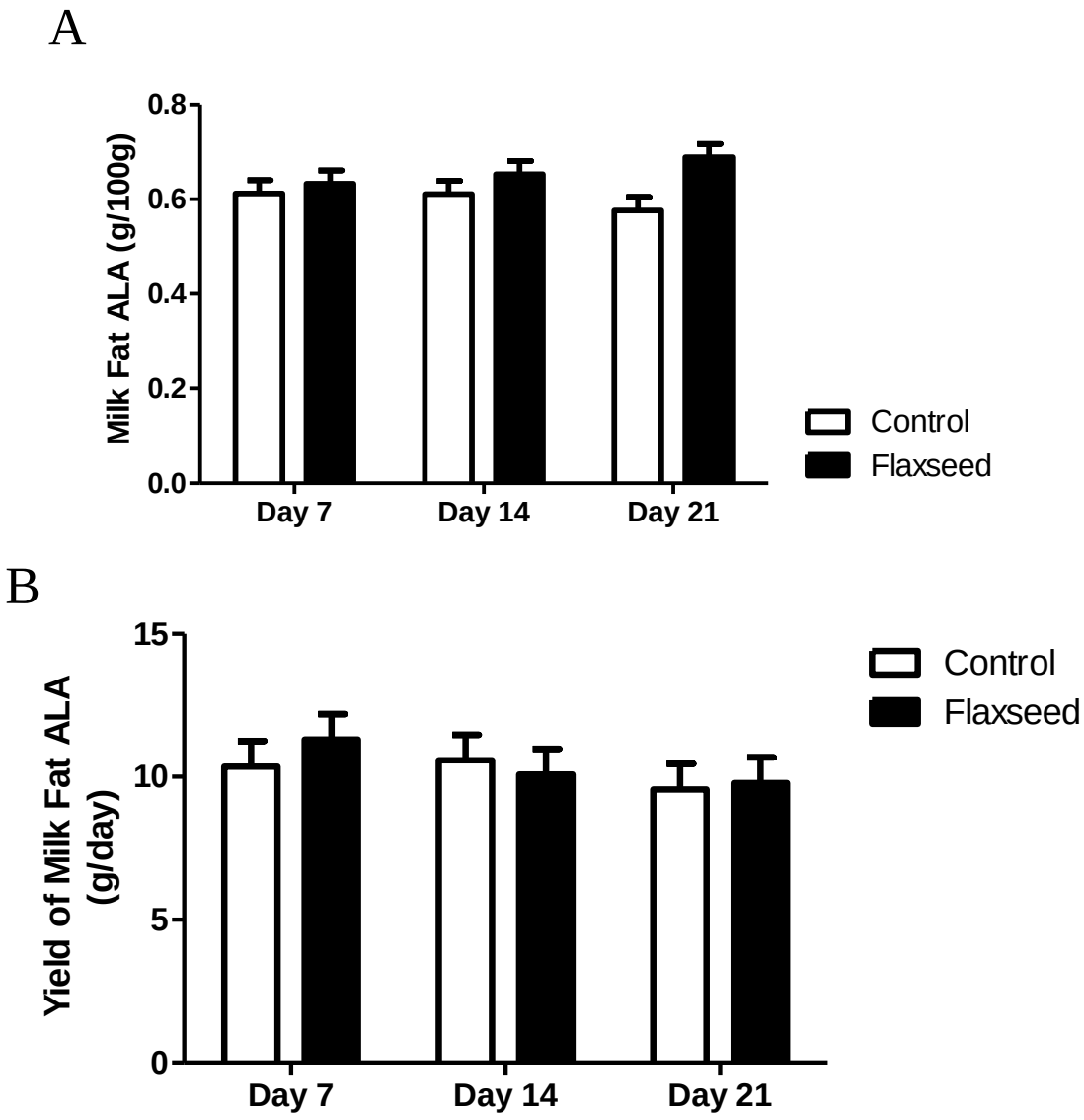


Figure 2.10: Alpha-linolenic Acid (ALA) in Milk Fat (A) and Yield of ALA in milk Fat (B)

There was a tendency for flaxseed cows to have more ALA in milk fat compared to controls (Treatment: P=0.06). A tendency was seen for a treatment by day interaction as the amount of ALA in milk increased for flaxseed cows over time but decreased in control cows (Trt x Day: P=0.07). There was no difference between the groups for yield of ALA in milk fat (Treatment: P>0.1).

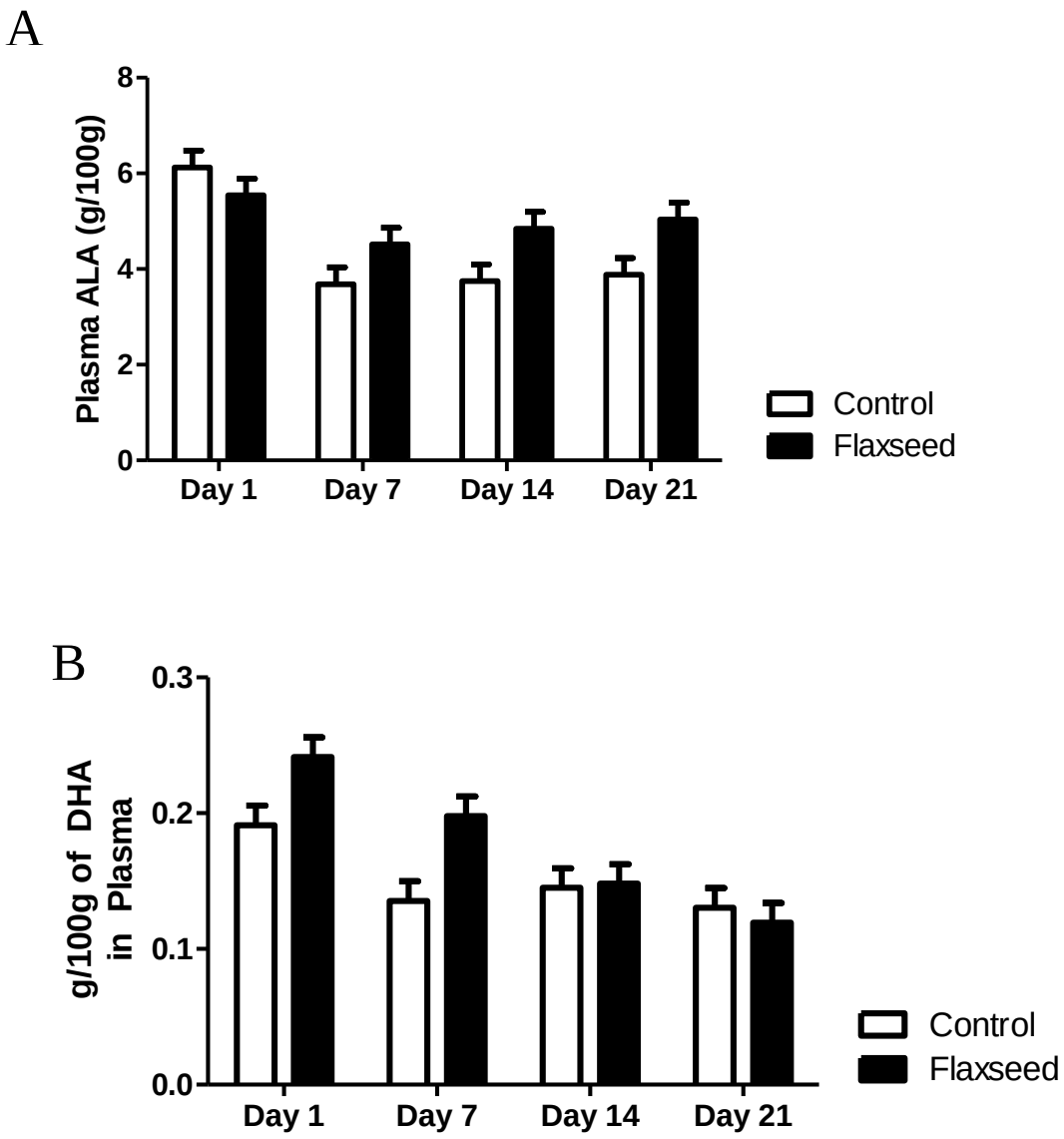


Figure 2.24: Alpha-linolenic Acid (ALA; A) and Docosahexaenoic Acid (DHA; B) in Plasma

Control cows tended (P=0.09) to have less ALA (4.4 g/100g) in their plasma compared to treated cows (5.0 g/100g; Treatment: P=0.09). There was a tendency for the flaxseed cows to have more DHA in plasma compared to controls (Treatment: P=0.09). A tendency was seen for a treatment by day interaction (Trt x Day: P=0.06) and overall DHA decreased over time in the plasma for both groups (Day: P<0.01).

Tables

Table 2.1: Primer sequences, annealing temperatures, and size of amplicons analyzed with qPCR.

Gene	FWD Sequence	REV Sequence	Temp ˚C	Size (bp)
RPL19	ATCGATCGCCACATGTATCA	GCGTGCTTCCTTGGTCTTAG	60	168
IL10	CTGACAGCAGCTGTATCCACTTG	GTGCAGTTGGCCTTCATTTGA	60	110
TNF	AGAGGGAAGAGTCCCCAGGTG	CCCCGGAGAGTTGATGTCG	60	125
IL6	AGCGCATGGTCGACAAAATC	TCGCCTGATTGAACCCAAGAT	60	156

Table 2.2 Feed Ingredients and Diet Composition
Ingredients and dry matter composition of diets.

	Diets	
	Control	Flaxseed
Ingredients, %		
Corn silage	32.54	32.54
Alfalfa haylage	12.80	12.80
Grass hay	2.67	2.67
Cereal straw	2.67	2.67
Shell corn dry	11.95	10.24
Candy meal	9.17	9.17
Cottonseed hull	2.13	2.13
Canola meal	10.67	10.03
Whole roasted soybean	4.80	1.07
NPN (Optigen)	0.53	0.53
Min\Vit Mix	3.35	3.35
LinPro™-R (flaxseed)	0.00	3.52
Expeller soybean meal	0.00	2.56
Salt	0.32	0.32
Molasses	6.40	6.40

Table 2.3: Fatty acid composition (g/100g) of milk fat

Fatty acids	Day 7		Day 14		Day 21		P value		
	Cont	Flax	Cont	Flax	Cont	Flax	Cont v. Flax	Day	Trt x Day
C4:0	6.03	5.54	5.72	5.17	5.55	5.36	0.15	0.01	0.23
C6:0	2.40	2.16	2.46	2.17	2.38	2.33	0.30	0.68	0.37
C8:0	1.12	0.99	1.18	1.03	1.17	1.11	0.41	0.23	0.64
C10:0	34.35	30.56	40.03	31.93	33.31	34.11	0.46	0.55	0.41
C11:0	0.03	0.02	0.04	0.03	0.03	0.03	0.59	0.09	0.10
C12:0	2.20	1.84	2.35	2.02	2.30	2.15	0.42	0.18	0.61
C13:0	0.05	0.04	0.07	0.06	0.06	0.06	0.53	0.00	0.13
Ci14:0	0.06	0.06	0.07	0.05	0.08	0.06	0.14	0.08	0.59
C14:0	7.47	6.76	7.80	6.69	7.72	7.55	0.41	0.28	0.26
i15:0	0.16	0.15	0.18	0.15	0.18	0.16	0.16	0.14	0.47
a15 0	0.28	0.25	0.33	0.27	0.34	0.29	0.15	0.00	0.39
C14:1	0.37	0.43	0.47	0.54	0.49	0.53	0.21	<.0001	0.86
C15:0	0.66	0.59	0.72	0.66	0.69	0.69	0.59	0.00	0.06
i16:0	0.18	0.16	0.17	0.15	0.19	0.16	0.06	0.16	0.29
C16:0	23.52	24.10	22.75	24.77	22.71	24.24	0.03	0.68	0.23
i17 0	0.26	0.24	0.25	0.24	0.25	0.24	0.03	0.90	0.71
C16:1	1.49	1.63	1.47	1.67	1.44	1.53	0.43	0.15	0.30
a17 0	0.44	0.41	0.44	0.42	0.44	0.40	0.03	0.64	0.37
C17:0	0.70	0.69	0.64	0.66	0.62	0.61	0.99	0.00	0.47
C17:1	0.36	0.37	0.34	0.39	0.34	0.34	0.58	0.04	0.01
C18:0	14.45	14.03	14.53	12.82	14.36	13.05	0.02	0.08	0.09
t4	0.04	0.03	0.04	0.03	0.04	0.03	0.04	0.29	0.84
t5	0.03	0.02	0.03	0.02	0.03	0.02	0.02	0.94	0.55
t6-8	0.42	0.36	0.40	0.35	0.40	0.34	0.00	0.00	0.78
t9	0.22	0.27	0.23	0.23	0.23	0.26	0.50	0.85	0.76
t10	0.07	0.09	0.07	0.10	0.07	0.06	0.88	0.81	0.66
t11	1.14	1.47	1.31	1.08	0.87	0.98	0.78	0.19	0.25
t12	0.07	0.20	0.00	0.06	0.00	0.06	0.14	0.09	0.75
C18:1c9	26.78	28.71	26.88	30.78	27.80	28.79	0.28	0.23	0.07
t15	0.40	0.27	0.43	0.15	0.32	0.21	0.01	0.35	0.33
C18:1c11	1.13	1.09	1.11	1.12	1.07	1.04	0.72	0.09	0.58
C18:1c12	0.46	0.45	0.50	0.47	0.50	0.47	0.44	0.15	0.65
LA	3.08	2.85	3.09	2.92	3.04	2.97	0.37	0.77	0.53
C20:0	0.13	0.12	0.14	0.11	0.14	0.12	0.04	0.06	0.02
C20:1	0.06	0.07	0.08	0.07	0.09	0.09	0.55	<.0001	0.09
ALA	0.61	0.61	0.61	0.65	0.69	0.57	0.06	0.85	0.08
9,11cla	0.53	0.48	0.53	0.48	0.54	0.49	0.23	0.65	0.99
C22:0	0.03	0.03	0.04	0.02	0.05	0.02	0.04	0.35	0.11
C20:3	0.08	0.07	0.08	0.07	0.10	0.07	0.01	0.20	0.04
C20:4	0.21	0.19	0.19	0.17	0.18	0.16	0.01	<.0001	0.88
C20:5	0.05	0.05	0.04	0.04	0.04	0.04	0.20	0.03	0.57
C24:0	0.01	0.01	0.02	0.01	0.03	0.02	0.33	0.02	0.40
C22:4	0.02	0.02	0.02	0.01	0.02	0.01	0.29	0.26	0.42
C22:5	0.10	0.08	0.09	0.07	0.09	0.07	0.01	0.02	0.25

Table 2.4: Fatty acid composition (g/100g) of plasma

	Day 1		Day 7		Day 14		Day 21		P value		
Fatty acids	**Cont**	**Flax**	**Cont**	**Flax**	**Cont**	**Flax**	**Cont**	**Flax**	**Cont v Flax**	**Day**	**Trt x Day**
C14:0	1.58	1.60	1.02	0.98	0.91	0.76	0.79	0.82	0.53	<.0001	0.59
i15:0	0.46	0.43	0.21	0.21	0.18	0.18	0.18	0.19	0.72	<.0001	0.66
a15 0	0.45	0.43	0.24	0.23	0.22	0.22	0.22	0.22	0.72	<.0001	0.90
C14:1	0.16	0.16	0.15	0.15	0.15	0.13	0.14	0.13	0.46	0.10	0.46
C15:0	0.70	0.68	0.43	0.41	0.40	0.39	0.40	0.39	0.58	<.0001	0.91
i16:0	0.24	0.23	0.18	0.17	0.16	0.15	0.16	0.14	0.30	<.0001	0.98
C16:0	14.30	14.49	13.97	13.34	12.49	12.07	11.97	11.60	0.58	<.0001	0.43
i17 0	0.36	0.35	0.24	0.24	0.22	0.24	0.24	0.20	0.81	<.0001	0.78
C16:1	1.45	1.46	1.21	1.23	1.08	1.11	1.02	1.07	0.59	<.0001	0.97
a17 0	0.36	0.36	0.30	0.29	0.29	0.27	0.30	0.27	0.45	<.0001	0.73
C17:0	0.61	0.61	0.52	0.48	0.50	0.46	0.55	0.47	0.03	<.0001	0.26
C17:1	0.99	0.80	0.75	0.72	0.64	0.64	0.68	0.57	0.32	0.04	0.70
C18:0	13.75	14.19	15.14	15.14	14.75	14.16	14.88	13.83	0.19	0.01	0.11
t6-8	0.06	0.06	0.12	0.11	0.09	0.10	0.09	0.08	0.75	<.0001	0.61
t9	0.03	0.04	0.10	0.09	0.08	0.08	0.08	0.07	0.92	<.0001	0.72
t10	0.07	0.08	0.16	0.12	0.11	0.11	0.12	0.10	0.57	0.00	0.41
t11	0.72	0.74	0.73	0.84	0.58	0.72	0.55	0.58	0.08	<.0001	0.20
t12	0.29	0.31	0.35	0.36	0.33	0.33	0.30	0.30	0.62	<.0001	0.81
C18:1c9	13.91	14.45	13.63	14.04	12.09	11.67	11.42	10.80	0.97	<.0001	0.63
C18:1c11	0.93	1.01	0.97	0.99	0.94	0.90	0.86	0.80	0.93	<.0001	0.14
C18:1c12	0.33	0.33	0.53	0.52	0.54	0.54	0.53	0.53	0.92	<.0001	0.96
LA	28.06	27.20	35.45	33.54	39.69	38.42	41.06	40.21	0.24	<.0001	0.80
C20:0	0.05	0.04	0.05	0.06	0.05	0.05	0.05	0.05	0.47	0.72	0.59
GLA	0.53	0.55	0.49	0.46	0.50	0.52	0.49	0.56	0.78	0.41	0.83
ALA	6.13	5.54	3.69	4.51	3.75	4.85	3.88	5.04	0.10	<.0001	0.00
C20:1	0.02	0.02	0.02	0.06	0.05	0.05	0.05	0.04	0.47	0.06	0.09
9,11cla	0.21	0.20	0.20	0.21	0.15	0.16	0.15	0.14	0.88	<.0001	0.25
C20:3	1.62	1.67	1.17	1.16	1.31	1.33	1.38	1.36	0.95	<.0001	0.95
C20:4	3.83	3.94	3.06	2.97	2.78	2.89	2.63	2.75	0.64	<.0001	0.55
C20:5	1.07	1.08	0.78	0.80	0.67	0.76	0.60	0.72	0.35	<.0001	0.69
C24:0	0.04	0.01	0.01	0.02	0.02	0.03	0.02	0.03	0.64	0.46	0.06
C22:4	0.37	0.38	0.27	0.25	0.25	0.21	0.28	0.20	0.07	<.0001	0.01
C22:5	1.28	1.35	1.07	1.03	0.99	0.91	0.96	0.82	0.35	<.0001	0.14
C22:6	0.19	0.24	0.14	0.20	0.15	0.15	0.13	0.12	0.09	<.0001	0.06

Table 2.5: Fatty acid composition (g/100g) of red blood cells

Fatty acids	Day 1		Day 7		Day 14		Day 21		P value		
	Cont	Flax	Cont	Flax	Cont	Flax	Cont	Flax	Cont v Flax	Day	Trt x Day
C12:0	0.15	0.18	0.13	0.14	0.17	0.19	0.16	0.20	0.63	0.30	0.90
C14:0	0.91	1.16	1.06	1.32	1.08	1.30	1.03	1.30	0.02	0.34	0.99
i15:0	0.03	0.07	0.02	0.03	0.02	0.03	0.03	0.02	0.27	0.05	0.31
a15 0	0.21	0.15	0.09	0.05	0.15	0.11	0.11	0.12	0.57	0.05	0.95
C15:0	0.23	0.22	0.19	0.18	0.16	0.18	0.18	0.13	0.47	0.04	0.57
i16:0	0.03	0.05	0.03	0.05	0.03	0.05	0.03	0.02	0.48	0.68	0.71
C16:0	6.61	6.81	6.73	6.86	6.22	7.50	6.53	6.87	0.25	0.95	0.19
i17 0	0.12	0.14	0.10	0.12	0.12	0.12	0.13	0.12	0.64	0.78	0.88
C16:1	1.08	1.21	1.07	1.15	1.09	1.11	1.04	1.11	0.31	0.12	0.38
C17:0	0.54	0.43	0.54	0.41	0.48	0.38	0.50	0.43	0.15	0.05	0.47
C17:1c10	1.31	1.85	1.57	1.19	2.09	2.88	1.64	2.28	0.22	0.08	0.56
C18:0	13.96	13.50	14.65	13.82	13.90	13.77	14.42	14.14	0.38	0.42	0.87
t6-8	0.03	0.01	0.03	0.03	0.01	0.03	0.02	0.03	0.91	0.84	0.35
t9	0.07	0.08	0.08	0.09	0.06	0.09	0.07	0.06	0.60	0.72	0.51
t10	0.12	0.10	0.09	0.10	0.12	0.14	0.10	0.09	0.96	0.18	0.75
t11	0.42	0.38	0.34	0.38	0.33	0.50	0.27	0.47	0.16	0.36	0.12
t12	0.21	0.19	0.21	0.20	0.19	0.21	0.20	0.19	0.92	0.82	0.21
C18:1c9	29.72	29.46	30.00	30.25	30.31	27.43	30.00	29.23	0.29	0.23	0.07
C18:1c11	0.73	0.72	0.72	0.69	0.68	0.71	0.74	0.67	0.49	0.62	0.24
LA	20.31	19.85	19.62	20.37	19.78	19.20	20.53	19.77	0.66	0.44	0.36
C20:0	0.12	0.11	0.13	0.12	0.12	0.12	0.10	0.11	0.46	0.01	0.20
GLA	0.13	0.17	0.13	0.16	0.14	0.17	0.13	0.16	0.02	0.68	0.90
ALA	1.65	1.59	1.42	1.57	1.40	1.58	1.42	1.53	0.38	0.52	0.67
C20:1	0.12	0.08	0.11	0.06	0.09	0.11	0.08	0.07	0.39	0.22	0.04
9,11cla	0.79	0.77	0.84	0.82	0.86	0.70	0.80	0.78	0.56	0.64	0.39
C20:2	0.16	0.16	0.17	0.12	0.16	0.18	0.14	0.19	0.82	0.91	0.45
C20:3	1.03	1.13	0.90	1.04	0.87	0.93	0.92	0.99	0.14	<.0001	0.44
C20:4n6	7.03	7.45	6.75	7.81	6.52	6.87	6.86	7.18	0.04	0.01	0.09
C20:4n3	0.41	0.42	0.31	0.39	0.38	0.52	0.51	0.54	0.43	0.32	0.85
C20:5	1.14	1.21	1.15	1.27	1.17	1.14	1.16	1.23	0.28	0.37	0.21
C24:0	0.98	0.95	0.88	1.06	1.09	0.72	1.05	1.12	0.78	0.49	0.10
C24:1	0.16	0.15	0.07	0.18	0.19	0.14	0.16	0.17	0.53	0.52	0.10
C22:0	0.26	0.17	0.22	0.24	0.24	0.17	0.26	0.25	0.33	0.56	0.40
C22:4	0.54	0.47	0.54	0.52	0.35	0.44	0.42	0.43	0.96	0.01	0.31
C22:5	0.74	0.64	0.68	0.70	0.60	0.71	0.72	0.62	0.78	0.91	0.22
C22:6	0.03	0.01	0.03	0.04	0.04	0.05	0.04	0.04	0.95	0.43	0.71

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Chapter 3: Effects of feeding methane inhibitor on return of ovarian activity in transition dairy cows

Abstract

Methane is a major contributor to greenhouse gases. Enteric fermentation of feed by ruminants is the largest source of anthropogenic methane emissions accounting for 25% of global methane emissions. On average, from 2000-2009, agriculture accounted for 12.7 Gt CO_2 eq yr^{-1} of greenhouse gas emissions. In the United States, 6.3% of greenhouse gas emissions are attributed to enteric fermentation by ruminant livestock species. Methane production by microbes in the cow's rumen accounts for energy loss to the cow which could otherwise be used for productive functions. Work done by Hristov et al. (2015) revealed lactating dairy cows receiving a methane inhibitor (3-nitroxypropanol; 3NOP) in their feed exhibited an approximate 30% reduction in methane production and had increased body weight gain compared to cows on a control diet. Based on those results the purpose of this study was to determine if feeding 3NOP to transition dairy cows would affect return to ovarian activity after parturition in high-producing Holstein dairy cows.

Holstein dairy cows from the Penn State herd were blocked based on parity and randomly assigned to receive 3NOP or a vehicle containing SiO_2 and propylene glycol (control) at 60 mg/kg of feed on a dry matter basis within blocks. Milk samples were collected every Monday, Wednesday, and Friday starting week 2 to week 8 after parturition. Milk samples were assayed via ELISA to determine progesterone concentration. Progesterone

concentration was analyzed over time for each cow to determine evidence of a first CL, length of first luteal phase, interval to second CL, and days to the second CL.

There were no differences observed between treatment groups for any of the reproductive parameters. Days to first CL was 32 ± 2.5 days in control cows and 32 ± 2.3 days in 3NOP cows ($P>0.1$). The average length of the first luteal phase in control cows was 13 ± 1.6 days and 12 ± 1.6 days in 3NOP cows ($P>0.1$). Eleven control cows and sixteen 3NOP cows expressed evidence of a second luteal phase and CL. Of these cows, the average days to evidence of a second CL from calving was 48.7 ± 2.2 and 49.7 ± 1.9 days in control and 3NOP cows, respectively ($P>0.1$). The interval from the end of the first luteal phase to the second was 8.3 ± 0.8 days in the control group and 9.9 ± 0.8 days in the 3NOP group ($P>0.1$).

Introduction

In the United States, 6.3% of greenhouse gas emissions are attributed to enteric fermentation by ruminant livestock species and globally it is the largest source of anthropogenic methane emissions accounting for 25% of methane emissions (IPCC-Mitigation of Climate Change, 2014; Hristov et al., 2015). Methane is a major contributor to greenhouse gases and is of concern relative to its climate warming potential (Hill et al., 2016). Methane production by microbes in the cow's rumen that is lost to the atmosphere is energy loss from the cow that could otherwise be used for productive functions such as increased milk yield or reproduction functions (Hristov et al, 2015). It is especially important to manage cows during the transition period (three weeks prior to three weeks after calving) to reduce negative energy balance. One approach to increase energy available to the cow is to decrease methanogenesis by rumen microbes (Hristov et al., 2015).

Negative energy balance during the transition period can increase calving interval (Wathes et al., 2007). The optimal calving interval in cows is 12-13 months for producers to maximize profit (Arbel et al., 2001). After calving, if cows are delayed in returning to ovarian activity, and thus cyclicity, this will cause an undesirable increase in calving interval (Wathes et al., 2007). In general, cows should begin cycling within 45 days after calving (Lamming and Darwash, 1998; Taylor et al., 2004). Cows which fail to cycle within this time are classified as having delayed ovulation and will most likely not conceive to first service (Taylor et al., 2004).

Measuring progesterone concentrations in blood and milk is an effective way to determine phases of the estrous cycle (Dolezel et al., 1993; Arnstadt and Cleere, 1981). Measuring progesterone in blood or milk is an indicator of presence or absence of a corpus

luteum (Banu et al., 2012). Previous studies estimated a correlation coefficient of 0.88 between the concentrations of progesterone in milk and plasma (Etherington et al., 1991). One study reported a 5-fold difference in progesterone concentrations between plasma, whole milk, and skim milk (Faustini et al., 2007). Progesterone is fat-soluble so concentrations are greater in plasma and whole milk than in skim milk (Faustini et al., 2007).

The objective of this study was to determine if a methane inhibitor would affect the time of return to ovarian activity following parturition in high-producing Holstein dairy cows. Based on the increased body weight observed in the Hristov et al. (2015) study we hypothesize that a reduction in methane production and improved energy balance would allow for energy to be partitioned to reproductive functions and hasten the onset of ovarian activity.

Materials and Methods

ANIMALS

All procedures involving animals were reviewed and approved by the Pennsylvania State University Institutional Animal Care and Use Committee (protocol #46710) and complied with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS Ag Guide, 2010). Fifty-four Holstein dairy cows from the Penn State herd were housed in a tie stall barn and milked twice a day at 0700 and 1900 h. Milk yields were recorded at each milking. The experiment was carried out in two phases due to housing limitations. The cows were blocked based on their previous lactation milk yield or Predicted Transmitting Ability for Milk Yield (for the primiparous cows). Cows within blocks were randomly assigned to treatments. Control cows (n=27) received a vehicle containing SiO_2 and propylene glycol at 60 mg/kg of feed and treated cows (n=27) received the methane inhibitor (3-nitroxypropanol; 3NOP) at 60 mg/kg of feed. The cows were fed a diet balanced for early lactation cows (NRC, 2007) for the first three weeks after calving and were switched to a lactation diet for the subsequent weeks. The diets were formulated to meet National Research Council (NRC) requirements for fresh and lactating cows.

Milk samples were collected every Monday, Wednesday, and Friday morning starting two weeks after calving until 8 weeks after calving. Milk samples were defatted by centrifuging each sample at 2511 x g for 15 minutes at 4°C. The fat cake was removed and the defatted milk was stored at -20°C until analysis.

ELISA Validation and Enzyme-Linked Immunoassay

The ELISA procedure used for validation and subsequent determination of milk progesterone concentration followed methods previously described by Munro and Stabenfeldt (1984).

To validate this assay both whole and defatted milk samples were tested. Milk was collected from a cow known to have low progesterone. Half of the milk was defatted and the other half kept as whole milk. Progesterone was extracted from both whole and defatted milk. Samples of whole and defatted milk were spiked with 1 ng/mL and 5 ng/mL of progesterone (482604; Cayman Chemical. Ann Arbor, MI). Both concentrations were well within the linear detection range of the standard curve. The defatted and extracted samples yielded the most accurate results with an average variance of 0.08 ng/mL and 100% recovery (Table 3.1 Results of Validation Assay).

The validation assay and subsequent assays were carried out as follows. Goat anti-mouse IgG secondary antibody (401211; Calbiochem, La Jolla, CA) was diluted with coating buffer (0.05M sodium carbonate-bicarbonate; pH 9.6) at 2 ug/mL. To each well of a 96-well plate (3369; Corning, Kennebunia, ME) 100 uL of the diluted secondary was added. Plates were incubated at room temperate for 5 minutes on a plate shaker. Plates were then incubated overnight at 4°C. Coated plates could be stored for up to four weeks at 4°C without decanting the secondary. The plates were washed four times with 100 uL of wash buffer (5X MOPS + 10% Tween 20; pH 7.2) to remove excess secondary antibody. A monoclonal progesterone primary antibody (P01-92-11MP; Biostride, Redwood City, CA) was diluted 1:50,000 with assay buffer (0.04 M MOPS + 0.12M NaCl + 0.01M EDTA + 0.05% Tween 20 + 0.005% chlorhexidine digluconate + 0.1% gelatin; pH 7.4). To each well, except the non-specific

binding (NSB) wells, 100 uL of the diluted primary antibody was added and the plate was incubated at room temperature for 90 minutes with shaking. The extracted samples were prepared by adding 250 uL of assay buffer to each sample and mixing for 15 seconds. The samples were incubated at 37°C for 30 minutes. The standard curve was prepared using a progesterone kit standard (482604; Cayman Chemical. Ann Arbor, MI). The stock concentration of the progesterone standard was 100 ng/mL and this was diluted to 10 ng/mL for the most concentrated standard in each assay. The range of our standard curve was 10 ng/mL to 0.16 ng/mL.

After primary antibody incubation the plate was washed four times with wash buffer to remove excess primary antibody and 100 uL of each sample and standard were added in duplicate. The NSB wells contained assay buffer only. The plate was incubated at room temperature for 90 minutes on a plate shaker. The progesterone-3-horseradish peroxidase conjugate (P3-P4 HRP; kindly supplied by Dr. Francisco Diaz, Penn State), was diluted 1:400,000 with assay buffer and 50 uL of the diluted P3-P4-HRP conjugate was added to the samples and standards. The plate was incubated at room temperature for another 90 minutes on a plate shaker. After the final incubation the plate was washed four times with wash buffer to remove excess conjugate. To each well, 125 uL of substrate buffer (0.05 M sodium acetate; + 0.5M H_2O_2 + 20 mg/mL tetramethylbenzidine, pH 4.4) was added and the plate was covered with aluminum foil and incubated at 37°C for 15 minutes. After 15 minutes, 50 uL of stop solution (0.5 M $H_2SO_{4;}$) was added to each well and the samples were immediately assayed on a VICTOR^3 1420 Multilabel Counter using a 450 nm filter (PerkinElmer, Waltham, MA) using the Wallac 1420 Manager software.

Data Analysis

Progesterone concentrations over the six week sampling period were plotted for each cow and data were analyzed for evidence of a first corpus luteum (CL), length of the first luteal phase, interval of first to second luteal phase, and evidence of a second CL. To determine evidence of a CL, cows had to exhibit milk progesterone concentrations of 0.5 ng/mL or greater for two consecutive samples. The cut-off concentration used was 0.5 ng/mL because it fell within the accurate detection range of our standard. Furthermore, progesterone concentrations in whole milk and plasma at 2 ng/mL and greater have been previously cited as evidence of a functional CL (Bulman and Lamming, 1978; Etherington et al., 1991; Lamming and Darwash, 1998; Taylor et al., 2004). Previous literature reported plasma progesterone concentrations are five-fold greater than in skim milk (Faustini et al., 2007). The cut-off value of 0.5 ng/mL in skim milk would be equivalent to approximately 3 ng/mL in plasma. The length of luteal phases was determined as the period of time progesterone concentrations remained at or above 0.5 ng/mL and the interval between two luteal phases was the time progesterone concentrations remained below 0.5 ng/mL after evidence of the first CL.

Data were analyzed using MIXED procedures of SAS (SAS v 9.4). Statistical significance was declared for p-values less than 0.05 and tendencies for p-values between 0.05 and 0.1. In cases where data were not normally distributed the data were log or square root transformed and normal distribution was confirmed. Least square means with pooled standard errors were plotted on graphs. All graphs were created using GraphPad (Version Prism 5; GraphPad Software Inc., La Jolla, CA)

Results

Evidence of CL and Length of Luteal Phase

The progesterone ELISA yielded intra- and inter-assay coefficients of 3.2% and 12.8%, respectively. The limit of sensitivity for the ELISA, using a 100 uL defatted and extracted milk, samples was 0.16 ng/mL. Results showed that there was no difference between control and 3NOP cows in any of the measured reproductive parameters. Days to first CL was 32 ± 2.5 days in control cows and 32 ± 2.3 days in 3NOP cows (P>0.1). There were six cows in the control group and three in the 3NOP group that never showed evidence of a CL so these cows were excluded from the analysis. The average length of the first luteal phase in control cows was 13 ± 1.6 days and 12 ± 1.6 days in 3NOP cows (P>0.1). Eleven control cows and sixteen 3NOP cows expressed evidence of a second luteal phase and CL. Of these cows, the average days to evidence of a second CL from calving was 48.7 ± 2.2 and 49.7 ± 1.9 days in control and 3NOP cows, respectively (P>0.1). The interval from the end of the first luteal phase to the second was 8.3 ± 0.8 days in the control group and 9.9 ± 0.8 days in the 3NOP group (P>0.1).

Discussion

The main objective of this study was to determine if 3-nitrooxypropanol (3NOP), a methane inhibitor, had any effect on return to cyclicity in dairy cattle. Based on the results there was no effect of the methane inhibitor on any reproductive functions when compared to control cows. We determined return of ovarian activity to be when cows exhibited progesterone concentrations of >0.5 ng/mL in milk on two sampling days. In our study, there was no difference between treatment groups to resumption of ovarian activity. Cows that cycle within 45 days after calving are more likely to conceive and maintain a pregnancy to fewer inseminations than cows that begin cycling after 45 days (Bulman and Wood, 1980; Lamming and Darwash 1998). In one study of 533 cows, half of the animals resumed ovarian activity by 20 days postpartum and 92.4% by 40 days (Bulman and Wood, 1980). There was no difference in return to ovarian activity in our study and both treatment groups returned to cyclicity within the normal time period. Although the 3NOP supplement did not negatively affect the parameters measured, it would appear reducing methane production did not allow cows to partition energy to be used for reproductive function.

Normal luteal phases are typically 12 to 19 days (Robertson, 1972; Mather et al., 1978; Taylor et al., 2004). However, after calving, the first estrous cycle tends to be shorter than normal with a luteal phase that may only last for approximately 8 days (Usmani et al., 1990; Perry et al., 1991; Qureshi et al., 1998). The average length of the first luteal phase in this study was within the normal length of a luteal phase, although there were cows in both treatment groups which had shortened first luteal phases. Because progesterone concentrations are significantly lower in skim milk compared to plasma, the assay may have

lacked the sensitivity to detect shortened luteal phases with low progesterone concentrations (Faustini et al., 2007).

The average days to evidence of a second CL did not differ between control and 3NOP cows, nor did the interval from the end of the first luteal phase to the second. Our values for these parameters are comparable to published results that reported an average of 51 days to a second cycle postpartum with an average of 10 days from the end of one luteal cycle to the second (Etherington et al., 1991). Compared to the first luteal phase, which tends to be shortened after parturition, the second luteal phase is longer with increased progesterone concentrations (Dolezel et al., 1993). Producers want cows to start cycling as early as possible after parturition because this is highly correlated with increased conception rates and improved reproductive performance (Thatcher and Wilcox, 1973; Lamming and Darwash, 1998).

The ability to measure progesterone in milk provides a convenient method for producers and researchers to monitor ovarian activity in cattle. It was shown that the concentration of progesterone in milk reflects that in plasma with a previously reported correlation coefficient of 0.88 (Arnstadt and Cleere, 1981; Etherington et al., 1991). Whole milk (Dolezel et al., 1993), skim milk (Qureshi et al., 1999), milk fat (Mather et al., 1978), and milk whey (Faustini et al., 2007) have all been used to measure progesterone. In plasma, during the follicular phase, average progesterone concentrations were 0.44 ng/ mL and during the luteal phase 6.8 ng/mL (Donaldson et al., 1970; Robertson, 1972.). In whole milk, previous findings reported minimum concentrations of 4 ng/mL and up to 18 ng/mL in the presence of a functional CL (Zaied et al., 1979; Etherington et al., 1991; Taylor et al., 2004). In skim milk, concentrations as low as 1.5 ng/mL were reported in buffaloes with a

developing CL (Qureshi et al., 2000). The concentration of progesterone in skim milk is expected to be less than that found in whole milk because progesterone is fat soluble (Faustini et al., 2007). The variability in the published results for progesterone concentrations in whole milk is thought to be due, in part, to the fact that progesterone is fat soluble (Faustini et al., 2007). To reduce the variability caused by milk fat, using defatted milk or milk whey is suggested (Qureshi et al., 1999; Faustini et al., 2007).

It is important to remember that ovarian activity is highly influenced by uterine function (Brinsfield and Hawk, 1968; Boyd et al., 1977; Etherington et al., 1991; Kindahl et al., 1992; Royal et al., 200;). Dystocia, retained placenta, or any form of uterine infection after parturition contributes to delayed onset of ovarian activity (Marion and Gier, 1968; Boyd et al., 1977; Etherington et al., 1991; Royal et al., 2002). Uterine health and overall cow health is largely determined by energy balance after parturition (Wathes et al., 2007). Strategies to mitigate negative energy balance after parturition are being studied to reduce metabolic diseases and subsequently improve fertility.

Metabolic hormones, including IGF-1 and insulin, play a significant role in regulating reproductive function (Taylor et al., 2004). Insulin was shown to stimulate proliferation of granulosa cells and IGF-1 and insulin are both responsible for regulating luteinizing hormone pulses from the pituitary (Beam and Butler, 1999; Adam et al., 2000; Taylor et al., 2004). After parturition, dry matter intake is often not sufficient to support high milk production, which causes alterations in metabolic hormones including decreased insulin, leading to negative energy balance (Diskin et al., 2003; Samarutel et al., 2008). This is especially evident in primiparous cows as these cows not only need energy for milk production but growth as well (Boyd et al., 1977; Bulman and Lamming, 1978; Wathes et al., 2007). In

most cows, resumption of ovarian activity does not occur until return to a positive energy balance (Canfield and Butler, 1991; Beam and Butler, 1999; Samarutel et al., 2008). Increased dry matter intake by cows after parturition was strongly correlated to earlier onset of cyclicity (Patton et al., 2007).

There are discrepancies in the literature as to whether or not milk production plays a role in level of negative energy balance and return to ovarian activity after parturition. One study reported a longer interval to first ovulation in high producing cows (36.9 days) when compared to low producing cows (28.4 days) (Johnson et al., 1966). However, another study reported no effect of milk production on resumption of ovarian activity (Boyd et al., 1977). It is more commonly agreed in the literature that delayed cycling and irregular cycles are primarily due to negative energy balance and that the causes of negative energy balance may or may not be attributed to milk production (Taylor et al., 2004; Esposito et al., 2014; Boldt et al., 2015).

In the study by Hristov et al., (2015) that served as the basis for the present work, cows that received methane inhibitor had 80% greater body weight gain over the 12 week trial and gained 168 g/day more than the control cows. It was speculated that production of methane in the cow reduces feed efficiency and causes energy loss to the cow (Hristov et al., 2015). Reducing methane output could spare energy for milk and reproductive function (Hristov et al., 2015). This is supported by previous findings in which cows with greater loss of body weight and body condition had impaired fertility (Butler, 2000; Taylor et al., 2004). In this study we did not observe any differences in return of ovarian activity in the cows that received methane inhibitor. However, it is an area worthy of further pursuit based on the

evidence in the literature that increasing energy available to the cow after parturition leads to overall improved reproductive health and performance.

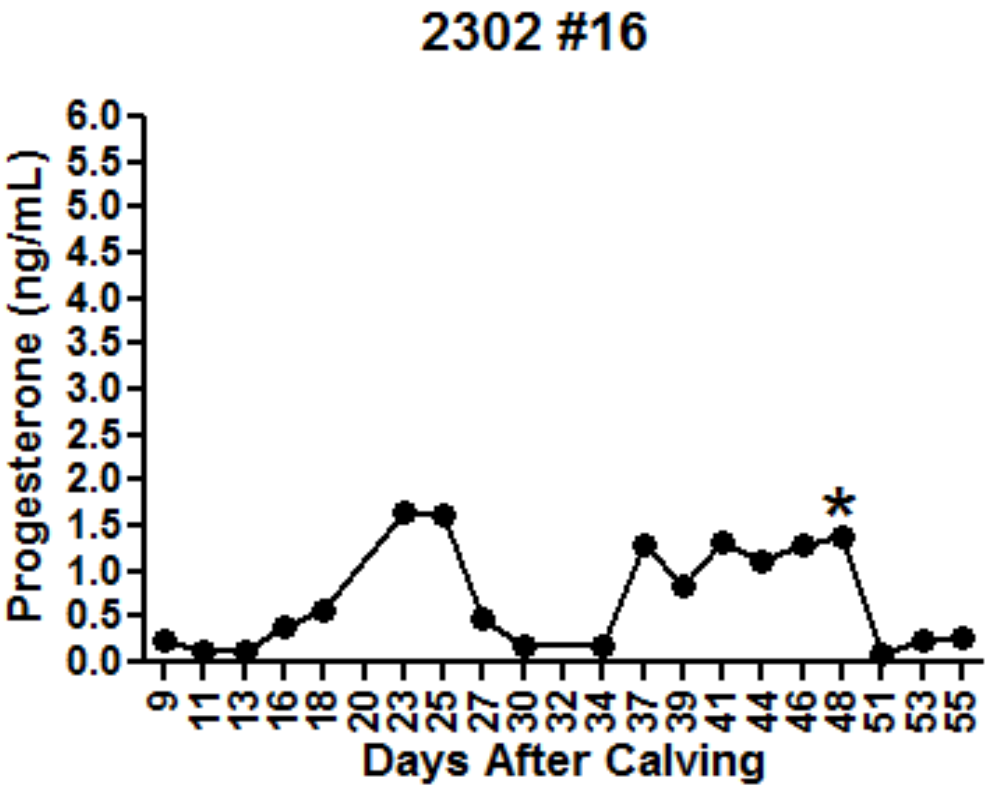

Figure 3.1: Representative graph of a cow which began cycling around 20 days after calving and had a short first cycle followed by a second, longer cycle. The asterisk represents when the cow received a PGF injection to begin the ovsynch program for breeding.

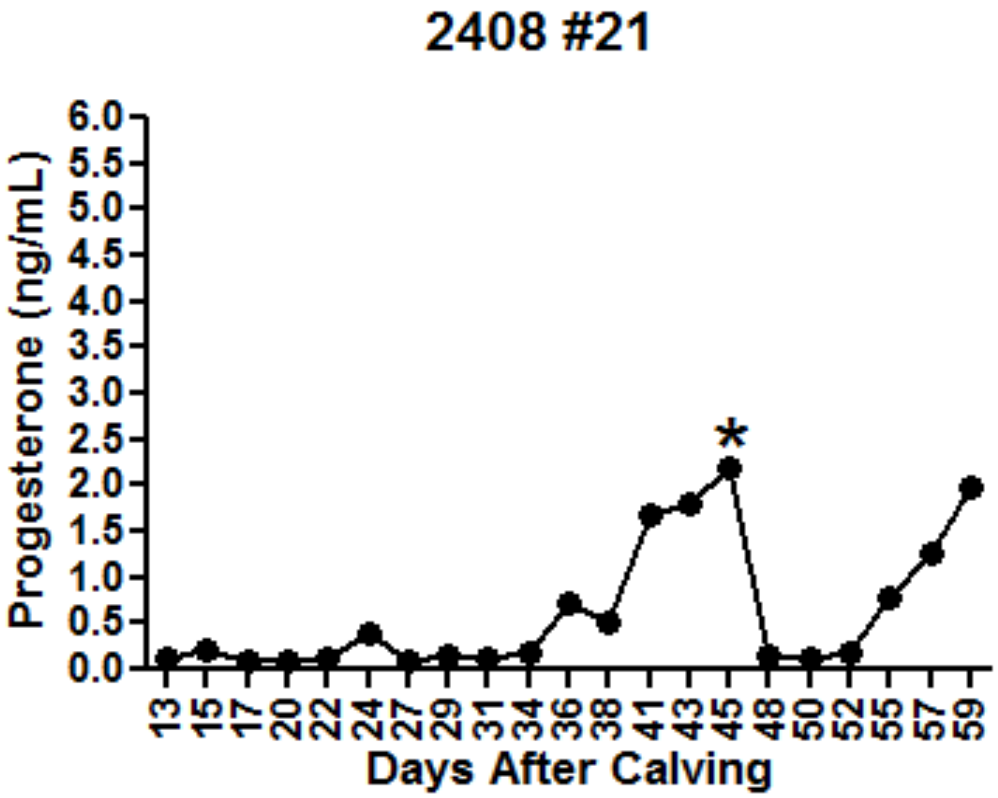

Figure 3.2: Representative graph of a cow exhibiting delayed onset of ovarian activity. The asterisk represents when the cow received a PGF injection to begin the ovsynch program for breeding. In this case the start of a second luteal phase can be observed in response to the synchronization program.

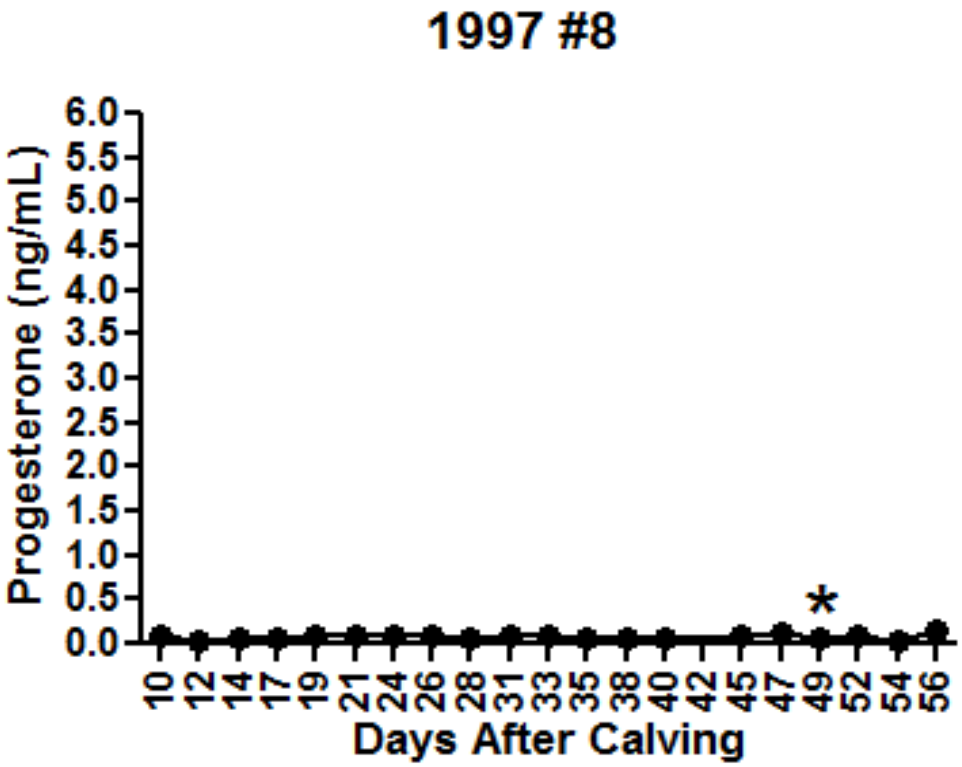

Figure 3.3: Representative graph of a cow that failed to resume ovarian activity following calving.

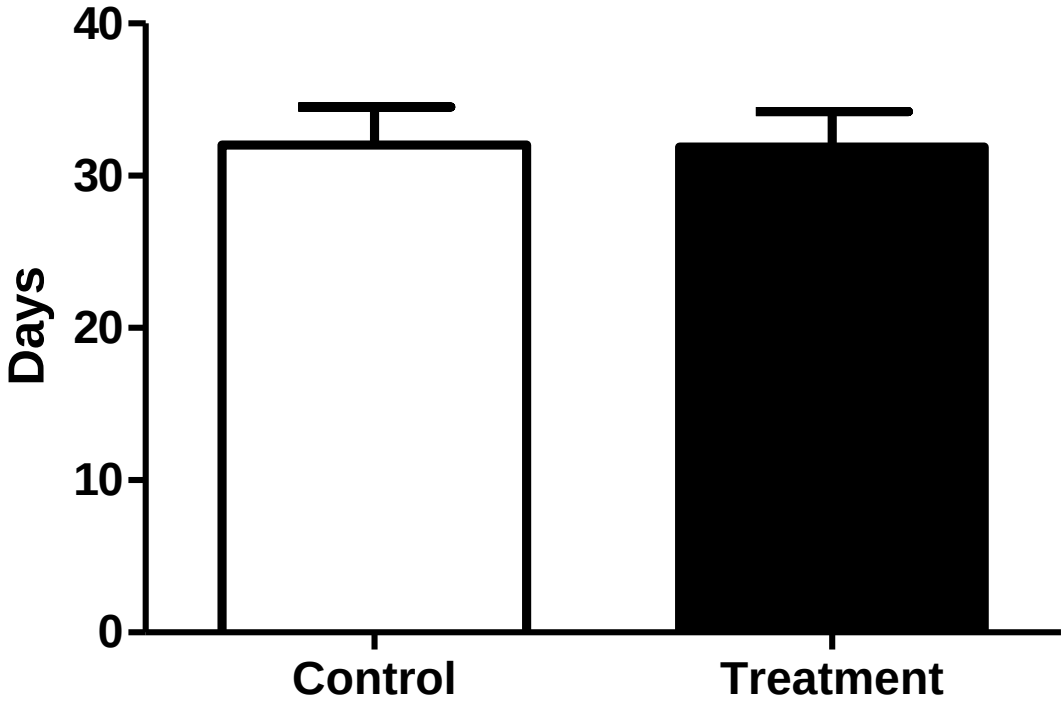

Figure 3.4: Days after calving to first corpus luteum (CL)
There was no difference observed between treatments (P>0.1).

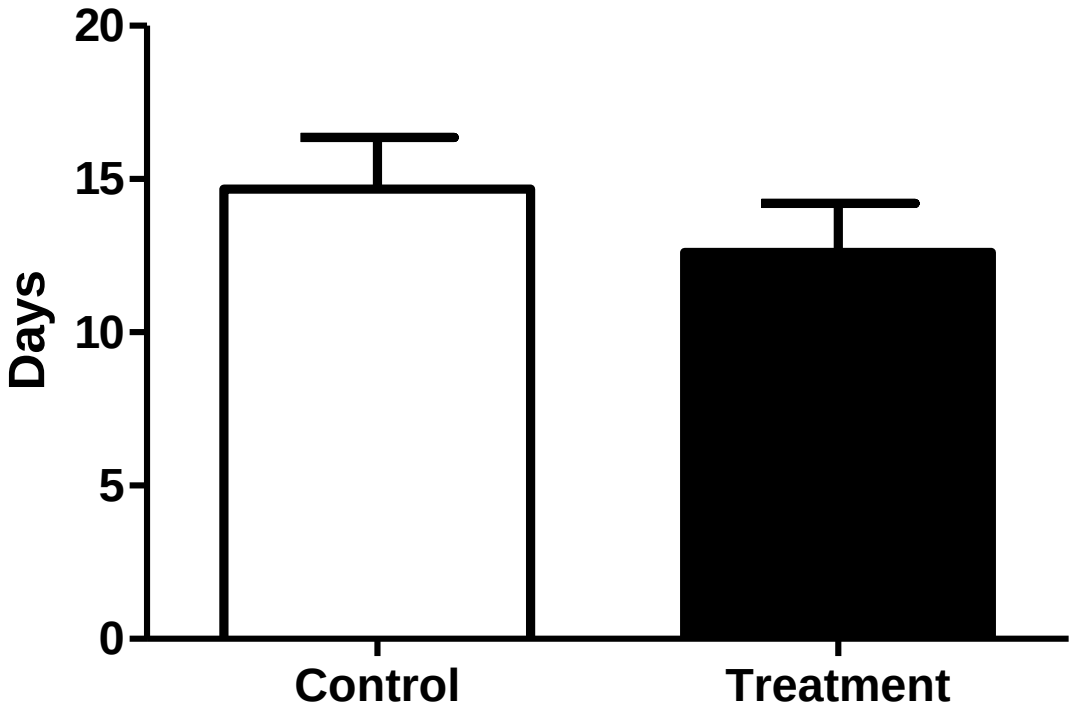

Figure 3.5: Length of first luteal phase
Only cows which expressed a luteal phase that was not interrupted by PGF injection were included in the analysis. No difference was observed between the two groups (P>0.1).

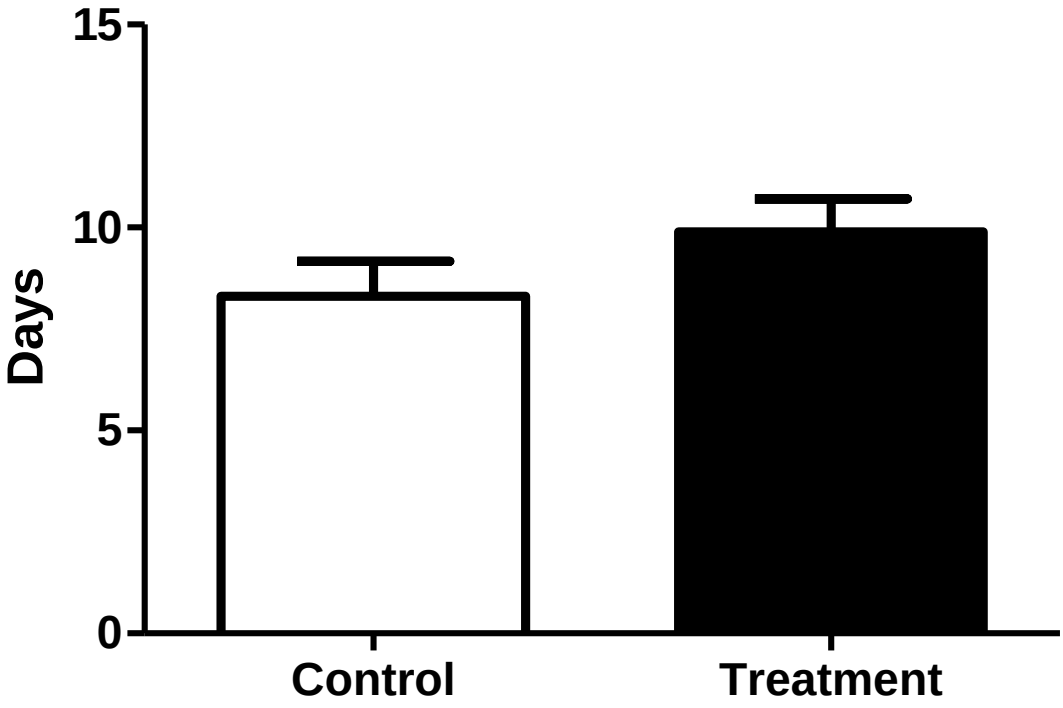

Figure 3.6: Interval between luteal phases
The average interval from the end of the first luteal phase to the start of the second. No difference was observed between treatments (P>0.1).

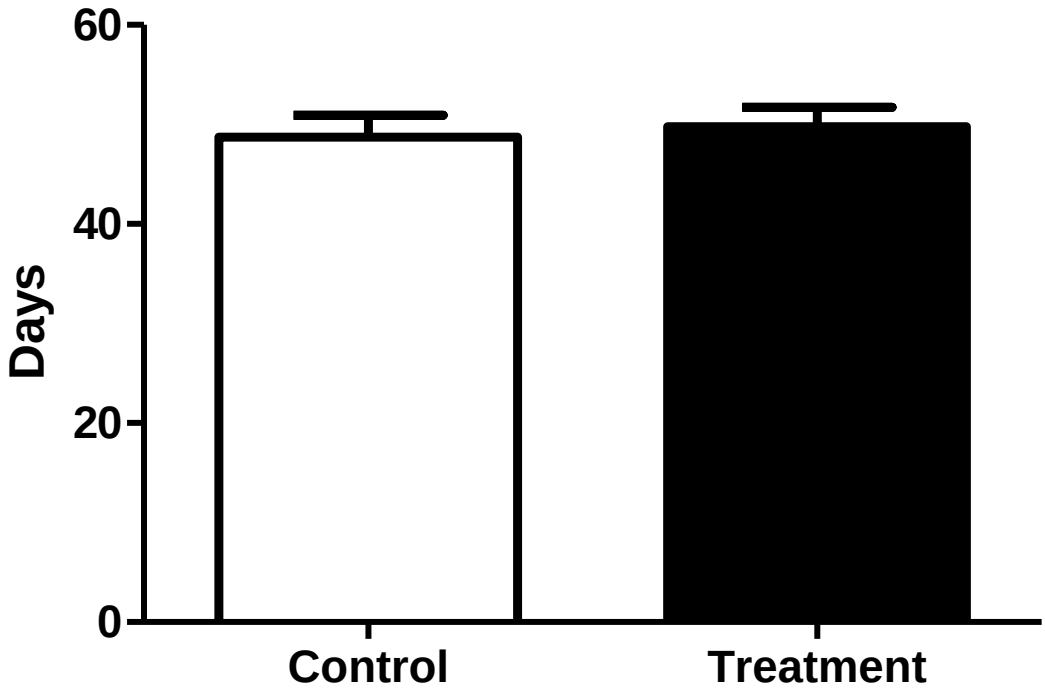

Figure 3.7: Days from calving to formation of a second corpus luteum (CL) There were 11 cows in the control group and 16 in the 3NOP group that had formation of a second CL. There was no statistical difference observed between treatments groups (P>0.1).

Table 3.1: Results of validating an ELISA for milk samples

	Not Spiked		Spiked 1ng/mL P4		Spiked 5 ng/mL P4	
	Not Extracted	Extracted	Not Extracted	Extracted	Not Extracted	Extracted
Defatted Milk	0.014±0.01	0.015±0.008	1.13±0.01	1.28±0.05	1.13±0.23	5.04±0.1
Whole Milk	0.61±0.33	0.07±0.01	0.38±0.52	0.089±0.009	0.75±0.66	0.09±0.04

An ELISA which was previously validated for plasma was used to measure progesterone concentrations in milk. A validation was carried out in which both whole and defatted milk samples were spiked and extracted. The defatted and extracted milk samples had the best recovery and least amount of variation compared to whole milk samples.

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Chapter 4: General Discussion

The transition period is perhaps the most dangerous and critical time in the production cycle of the dairy cow. A thorough understanding of the intricate interactions between nutrition, the immune system, and reproduction is necessary to implement effective management practices. The focus of these studies was to understand how health of the dairy cow can be improved during the transition period to allow for more efficient milk production and improved reproductive performance. We tested the hypothesis that feeding a flaxseed supplement effects immune cell function and incorporation of omega-3 fats in milk fat, plasma, and red blood cells. The second study addressed whether improved energy balance would affect measures of reproductive function. This study was based on prior work showing that feeding an inhibitor of methane production resulted in increased body weight gain in high producing dairy cows. In both studies, measurements of milk production and components and plasma concentration of glucose and non-esterified fatty acids where taken to assess energy balance.

Negative energy balance (NEB) is at the root of negative health events during the transition period. Negative energy balance results in fat mobilization and changes in metabolic hormones that can reduce reproductive function (Wathes et al., 2007). After parturition high producing dairy cows are typically not able to consume enough feed to offset the high energy demand of lactation. This is why we chose to measure plasma metabolites which are measures that can indicate energy status of a cow. In the flaxseed study, plasma concentration of NEFA and glucose were measured as indirect measures of energy balance. Non-esterified fatty acids are released from fat stores in the body and the liver oxidizes them

to carbon dioxide and ketone bodies like beta-hydroxybutyrate (BHBA; Wathes et al., 2007). Beta-hydroxybutyrate is a ketone body that is synthesized in the liver and can provide important energy balance data on the amount of fatty acid oxidation occurring in the cow (Wathes et al., 2007). Although no direct measurements for energy balance were taken in the reproductive part of the methane study, measuring reproductive functions is an indirect way of assessing energy balance. Intake and bodyweight were recorded by other researchers in this study and cows receiving the methane inhibitor had decreased dry matter intake, gained less weight, and produced more milk than the control cows. The methane inhibited cows exhibited better feed efficiency but experienced greater negative energy balanced compared to the control cows. Cows need to be in positive energy balance to cycle, conceive, and maintain pregnancy; therefore cows that cycle sooner after parturition are in a more positive energy balance than those who take longer to cycle (Canfield and Butler, 1991; Beam and Butler, 1999; Samarutel et al., 2008). One question that remains in regard to energy balance and methane production is how much energy is lost to methane production, and if cows can partition this energy to other productive functions if methane production is reduced.

There is a connection between negative energy balance and impaired immune function. Immunosuppression is thought to be caused, in part, by negative energy balance, because glucose concentrations are low. Immune cells require glucose for energy to carry out normal functions (Sordillo and Rapheal, 2013; Ingvartsen and Moyes, 2015). During this time the immune system is unable to mount an adequate response if threatened by pathogens due to lack of cell numbers and energy required to support activation. Equally as dangerous is a lack of self-regulation to resolve inflammation by the immune system during the transition period (Sordillo et al., 2009). Results presented show that the number (or percentage) of neutrophils

producing reactive oxygen species and exhibiting phagocytosis was reduced when cows were supplemented with flaxseed. A reduction in neutrophil function is generally associated with an anti-inflammatory state but could also be due to immunosuppression if neutrophil numbers are low (Asehnoune, et al., 2004; Silvestre et al., 2011). Unfortunately, we did not count neutrophil numbers for each cow. Having a count of immune cells would have provided further insight to the immune status of each cow and could have allowed for more in depth analysis of our neutrophil results. Despite this pitfall, our neutrophil data is provides further information about immune responses in transition cows. Neutrophils have been studied in the transition cow and their numbers and function were shown to be reduced by NEFA and BHBA (Suriyasathaporn et al., 1999; Scalia et al., 2006).

Another key finding of this study is that cows fed the omega-3 diet did not exhibit milk fat or protein depression or decreased milk yield. In fact, milk fat percentage and the amount of omega-3 fats in milk fat both increased in the supplemented cows. Previous studies reported that bio-hydrogenation of omega-3 fats in the rumen reduce the amount of these fats available to the mammary gland (Palmquist, 1993; Syadiati et al., 2012). To increase the amount of omega-3 fats in the milk, researchers fed fat at such a high concentration that it caused milk fat depression (Romo et al., 1996; Petit, 2002; Mustafa et al., 2003; Petit et al., 2007; Zachut et al., 2010). While milk fat depression improves energy balance, it also results in reduced value of the milk (Schingoethe and Casper, 1991; Cant et al., 1996; Zachut et al., 2010; Gandra et al., 2016). In one study increasing amounts of conjugated linoleic acid were supplemented to cows and milk fat yield was decreased but energy balance increased (Odens et al., 2007). Increasing omega-3 fats in the milk allows producers to market their products to a wider audience because of the rising interest in increasing omega-3 fats in diets (Cox et al.,

2007; Siro et al., 2008 Simopoulos, 2009). Based on the success of Eggland's Best® Eggs, which saw a 37% increase just two years after the launch of their omega-3 enriched eggs, there is promise for a market for omega-3 enriched milk (Laymon, 2004). This, along with the increased milk fat percentage, may justify the extra cost of the flaxseed supplement, and could possibly increase revenue for producers (Laymon, 2004; Welter et al., 2016).

The main limitation of both studies was small sample size. In almost all large animal studies the limiting variable to having enough power is sample size. To detect statistical differences on reproductive parameters very large sample sizes are needed (Chapman and Seidel, 2007). With only 15 cows on the flaxseed trial it is difficult to draw definitive conclusions on the effects of omega-3 fats on immune function in the transition period. Another limitation is the variability between cattle and variability during the transition period in general. However, to account for this variability, cows were used as a random variable in the statistical analysis. Some cows seem to have few problems during the transition period whereas others suffer from severe periparturient diseases and NEB. Another flaw in the study was that we did not obtain a blood sample from the cows before they calved. The Day 1 sample was collected approximately 17 hours postpartum after cows had already began to consume their respective diets. Because of this, there was no true time 0 sample taken without effect of diet. Despite this flaw, because both groups of cows began consuming the diet before sampling and the average time until first sampling was the same for both groups it did not inhibit the ability to draw conclusions from this study. However, it would be important in future studies to obtain samples from cows before calving and before they begin consuming the diet. Data obtained before cows begin the diet provides a starting point for each cow.

Samples from cows before, during, and after the transition period would provide data on the changes that occur in energy balance and immune function.

Results generated from supplementing transition dairy cows with flaxseed has given further insight into immune function in transition dairy cows, but has also led to more questions which will need to be addressed. It will be important to investigate the direct effects of omega-3 fats on neutrophils to determine if they induce anti-inflammatory responses. It is also not known if omega-3 fats aid immunosuppression by decreasing neutrophil numbers or functions or both. Furthermore, definitive conclusions on effects of omega-3 fats on health and fertility will require recording incidences of periparturient disease and conception and pregnancy rates. These studies will also need to partition effects on primiparous and multiparous cows as both have unique problems associated with NEB, immune function, and fertility (Gierek et al., 2006; Wathes et al., 2007). Primiparous cows are generally believed to suffer more from NEB after their first calf due to the fact that they are still growing, but immune cell numbers have been shown to be decreased in older cows compared to primiparous cows (Gierek et al., 2006). To fully understand the effects of omega-3 fats on reproduction, it would also be beneficial to investigate if there are any direct effects on the corpus luteum. Some studies suggest that the inhibitory mechanism of PUFA on uterine prostaglandin synthesis prolongs the life of the corpus luteum (Staples et al., 1998; Cheng et al., 2001; Leroy et al., 2014). Fatty acids in the diet increase cholesterol synthesis, and cholesterol is the precursor of steroid hormones, therefore there may be an effect of omega-3 fats on ovarian function (Petit et al., 2002). Furthermore, fatty acid content in follicular fluid has been found to be correlated with dietary fatty acid content (Adamczyk et al., 2016; Leroy et al., 2014).

Overall, these studies provide further insight to the transition period of dairy cattle. We were able to provide new data on the effects of omega-3 fats on neutrophil function and show that supplementing cows with fats does not have to lead to milk fat depression. Furthermore it was shown that there were no negative effects of a methane inhibitor on reproductive function. Results of these studies can be utilized to further examine ways to combat NEB during the transition period, and thus improve dairy cow health and production.

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