EARLY LIFE CALF NUTRITION AND MANAGEMENT AND THEIR IMPACTS ON HEALTH AND PRODUCTIVITY

by

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To Nate,

I can't wait to continue chasing our dreams together

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ABSTRACT

The first four months of a dairy calf's life provide the foundation for future production and health. Therefore, it is critical that calves are managed to optimize both health and growth, to maximize their potential. Calf morbidity, which leads to less productive animals and is an economic impact on producers, continue to be areas of opportunity in the dairy industry. In addition, the animal agricultural community is working to reduce the use of antibiotics, due to a growing concern regarding antibiotic resistant bacteria. This provides researchers with the challenge of identifying strategies to reduce calf morbidity and mortality, while also decreasing reliance on antibiotics. The objective of this dissertation was to evaluate nutritional strategies supplemented to calves as well as maternal factors that impact colostrum. The studies focused on strategies to improve the innate and adaptive immune responses and growth of the calf, ultimately reducing reliance on antibiotics. Chapters 2 and 3 discuss feeding dairy calves *Saccharomyces cerevisiae* fermentation products in milk replacer and solid feeds until 4 months of age. This study concluded that feeding *Saccharomyces cerevisiae* fermentation products to calves improves average daily gain and feed efficiency post-weaning and reduces antibiotic treatment incidence for respiratory disease. It also increases the evenness of the fecal microbiome and the acute innate immune response, as determined by increased TNF-α, glucose, and respiration rate during a lipopolysaccharide challenge. Chapter 4 evaluated the effects of feeding dairy calves medium chain fatty acids (C8:0 and C10:0) in milk replacer until 60 days of age. Feeding C8:0 and C10:0 to calves reduced plasma NEFA concentrations around weaning, suggesting the mobilization of less adipose tissue to meet the energy demands of the calf. This trial also determined that vaccinating calves at 3 weeks of age with ovalbumin combined with an aluminum hydroxide adjuvant, is an effective way to evaluate their adaptive immune responses.

Supplementing calves directly is not the only way to impact calf growth and health, maternal factors also impact calf nutrition through colostrum consumption. Last, chapter 5 explored maternal factors that influence the lipidome of colostrum and therefore the lipid intake of the newborn calf. This study concluded that the metabolic status of the cow affects circulating lipids and the lipid content of colostrum. Also, the lipidome of colostrum is distinct from the circulating lipidome of the calf, which is similar to the circulating lipidome of the cow, except for phosphatidylglycerol, where it appears that colostrum serves as the source for the

phosphatidylglycerol present in the circulation of the calf. There are many different nutritional strategies that can impact the health and productivity of calves. Calves can be directly supplemented with nutraceuticals like *Saccharomyces cerevisiae* fermentation products or medium chain fatty acids, or calf nutrition can be influenced by the maternal factors through the consumption of colostrum.

CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

Dairy calves are highly susceptible to disease due to calves being born immunonaïve and experiencing various stressors (environmental, nutritional, transportation, handling, etc.) early in life, that increase the risk of disease occurrence (Barrington and Parish, 2001). Dairy calves are considered immunonaïve at birth because of the placenta type, epitheliochorial, prevents the passage of maternal immunoglobulins to the fetus. For many placental mammals, immunoglobulins pass through the placenta from the maternal to the fetal blood circulation, providing some protection against environmental pathogens at birth. Cows have a synepitheliochorial placenta, which means that there are six layers of tissue that separate the fetal blood supply from the maternal, three layers from the fetal side and three from the maternal side (Senger, 2005). The six tissue layers prevent the maternal blood supply from coming into contact with the fetal blood supply, therefore maternal immunoglobulins are unable to be passed to the fetus during gestation. Moreover, newborn calves are unable to successfully initiate an immune response (Barrington and Parish, 2001). The problem is that not only do calves not have passive immunity at birth from the mother, but they also have immature immunological mechanisms so they cannot mount an adaptive immune response. The calf's innate and adaptive immunity, which starts to develop *in utero*, is suppressed during parturition due to placental progesterone and prostaglandin, maternal cortisol and estrogen, and fetal cortisol (Chase et al., 2008). Therefore, calves rely on colostrum to receive immunoglobulins from their dam, through the passive transfer of immunity, until their own immune system is developed to sufficiently protect them from pathogens.

Unfortunately, ensuring calves receive immunoglobulins via colostrum is not enough to ensure that they remain healthy and grow efficiently through weaning. Calves remain highly susceptible to disease during the first months of life as their immune system continues to develop, and again after being weaned because of stress (Chase et al., 2008). The passive transfer of immunity from the dam protects the calf initially, but that passive immunity starts decreasing as maternal antibodies start to decay (Figure 1.1). As passive immunity starts decreasing, the calf's own immune system starts to develop, however, there is still a window of susceptibility where the

passive immunity is no longer high enough to resist an infection, and the innate and active immune systems of the calf is not developed enough to fully protect the calf from disease. Furthermore, weaning is a particularly stressful time for calves because of diet changes causing reduction in total immunity (Chase et al., 2008).

It is estimated that in the U.S., prior to weaning 1 in 5 and 1 in 4 calves will experience bovine respiratory disease (BRD) or digestive illnesses, respectively (NAHMS, 2012; Walker et al., 2012). Approximately, 80-90% of those unhealthy calves are treated with antibiotics. After weaning, it is estimated that 1 in 10 calves experiences BRD, and about 80% of those unhealthy calves are treated with antibiotics (NAHMS, 2012). The use of antibiotics to treat disease is a concern because of the increasing prevalence of antibiotic resistant bacteria in human and animal medical fields (Langford et al., 2003; Loo et al., 2019). Also, many diseases are caused by viruses not bacteria, and antibiotic treatments are commonly administered prior to knowing the exact cause of the illness, therefore reducing the efficacy of antibiotics. The United States Food and Drug Administration sought to address this issue by revising the Veterinary Feed Directive (VFD) that was a part of the Animal Drug Availability Act of 1996. The revision stated that feedstuffs that included medically important antimicrobials were required to be authorized by a licensed veterinarian. The growing concern regarding antibiotic resistant bacteria and the revised VFD has increased interest in strategies to reduce morbidity and mortality in calves without relying solely on antibiotics.

Throughout the remainder of this chapter, possible strategies to improve the health and productivity of calves through the use of nutritional and management strategies will be discussed. While there is considerable focus on the immunoglobulin concentrations in colostrum, colostrum lipids need to be further explored because they have essential roles in thermogenesis and immune function of the calf. An increased understanding of factors that influence the inclusion of lipids in colostrum is necessary to ensure calves receive an optimal lipid profile for health. In addition, the term nutraceutical describes the use of nutrition to improve health status. This paper will discuss how the immune status and productivity of calves are influenced when fed *Saccharomyces cerevisiae* fermentation products (SCFP) and medium chain fatty acids (MCFA) as nutraceuticals.

1.2 The Immune System

The immune system is a network of cells and tissues, and the substances they secrete, that work together to defend the body from the invasion of foreign pathogens (bacteria, viruses, fungi, and parasites) that are deemed harmful to the body. The immune system is divided into three levels (Figure 1.2). The first level is barriers, which can be chemical, physical, or microbiological. The second level is innate immunity, which is made up of cells and soluble factors that can recognize tissue damage and pathogens and act immediately to repair the damage and clear the pathogen. Innate immune cells include macrophages, neutrophils, eosinophils, natural killer (NK) cells, mast cells, and dendritic cells, while the soluble factors include acute phase proteins, cytokines, and complement proteins. It is currently believed that innate immune system does not have the ability to remember past responses, so every incidence will have the same reaction time and intensity. The third level is adaptive immunity, which includes B lymphocytes (B cells) and T lymphocytes (T cells). The adaptive immune system has a delayed response compared to the innate, but it is responsible for producing B and T cells which remember past pathogens, allowing for a faster and stronger response during re-exposure.

1.2.1 Barriers

The first line of defense against pathogens incudes physical, chemical, and biological barriers that work to protect the body without activating an immune response. Physical barriers include the epithelium, specifically tight junctions between epithelial cells that prevent pathogens from penetrating the body, the mucosal layer on the epithelium of the gastrointestinal, respiratory, and genitourinary tracts that minimize contact between pathogens and epithelial cells, and the epithelial cilia that sweeps away the contaminated mucosal layer, removing trapped particles from the body, and allowing for fresh mucus to take its place (Chaplin, 2010). Chemical barriers include the antimicrobial enzyme lysozyme found in tears, saliva, mucus, and sweat, sebaceous glands on the skin that secrete acids, digestive enzymes, and stomach acid. The final type of barrier is biological, which are organisms that protect against invading pathogens. These harmless organisms live on the skin and in the reproductive, digestive, and urinary tracts and prevent pathogens from colonizing by using up the food sources and secreting substances that make the environment less hospitable for pathogens (Chaplin, 2010).

1.2.2 Innate Immunity

If the barrier level of the immune system is unsuccessful at preventing the invasion of pathogens, the next line of defense is the innate immune system. The innate immune response is divided into two steps, first, recognizing a pathogen and second, recruiting defense cells to eliminate the pathogen (Parham, 2014). Immune cells, such as neutrophils, dendritic cells, macrophages, and natural killer (NK) cells, express pattern-recognition receptors (PRRs), which are proteins that detect pathogens via pathogen-associated molecular patterns (PAMPs) and recognize damaged or injured cells via damage-associated molecular patterns (DAMPs). There are four families of PRRs, including Toll-like receptors (TLRs), Nucleotide-binding oligomerization domain-like receptors (NLRs), RIG-1 like receptors (RLRs), and C-type lectin receptors (CLR; Kawasaki and Kawai, 2014). It is important to note that PRRs recognize molecular structures that are shared by a variety of pathogens and not found in mammalian cells. They do not recognize specific antigens, which is why the innate immune system is referred to as non-specific, meaning the innate system recognizes structures as pathogenic, but not the particular pathogen present (Hato and Dagher, 2015). The most researched and well known PAMP is lipopolysaccharide (LPS), which is found in the outer membrane of gram-negative bacteria and is recognized by the PRR TLR4.

After PRRs bind to PAMPs or DAMPs, immune cells are activated and respond by producing inflammatory cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α), inducing a protective pro-inflammatory response. Some cytokines produced from macrophages are chemokines, meaning that they attract other immune cells like neutrophils to the site of infection (Roberts et al., 2002). Both macrophages and neutrophils are phagocytic cells, meaning they secrete enzymes that are highly destructive to pathogens, which allows them to then engulf and digest the damaged organism (Nicholson, 2016). Pro-inflammatory cytokines IL-1, IL-6, and TNF-α travel through the blood stream to the liver and stimulate hepatocytes to secrete acute phase proteins, such as serum amyloid A, haptoglobin, and fibrinogen, which help by killing infectious microbes, repairing damaged tissue, and restoring the body to a healthy state (Murata et al., 2004).

In addition to the cell mediated components of the innate immune system, there are also humoral components, including C-reactive protein, complement proteins, mannose-binding lectin, LPS binding protein, and antimicrobial peptides, all of which are involved in identifying microbes

and clearing infections (Turvey and Broide, 2010). Within a few minutes of the body being exposed to a pathogen, this pro-inflammatory response can be activated, which is why the innate immune response is considered rapid. If the innate immune system is unable to neutralize the infection, dendritic cells, which are referred to as antigen-presenting cells, will take antigens from the pathogen that is invading the body, to a lymph node. There it will present the antigen to lymphocytes, therefore activating an adaptive immune response to clear the invading microbes (Roberts et al., 2002).

1.2.3 Adaptive Immunity

The adaptive immune system, sometimes referred to as the acquired immune system, contains both a cellular and a humoral component, just like the innate system (Turvey and Broide, 2010). Adaptive immune responses are facilitated by white blood cells called lymphocytes. Cellular-mediated responses are carried out by T and B cells, and humoral-mediated responses are carried out by antibodies, which are produced from B cells (Turvey and Broide, 2010). Both T and B cells derive from multipotent hematopoietic stem cells in bone marrow. After B cells are made, they mature in the bone marrow, and then are released into the lymphatic system and move throughout the body. At this point they are called naïve B cells and have what are referred to as membrane-bound antibodies on their cell surface (Chaplin, 2010). Antibodies are proteins that are also referred to as immunoglobulins. When naïve B cells come into contact with an antigen that fits its membrane-bound antibody, it will bind the antigen and then divide and differentiate into a memory B cell or an effector B cell. An antigen is defined as any molecule that elicits an adaptive immune response (Roberts et al., 2002). Memory B cells possess the same membrane-bound antibody as the naïve B cell. Effector B cells also have the same antibody that the naïve B cell had, the only difference is that antibody is no longer membrane bound. Effector B cells secrete antibodies which will move throughout the body and bind to the pathogen that stimulated its production. When an antibody binds to an antigen, it both inactivates the pathogen's ability to bind to receptors on hosts cells and labels the pathogen for phagocytosis by innate immune cells (Nicholson, 2016), also referred to as humoral immunity.

Even though T cells are made in bone marrow, they migrate to the thymus to mature. All T cells have a T cell receptor and either a CD4 receptor, CD8 receptor, or both a CD4 and a CD25 receptor with the receptors they possess determining their role. After T cells mature, they are

released from the thymus. T cell receptors are not capable of binding to antigens like antibodies, they recognize antigens that are bound to antigen-presenting cells. Helper T cells have the CD4 receptor and activate cytotoxic T cells and B cells. Cytotoxic T cells have the CD8 receptor and remove infected host cells and pathogens through apoptosis. T regulatory cells have the CD4 and CD25 receptors and help the immune system differentiation between host and foreign molecules (Chaplin, 2010), which is called cellular-mediated immunity.

Once B and T cells are activated after encountering a specific antigen, they will continue to proliferate in order to mount an effective immune response (Chaplin, 2010). It is important to note that adaptive immune responses are destructive to cells and tissues, therefore it is vital that the adaptive immune system is only activated in response to harmful foreign invaders. Many foreign molecules are harmless and activating an adaptive immune response would be unnecessary and potentially harmful to the host, an example of this would be an allergic reaction (Roberts et al., 2002). However, a successful adaptive immune response can take days to mount a response after initial exposure to the pathogen, because T and B cells expressing the receptor specific to the invading pathogen must replicate first.

The immune system is comprised of three levels which each play vital roles in protecting the host from harm. The first level is chemical, physical, or biological barriers, which minimizes the host's exposure of invading pathogens. The innate system, which is fast acting but not specific or diverse and lacks memory. Last, the adaptive system, which is specific, highly diverse, and contains memory, but has a slow response time. Having a better understanding about the role of the immune system will allow for nutrition and management strategies to be targeted to improve calf health by impacting the innate and adaptive immune responses.

1.3 Colostrum

Colostrum is the first secretion from the mammary gland after parturition. It contains high levels of immunoglobulins and nutrients like proteins, fats, vitamins, and minerals that are essential for the growth and health of the newborn. Compositionally, bovine colostrum varies based on breed, parity, nutrition of the dam during late-gestation, and length of the non-lactating period prior to parturition (Parrish et al., 1947; Parrish et al., 1948). In general, bovine colostrum contains a greater percentage of total solids, fat, protein, minerals and vitamins, hormones, cytokines, and growth factors and a lower percentage of lactose compared to mature bovine milk

(Uruakpa et al., 2002; Godden, 2008). Due to the distinct differences between colostrum and mature bovine milk, it is important to understand the biological reasons and physiological effects of these differences. In the next sections, colostrogenesis, the composition of colostrum, and colostrum management will be discussed.

1.3.1 Colostrogenesis

Colostrogenesis is defined as the process of forming colostrum, it occurs prior to parturition, and is partially regulated by lactogenic hormones such as progesterone, estrogen, and prolactin (Barrington et al., 2001). During colostrogenesis there is a mass transfer of immunoglobulins and other nutrients from maternal circulation into mammary gland secretions (i.e. colostrum; Baumrucker and Bruckmaier, 2014). Colostrogenesis in ruminants starts 3-5 weeks before parturition and ends just prior to parturition (Brandon et al., 1971). This process is a distinct stage in mammary gland development and differs from the mammary gland's primary role, which is milk production. The stages of mammary gland development include mammogenesis, colostrogenesis or lactogenesis I, secretory activation or lactogenesis II, lactation or lactogenesis III, and involution. It is well understood what regulates each stage of mammary gland development, with the exception of colostrogenesis (Dembinski and Shiu, 1987). Colostrum formation occurs prior to secretary activation, which is the onset of copious milk production stimulated by the drop in progesterone around parturition (Deis and Delouis, 1983; Nguyen et al., 2001), and prior to the closure of tight junctions between mammary epithelial cells (Hartmann et al., 1973). These leaky tight junctions before parturition allow for the transfer of large molecules such as antibodies and soluble immune factors into the colostrum (Kessler et al., 2019).

During milk fat synthesis, fatty acids (FA) are derived from one of two sources, either from *de novo* FA synthesis from volatile fatty acid (VFA) precursors (acetate and butyrate) in the mammary epithelial cells or preformed FA from plasma, which originate from the diet or the mobilization of adipose tissue (Clegg et al., 2001). Milk lipids from circulation are derived through the hydrolysis of triacylglyceride components of very low-density lipoprotein (VLDL) and chylomicrons, catalyzed by lipoprotein lipase (LPL) in the capillary bed of the mammary gland, or from the direct uptake of plasma NEFA after the mobilization of adipose tissue as a result of low blood glucose concentrations. In ruminants, short and medium-chain saturated FA primarily come from *de novo* FA synthesis (FA $\leq$ 16 carbons in length), whereas plasma lipids mainly

contribute longer-chain or preformed FA (FA ≥ 16 carbons in length; Clegg et al., 2001). The synthesis of *de novo* FA begins at secretory activation (Anderson et al., 2007), therefore it can be assumed that FA present in colostrum will be primarily from preformed lipids, indicating that diet and metabolic status can have a major influence on colostral lipids.

Research that has aimed to identify what mechanism is behind the regulation of colostrogenesis has primarily investigated immunoglobulin inclusion in colostrum (Guy et al., 1994; Barrington et al., 2001; Baumrucker et al., 2010). However, immunoglobulins are not the only component found in colostrum that are being moved from maternal circulation into mammary gland secretions during this time period. As mentioned above, colostrum has a unique composition compared to milk, as seen by the higher concentration of immunoglobulins as well as increased fat, vitamins, mineral, cytokines, growth hormones, etc. It is clear the importance that immunoglobulins in colostrum play in neonatal health and survivability (Barrington and Parish, 2001), but it should not be the only focus when trying to discover what regulates colostrogenesis and what factors might be impacting their inclusion rates. The following sections will shed light on the various components found in colostrum and how they impact calf health and productivity.

1.3.2 Nutritional Profile

Proteins

Cattle have a synepitheliochorial placenta, indicating there are six layers of tissue that separate the fetal blood supply from the maternal, three layers from the fetal side and three from the maternal side (Senger, 2005). The six tissue layers prevent the maternal blood supply from coming into contact with the fetal blood supply, therefore maternal immunoglobulins are unable to be passed to the fetus during gestation. Calves must then rely on colostrum to receive immunoglobulins via the passive transfer of immunity, in order to protect them from pathogens until their own immune system can protect them. In the dairy industry, research is heavily focused on the concentrations of immunoglobulins present in colostrum, particularly immunoglobulin G (IgG), because it is the most prominent immunoglobulin found in bovine colostrum (Butler, 1969). However, IgG is not the only immunoglobulin type found in colostrum, IgA and IgM are also present. Immunoglobulins are distinguished by on the type of heavy chain they have, IgG have gamma-chains, IgA have alpha-chains, and IgM have mu-chains. During a primary immune

response, IgM is the first antibody that is produced. It helps to activate the complement system, which increases the ability of antibodies to remove microbes and cells that are damaged from the body, and it promotes inflammation. IgA are produced by B cells in the mucous membranes and they help to clear viruses and toxins from the body and helps prevents microorganisms from binding to receptors on host cells (Pakkanen and Aalto, 1997).

Casein, which is the main protein found in both colostrum and milk, is present in higher concentrations in colostrum with concentrations decreasing with every milking post-partum (Puppel et al., 2019). During the digestion of colostrum/milk in the preruminant calf, casein clots in the abomasum due to the enzymatic actions of rennin and pepsin. This clot slows down the flow rate out of the abomasum and allows for the steady digestion and absorption of nutrients. However, casein is not only important for the nutrition of the calf, but it has also been investigated for its impacts on the immune system. Jiehui et al. (2014) performed a study looking at the effects that a bioactive peptide derived from casein has on lymphocytes. When a foreign antigen stimulates the body, lymphocytes are activated and proliferate to destroy the pathogen. The results of this study showed that casein-derived peptide QEPVL (Gln-Glu-Pro-Val-Leu) increased the activation and proliferation of lymphocytes both *in vivo* and *in vitro*. They also found that QEPVL has the ability to inhibit inflammation induced by LPS by increasing IL-4 and IL-10 (anti-inflammatory cytokines) and reducing IFN-γ and TNF-α (pro-inflammatory cytokines), regulating the release of nitric oxide, and increasing cAMP levels.

A glycoprotein in colostrum that has an immunological role and binds iron is lactoferrin. Bacteria growth is dependent upon iron, but lactoferrin binds iron making it unavailable for use, inhibiting the growth of microbes (Pakkanen and Aalto, 1997). Lactoferrin also promotes T-cell precursors to mature into helper T-cells, which activate macrophages and cytotoxic T-cells to attack pathogens, and immature B-cells to mature into antigen presenting cells (Zimecki et al., 1991; Actor et al., 2009). In summary, proteins in colostrum are essential for both the health and growth of the newborn calf.

Lipids

Bovine colostrum contains greater concentrations of lipids compared to mature milk (Foley and Otterby, 1978). Contarini et al. (2014) evaluated the lipid components in colostrum/milk for the first five days post-partum as well as 5 months into lactation. The results showed that fat

concentration did not change during the first 5 days after parturition but was reduced 5 months into lactation. At parturition, short-chain saturated fatty acids (SCSFA) were lower and increased with time while the opposite was true for long-chain saturated fatty acids (LCSFA), which started out higher and were reduced with time. It is believed that LCSFAs are higher in colostrum due to their association with fat mobilization, which is increased in a cow around parturition (Leiber et al., 2011). Polyunsaturated fatty acids (PUFA) were slightly increased at calving and then reduced by five days post-partum.

Omega-3 and omega-6 fatty acids (FA) are involved in the regulation of inflammation. Both types of FA start out at higher concentrations in colostrum but are reduced by five days post-partum (Contarini et al., 2014). Omega-3s are anti-inflammatory, meaning they can aid in reducing chronic or excessive inflammation (Calder, 2006), while omega-6s are pro-inflammatory, meaning they can induce the secretion of pro-inflammatory cytokines to clear the antigen that is causing the inflammatory response. If omega-6 (i.e. C18:2) levels are elevated and omega-3 (i.e. C18:3) levels are low, chronic inflammation can persist (Calder, 2015). If the opposite is the case, the body might not be able to clear the pathogen from the body. Omega-6 FA activates nuclear factor kappa B (NF-κB), which is an inflammatory transcription factor (Calder, 2012). Nuclear factor kappa B increases the expression of proteins like chemokines, cytokines, and proteins that are involved in an acute phase response, which each play a role in stimulating inflammation (Kumar et al., 2004). Omega-3 FA inhibits NF-κB, which inhibits the expression of these proteins that stimulate inflammation (Calder, 2012).

Concentrations of cholesterol and phospholipids (PL), specifically, sphingomyelin (SM), in milk are higher at parturition but decreased shortly after parturition (Contarini et al., 2014). PL include phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylglycerol (PG), and SM (Contarini et al., 2014). Cholesterol and phospholipids are the primary components of cell membranes, which are barriers that protect the gastrointestinal tract of calves from bacterial infections (Sprong et al., 2001). PL are recognized by innate-like T cells, which is important for the recognition of cancer, infection, and self-antigens (Van Rhijn et al., 2015; Godfrey et al., 2015). PS is translocated in the plasma membrane of cells during apoptosis and acts as a signal that ensures phagocyte recognition and the uptake of apoptotic cells (Martin et al., 1995), which then leads to the secretion of anti-inflammatory cytokines (Voll et al., 1997). If dead cells are not recognized and removed by phagocytes they will accumulate up

and can lead to chronic inflammation or autoimmunity (Gaipl et al., 2006). Cholesterol leads to the accumulation of macrophages and the promotion of inflammatory responses through increased TLR signaling and monocyte and neutrophil production. TLR signaling leads to further accumulation of cholesterol and heightens the inflammatory response (Yvan-Charvet et al., 2008), which is beneficial when an individual has an infection, but if the inflammatory response is heightened in response to disease, it can lead to chronic inflammation (Westerterp et al., 2014). This indicates that cholesterol is vital for the immune system, but too much of it can have negative implications and lead to chronic inflammation.

Carbohydrates

Lactose concentrations are relatively low in colostrum but soon after parturition, lactose levels steadily increase until they reach levels (~5%) present in mature milk (Parrish et al., 1948; Godden, 2008). Lactose is responsible for approximately 50% of the osmotic pressure present in mature milk, which regulates the volume of milk that is produced. However, colostrum contains lower concentrations of lactose, meaning less movement of water from the mammary epithelial cells into the secretory vesicles, making colostrum more viscous compared to milk (McGrath et al., 2016). Lactose is a disaccharide composed of the monosaccharides, glucose, and galactose. Glucose is the primary fuel source for immune cells and used to generate biosynthetic precursors (Calder et al., 2007). Lactose is also a good fuel source for bacteria that compete with IgGs to be absorbed, which could reduce IgG absorption efficiency. As the calf's immune system starts to develop, lactose levels in milk increase providing a preferential fuel source for immune cells. In addition to lactose, another carbohydrate type found in colostrum are oligosaccharides, which contain 3 to 10 monosaccharides linked by glycosidic bonds. Oligosaccharides can bind bacteria, therefore reducing the competition for absorption between bacteria and IgG that are present in colostrum. This then leads to improved apparent efficiency of absorption for IgG and increased successful passive transfer of immunity (Short et al., 2016).

Vitamins and Minerals

The ash fraction of colostrum contains greater concentrations of calcium (Ca), magnesium (Mg), zinc (Zn), manganese (Mn), iron (Fe), Vitamin A, Vitamin E, riboflavin, Vitamin B12, and

folic acid compared to mature milk (Foley and Otterby, 1978). Many minerals and vitamins found in colostrum, have direct effects on the immune system, for example, various immune functions are dependent on Ca influx like the proliferation of T cells (Quintana et al., 2005), the migration of T cells and macrophages (Bach et al., 2007), and oxidative burst in macrophages (Verma et al., 2011). When Mg concentrations in blood are low it causes low-grade systemic inflammation and oxidative stress (Rayssiguier et al., 2006). Magnesium aids in reducing oxidative stress by stabilizing DNA and promoting DNA transcription and replication (Rowe, 2012). Zinc is an antioxidant that helps to protect the body from reactive oxygen species as well as reactive nitrogen species (Wintergerst et al., 2006) and it aids in maintaining the integrity of the skin and mucosal membranes (Haryanto et al., 2015). Zinc is also required for the development and activation of T lymphocytes (Wintergerst et al., 2006) and helps support helper T-cell 1 (Th1) response (Haryanto et al., 2015). Iron forms hydroxyl radicals, which are very toxic to bacteria and, therefore, aid in the process of killing bacteria (Haryanto et al., 2015).

Vitamin A is essential for the proper functioning of adaptive immune system cells such as B and T lymphocytes and it plays a role in the development of Th1 and Th2 cells (Haryanto et al., 2015). Vitamin E is an antioxidant that helps protect cell membranes from free radicals that can cause it damage (NRC, 1989) and it increases the cytotoxicity of NK cells (Haryanto et al., 2015). Riboflavin (Vitamin B2) plays a role in the viability and activity of macrophages, and it is needed for macrophages to have an immune response. Vitamin B12 is essential for NK cells to function and is involved in T lymphocyte production (Saeed et al., 2016). Folic acid supports immune responses mediated by Th1 and it is needed for antibodies to efficiently respond to antigens (Saeed et al., 2016). Vitamins and minerals, found in colostrum, play essential roles in calf health and development.

Other Components

Bovine colostrum and milk contain many growth factors including epidermal growth factor (EGF; Yagi et al., 1986), insulin-like growth factor I (IGF-I; Collier et al., 1991), insulin-like growth factor II (IGF-II; Schams, 1994), transforming growth factor- β1 (TGF-β1; Ginjala and Pakkanen, 1998), and transforming growth factor- β2 (TGF-β2; Cox and Burk, 1991). Concentrations of growth factors are greatest in colostrum for a few hours post-parturition and then decline significantly as time progresses, for example IGF-I is 10-fold greater in colostrum

compared to milk at 6 d post-partum (Collier et al., 1991; Ginjala and Pakkanen, 1998). The most prominent growth factors in colostrum are IGF-I and IGF-II (Marnila and Korohnen, 2002). IGF-I is believed to be a major regulator in the development of the gastrointestinal tract (GIT) by stimulating mucosal growth, increasing brush-border enzyme production, increasing villus size, and stimulating intestinal DNA synthesis (Baumrucker et al., 1994). Insulin-like growth factor I, therefore, helps to protect the newborn from microbes within the intestines by strengthening the epithelium of the GIT and preventing microbes from passing through. Both IGF-1 and IGF-II promote tissues repair and wound healing (Wilson, 1998).

Another growth factor involved in regulating the immune system is transforming growth factor-β (TGF-β), which is believed to be involved in regulating CD4+ T-cell responses. Mice that lack TGF-β1 die from multi-organ inflammation very early in life (Shull et al., 1992), implying an anti-inflammatory role. Mice that have multi-organ inflammation because they are lacking TGF-β1 are saved when crossed with major histocompatibility complex (MHC) II knockout mice, suggesting that TGF-β plays a role in regulating the response of CD4+ T-cells (Letterio et al., 1996). TGF-β also induces regulatory T-cells (Tregs), which are a CD4+ T-cell that suppresses immune responses that can cause self-harm to the individual (Worthington et al., 2012). Growth factors present in bovine colostrum could also have a wide range of roles and impacts in the immune system of the newborn calf just like those seen in humans and mice.

In conclusion, bovine colostrum contains higher concentrations of total solids, lipids, protein, vitamins, minerals, hormones, cytokines, and growth factors compared to mature milk. All of these components elevated in colostrum have been illustrated to have important roles in the development of the calf and its immune system. Colostrum is a nutritious and essential meal to ensure newborn calves have the ability to fight off invading pathogens that hinder calf survivability.

1.3.3 Management

During passive transfer of immunity, immunoglobulins from the ingested colostrum are absorbed in the intestine and enter the calf's circulation. However, the effectiveness of passive transfer is influenced by many management factors, including the quality, bacteria contamination (cleanliness), and quantity of the colostrum, and how soon after birth colostrum is fed to the calf.

Successful passive transfer is dependent on the quality and cleanliness of the colostrum. Colostrum is considered high quality if it contains greater than 50 g/L of IgG, and the cut off for

cleanliness is less than 100,000 cfu/ml for total plate count, and less than 10,000 cfu/ml for total coliform count (McGuirk and Collins, 2004). High quality colostrum is necessary to ensure the calf received enough IgG to protect it from pathogens and reducing bacteria in colostrum can increase the availability of absorption sites for IgG to be absorbed (Saif and Smith, 1985).

It is recommended that calves receive at least 100 g of IgG in the first feeding of colostrum (Davis and Drackley, 1998) to achieve successful passive transfer. Timing is also very important because at birth calves have what is referred to as an 'open gut', meaning large molecules such as immunoglobulins, are able to pass through the intestinal enterocytes and enter the bloodstream (Broughton and Lecce, 1970). Gut closure is not instantaneous and the ability of the intestines to absorb large molecules slowly decreases from birth until complete closure at approximately 24 hours after birth (Weaver et al., 2000). Therefore, it is important that a newborn calf receives colostrum as soon as possible to improve the chances of successful passive transfer of immunity.

The serum IgG level of dairy calves can be measured to determine if relative transfer of passive immunity was successful. Passive transfer is deemed successful if serum IgG levels are greater than 10 mg/ml (Godden, 2008). Another way to estimate if a calf received enough IgG from colostrum is to measure serum total protein, which is correlated to serum IgG concentrations. Serum total protein can be measured using a refractometer and a value greater than 5.5 g/dL is an indication that the calf absorbed greater than 10 mg/ml of IgG from colostrum (Deelen et al., 2014; Elsohaby et al., 2015). Colostrum management is essential for the passive transfer of immunity from dam to calf, however, colostrum contains many other nutrients besides immunoglobulins that each have a unique role in the health and nutrition of the neonate. The next section aims to address why the lipids present in colostrum should be further explored.

1.4 Lipidome

The following section will dive deeper into the lipid content of colostrum and why the lipidome of colostrum is worth evaluating. As mentioned previously, when discussing the nutritional profile of colostrum, the fat content of colostrum is greater than that of mature bovine milk, about 6.7% vs 4.0%, respectively (Godden, 2008). Lipids in colostrum are an important nutrient and energy source for the newborn calf. When calves are born, they have relatively low energy reserves, only 3% of their birth body weight is adipose tissue (Morrill et al., 2012). Calves therefore rely on the fat in colostrum to meet their energy needs in order to maintain body

temperature, also known as thermogenesis (Contarini et al., 2014). This is especially important when a calf is born during temperatures below their thermoneutral zone (Davis and Drackley, 1998).

Lipids play important roles in the immune system function of calves. Calves supplemented with FA, such as butyrate (C4:0), medium chain fatty acids (MCFA; C6:0 to C12:0), and linolenic acid (C18:3) via milk replacer, had reduced inflammation, scouring, and medical treatments as well as increased serum titers after vaccination (Hill et al., 2011). Milk triacylglycerols (TG) are converted to monoglycerol and FA, through lipolytic activity in the gastrointestinal tract, both of which possess antimicrobial properties (Isaacs, 2001), that aid in protecting the host against gastrointestinal pathogens and infection (Sprong et al., 2002; Yoon et al., 2018). In both human and bovine milk, medium chained saturated FA have greater antibacterial and antiviral capability compared to MG and long-chain unsaturated FA (Isaacs et al., 1995). These essential roles that colostral lipids play in thermogenesis and immune function validate further exploration when evaluating colostrum quality and importance.

1.4.1 Lipid Classes and Structure

The term lipid is used to describe organic compounds that are soluble in organic solvents but insoluble in water (Smith et al., 1997). The major lipid constituents present in bovine colostrum include TG, FA PL, and cholesterol (Contarini et al., 2014). The chemical structure of a FA is a carboxylic acid group (-COOH) attached to an aliphatic chain (Figure 1.3). FA are classified based on on factors including length, whether it is saturated (no double bonds) or unsaturated (double bonds), if it has an even or an odd number of carbons, and whether it is linear or branched. A TG contains three FA esterified to a glycerol molecule (Figure 1.4). The term phospholipid refers to both glycerophospholipids and SM. Glycerophospholipids include PS, PE, PC, PI, and PG. Glycerophospholipids are similar to TG in that they contain a glycerol backbone, however, glycerophospholipids have two FA attached to the glycerol and a phosphate group in place of the third FA (Figure 1.5). However, SM contains one FA, a sphingosine, and a phosphocholine group (Figure 1.6). Cholesterol is a type of lipid referred to as a sterol and it is a vital structural compound found in cell membranes (Figure 1.7) with a chemical formula of C27H46O (Baez, 2013).

1.4.2 Metabolism and Absorption

In ruminant rations, cattle receive lipids from a variety of sources including, forages, cereal grains, oil seeds, and fat supplements. The type of fat in these sources does vary, for example, forages contain glycolipids, cereal grains and oil seeds contain TG, and fat supplements usually contain either FA or TG (Davis, 1990). Dairy cattle receive a relatively low amount of fat in the diet compared to monogastric animals, up to 5-6% in high producing cows, because excess fat can reduce rumen fermentation due to the coating effect of fat on feed particles (Bionaz et al., 2020). The metabolism of lipids starts in the rumen (Figure 1.8) with the processes of hydrolysis and biohydrogenation (Lock et al., 2006). Hydrolysis is the process in which microbial lipases hydrolyze ester linkages. As described above, TG and glycolipids contain either 3 FA or 2 FA and a sugar attached to a glycerol backbone. Hydrolysis cleaves the FA and sugar from the glycerol backbone with an extent of hydrolysis rate of >85%. However, many factors reduce the rate and extent of hydrolysis including increased level of dietary fat, reduced rumen pH, increased passage rate, and the presence of ionophores which inhibit the growth and activity of rumen bacteria (Lock et al., 2006). The glycerol backbones and sugar after hydrolysis are fermented by rumen bacteria into VFA. These VFA can be used by microbes and turned into microbial phospholipids, which then leave the rumen.

Unsaturated FA are toxic to many rumen bacteria, therefore the process of biohydrogenation removes the double bonds present in unsaturated FA, turning the unsaturated FA into a saturated FA (Lock et al., 2006). Unsaturated FA with one double bond are referred to as monounsaturated fatty acids, while unsaturated FA with multiple double bonds are referred to as polyunsaturated fatty acids (PUFA). The same factors, mentioned above, that reduce hydrolysis also reduce biohydrogenation. The extent of biohydrogenation rate (>80%) is less than hydrolysis, indicating some unsaturated FA do make it to the small intestine and are absorbed (Boerman et al., 2015). The FA that underwent hydrolysis and biohydrogenation attach to feed particles and exit the rumen. Sometimes cattle will be fed rumen-inert fats, which are able to pass through the rumen unaltered and are designed to not be detrimental to rumen bacteria (Maia et al., 2007).

The FA then progress to the first region of the small intestine called the duodenum, which is where digesta is mixed with bile and pancreatic juice. Lipids are hydrophobic, meaning they are insoluble in water, so in order for lipids to be absorbed they need to be solubilized with the help of micellar solutions (Freeman, 1984). Bile and pancreatic juices are vital for the formation of

micelles, bile supplies bile salts and lecithin, while pancreatic secretions provide phospholipases (enzyme needed to convert lecithin to lysolecithin). Bile salts and lysolecithin remove FA from the feed particles they were attached to when leaving the rumen, aiding in the formation of micelles (Figure 1.9). After micelles are formed, lipids are transferred across the unstirred water layer of the intestinal epithelial cells in the jejunum region of the small intestine and are absorbed. After being absorbed, in the intestinal epithelial cells, FA are re-esterified into TG and packaged into chylomicrons to be transported into circulation (Lock et al., 2006).

Once in circulation, lipoprotein lipases regulate the hydrolysis of TG in the chylomicrons to FA, as well as the uptake of FA by various tissues, such as mammary tissue (Fielding and Frayn, 1998). The FA that are taken up by the mammary epithelial cells from circulation are long-chain fatty acids (≥16 carbons), which are then esterified to LC-acyl-CoA. From here, LC-acyl-CoA are synthesized back to TG in the endoplasmic reticulum (ER; Bionaz and Loor, 2008). As TG leave the ER, they are coated in a phospholipid monolayer from the ER plasma membrane forming lipid droplets (Keenan and Mather, 2009). Lipid droplets move towards the apical membrane where they push out the apical membrane of the cell, causing the membrane to surround the droplet until it is pinched off and enters the lumen of the mammary gland. This phospholipid trilayer structure with a core of TG is referred to as a milk fat globule (Keenan and Mather, 2009).

Lipid digestion in calves is not the same as it is for mature cows. When calves are first born, they have an undeveloped rumen and therefore rely on enzymatic digestion in the abomasum early in life. In young calves, liquid feed bypasses the rumen, reticulum, and omasum, flowing directly into the abomasum via the esophageal groove. The esophageal groove can be stimulated by suckling or milk proteins and is formed from muscular folds in the reticulorumen (Heinrichs and Jones, 2003). When liquid feeds enter the abomasum, it forms a clot with the help of the enzymes, rennin and pepsin. The formation of a clot or curd allows for liquid feed to be digested and absorbed, because it slows down the rate that liquid feed flows out of the abomasum (Jones and Heinrichs, 2006). Pregastric esterase, which is a complex of lipolytic enzymes in saliva, starts the digestion of fat in the abomasum (Huber et al., 1961). It starts by hydrolyzing the TG present in the milk curd to diacylglycerides (DG) and FA (Hill et al., 1970). The length of the FA will determine how it is digested and absorbed. Short and medium chain FA can be directly absorbed in the small intestine and enter the portal drain viscera to be taken up by the liver where they will be converted to an energy source for the calf. Pregastric esterase will break some short and medium

chain FA down to shorter FA prior to absorption. Pancreatic lipase is needed to break down DG into MG and FA, in the small intestine. Pancreatic lipase is also needed to break down long chain fatty acids. However, the pancreas does not produce enough lipase to meet the demands for digestion until the calf is at least two weeks old (Gooden, 1973). Bile salts are required for micelle formation in order for MG and long chain FA to be absorbed by intestinal epithelial cells. After absorption FA are converted back to TG and packaged into chylomicrons just like in functioning ruminant animals.

1.4.3 Maternal Factors

As described above, colostral lipids are primarily coming from circulating lipids in the cow and these circulating lipids include dietary lipids and mobilized adipose tissue in the form of NEFA (Clegg et al., 2001). From late gestation to early lactation, dairy cattle experience a major shift in nutrient partitioning from late gestation fetal growth to early lactation milk synthesis, which both metabolically demanding processes. Also, during this transition period, it is common for dairy cattle to reduce dry matter intake, resulting in higher energy demands than what is consumed, leading to an energy deficit. To combat this deficit, adipose tissue, which is stored energy, will be mobilized as NEFA to help the cow keep up with demands. Therefore, the NEFA concentrations in circulation is a direct reflection of the relative amount of adipose tissue mobilization (McNamara, 1991), and the metabolic energy status of the animal. Increased plasma NEFA concentrations has been shown to alter the FA composition in milk (Jorjong et al., 2014; Jorjong et al., 2015), indicating that more research is needed to determine the influence that the metabolic status of the dam has on colostral fat amount and profile. This can then provide insight into what regulates the fat content of colostrum, and the possibility of manipulating the energy status of the dam to ensure the calf is receiving enough fat via colostrum for optimal health and growth.

Circulating lipids, however, do not solely come from NEFA when adipose tissue is mobilized, they are also comprised of lipids from the diet. Research looking at milk fat synthesis shows that diet can impact the fat content of milk. For example, cows that consume a diet high in PUFA, experience milk fat depression (Bauman and Griinari, 2001). This is because unsaturated FA are toxic to some rumen microbes (Maia et al., 2007), so in the rumen they are converted to saturated FA via biohydrogenation (Jenkins, 1993). This leads to the production of specific biohydrogenation intermediates (C18:2 *trans*-10, *cis*-12) that cause milk fat synthesis to be

decreased through the reduction of gene expression of enzymes associated with milk fat synthesis in the mammary gland (Harvatine and Bauman, 2011). In other species the FA composition of milk fat reflects the FA composition of the diet. Ruminants are unique in that the FA composition of the diet is altered by the rumen microbes. However, diet can still influence the microbial population in the rumen and therefore microbial processes, which can impact the FA composition and fat content of milk (Bauman and Griinari, 2003). This research, however, is specific to milk fat not colostrum fat, meaning more research is warranted to determine how dietary changes can influence FA composition and the fat content in colostrum as well as management strategies to ensure the optimal lipids are available to the calf via colostrum.

1.5 Calf Supplementation

1.5.1 Testing Immune Status

Two common ways to evaluate the responsiveness of the immune system is via an LPS challenge or a vaccine challenge. An LPS challenge model has been used on dairy calves (Kwon et al., 2011; Plessers et al., 2015; Benjamin et al., 2016), beef calves (Burdick Sanchez et al., 2020), adult ruminants (Greco et al., 2015), swine (Burdick Sanchez et al., 2018), and *ex vivo* using calf blood (Lopes et al., 2020). A vaccine challenge model has also been used frequently in dairy calves (Hill et al., 2011; Kim et al., 2011; Hill et al., 2012; Esselburn et al., 2013). There are many differences between LPS and vaccine challenges, which will be described in detail below.

An LPS challenge model is used to evaluate the animal's ability to mount an innate immune response. As discussed previously, LPS is an endotoxin and a component of the cell wall in gram-negative bacteria (Wang and Quinn, 2010). It is the LPS in the cell wall that is detected by immune cells during the invasion of the bacteria. Immune cells express PRRs, including TLRs, NLRs, RLRs, and CLRs, which detect PAMPs and LPS is a PAMP. The presence of LPS activates an immune response because LPS is not found in mammalian cells and is therefore foreign. When performing an LPS challenge, the animal receives an injection of LPS intravenously (Benjamin et al., 2016; Burdick Sanchez et al., 2020) or intramammary (Greco et al., 2015), exposing the animal to LPS, but not to bacteria. Immune cells recognize the presences of LPS and an acute immune response is initiated (Plessers et al., 2015). The utilization of an LPS challenge model allows researchers to evaluate how specific treatments effect the innate immune response to an acute

immune stimulus. The goal of an LPS challenge is to induce moderate morbidity without causing mortality, that is why this model is more advantageous than a live pathogen challenge. Another advantage of an LPS challenge is that the immune response is usually resolved within 24 h unlike if the animal was exposed to the pathogenic bacteria. When using LPS challenge models, it is common to measure blood parameters such as cytokines, acute-phase proteins, and energy balance markers to quantify how the animal is responding to the challenge (Plessers et al., 2015; Benjamin et al., 2016; Burdick Sanchez et al., 2020). This is one research method used in calves to evaluate their immune system when exposed to different treatments.

A vaccine challenge model is used to evaluate an animal's adaptive immune response. There are two ways to execute a vaccine challenge; one way is to vaccinate calves against a virus or bacteria that they are susceptible to, for example, bovine herpesvirus 1, bovine viral diarrhea virus 1, bovine viral diarrhea virus 2, parainfluenza-3 virus, and bovine respiratory syncytial virus (Hill et al., 2011; Hill et al., 2012; Esselburn et al., 2013). The second way is to vaccinate calves with a virus, bacteria, or protein that is not specific to bovine, for example porcine Hog cholera and *Erysipelothrix insidiossa* live vaccine (Kim et al., 2011) or ovalbumin (Nonnecke et al., 2012), which is an egg white protein. The benefit of this second method is to ensure the calf will not have any acquired immunity from natural exposure prior to the vaccine challenge. Regardless of which method is used, researchers can then evaluate the effects that experimental treatments have on antibody titer production for the virus, bacteria, or protein that the calf was vaccinated with, which will provide insight into how the calf's adaptive immune system responded to the vaccination. With vaccine challenge models, it is common to not only evaluate antibody production, but to also investigate cytokine production by peripheral blood mononuclear cells (PBMCs) after stimulation with the vaccine used and phytohemagglutinin (PHA), which is a known stimulator of the immune system. An increase in antibody and cytokine production would indicate an increased response by the adaptive immune system. Vaccine and LPS challenges evaluate different levels of the immune system, but they can both be used to determine if experimental treatments influence the immune response of a calf.

1.5.2 *Saccharomyces cerevisiae* Fermentation Products

During the anaerobic fermentation of *Saccharomyces cerevisiae*, commonly referred to as baker's yeast, the products produced are referred to as SCFP. Many metabolites are produced

during this fermentation process, including antioxidants, phytosterols, short chain fatty acids, organic acids, etc. (Deters et al., 2018). Feeding an active live yeast means that the yeast ferments nutrients in the gut and produces fermentation products, this differs from SCFP where the fermentation process takes place outside the animal and the animal is only fed the products produced during fermentation. A yeast culture is produced when cereal grains are fermented with baker's yeast and then the entire medium culture is dried down and fed whereas a yeast culture includes the yeast cell wall and SCFP does not (Alugongo et al., 2017). In the dairy industry, SCFP is commonly fed to cows to improve production parameters such as milk production, fat yield, and protein yield (Poppy et al., 2012). However, the impact that SCFP has on calf health and productivity is still being explored.

Brewer et al. (2014) evaluated the effects that feeding SCFP to calves has on salmonellosis. Salmonella organisms are commonly found on farms and are one of many organisms that cause scouring in calves. They observed that SCFP calves had less scouring and increased weight gain compared to control calves. They also observed that SCFP calves had greater rumen development, as indicated by longer papillae. Lastly, SCFP calves had reduced Salmonella intestinal colonization. Other studies also observed reduced scouring when calves are fed SCFP (Alugongo et al., 2017; Harris et al., 2017). An increase in starter intake has also been reported when feeding calves SCFP (Harris et al., 2017).

When feeding SCFP to calves to evaluate its effect on rumen fermentation, microbial community, and gastrointestinal morphology, Xiao et al. (2016) observed higher butyrate concentrations as well as higher *Butyrivibrio* (bacteria that produce butyrate) and lower *Prevotella* (bacteria that produce acetate and propionate) richness in the rumen of calves fed SCFP compared to control calves. Calves fed SCFP also had increased papillae length, which could be due to the increase in *Butyrivibrio* bacteria and greater butyrate production because butyrate is the preferred energy source for rumen epithelial cells and is an indicator of rumen development. Calves fed SCFP also had reduced crypt depth in the jejunum. Greater crypt depth coincides with lower villus height-to-crypt depth ratio which can lead to the malabsorption of nutrients, causing osmotic diarrhea (Pearson et al., 1978). Therefore, SCFP fed to calves can improve fecal scores, through improved intestinal absorption of nutrients, because of increased villus height-to-crypt depth ratio.

Studies have also focused on the effects that SCFP has on BRD when fed to calves. Mahmoud et al. (2020) specifically evaluated immune function and bovine respiratory syncytial

virus (BRSV) infection, which is a common virus involved in BRD, when calves are fed SCFP. This was conducted by challenging calves with BRSV via aerosol inoculation. They determined that SCFP reduced clinical disease scores and incidence of secondary bacterial infection compared to control calves. They also found, after isolating immune cells from blood and stimulating a TLR response, that SCFP calves produced more IL-6 and TNF-α compared to control calves. Calves fed SCFP also produced less pro-inflammatory cytokines in response to toll-like receptor stimulation of bronchoalveolar lavage cells. They concluded that SCFP is affecting both the systemic and mucosal immune systems of calves, which could explain the reduced clinical illness of SCFP calves.

Burdick Sanchez et al. (2020) evaluated the acute phase immune response following a LPS challenge of crossbred weaned steer calves fed SCFP for 21 d. They collected blood at various time points throughout the challenge and gave each calf a sickness behavior score at the same time points. SCFP calves had a greater rectal temperature multiple times between 6 and 24 h post LPS dosing compared to control calves. Sickness behavior scores were reduced for SCFP calves compared to control. Also, glucose, fibrinogen, and platelets were increased and TNF-α was reduced for SCFP calves compared with control. Cortisol peaked and recovered faster in SCFP calves as well. They concluded that due to the increased response (i.e. temperature, glucose, cortisol, platelets, and fibrinogen) of SCFP calves, combined with the reduced sickness behavior scores and pro-inflammatory cytokine production, SCFP might be priming the innate immune system, which may better prepare calves to handle pathogen exposure.

The exact mechanism behind the positive health and performance observed when feeding SCFP to calves is still unclear. The research discussed above would suggest that SCFP works by improving rumen fermentation, maintaining a stable gut microbiome, and increasing rumen papillae length. The establishment of a stable and beneficial gut microbiome, coupled with the prevention of pathogenic organisms, can lead to increased propionate concentrations in the rumen and decreased incidence of subacute ruminal acidosis, minimizing the establishment of pathogens that can cause scours and disease (Krehbiel et al., 2003). Feeding SCFP to calves may improve calf performance while reducing morbidity and mortality, therefore reducing antibiotic reliance on dairy farms.

1.5.3 Medium Chain Fatty Acids

Medium chain FA are FA that have a chain length of 6 to 12 carbons. Saturated MCFA include, caproic acid (C6:0), caprylic acid (C8:0), capric (C10:0), and lauric acid (C12:0), which all consist of a carboxyl group that is hydrophilic and a carbon chain what is lipophilic. Naturally occurring sources of MCFA include coconut oil, palm oil, milk fat, and cuphea oil, which are present in the form of medium chain triacylglycerides (Zentek et al., 2011).

As discussed above, in calves, during the digestion and metabolism of fats, FA will first be cleaved from glycerol by lipase, if they are consumed as TG. Medium chain triacylglycerides are more easily cleaved into MCFA and glycerol by pancreatic lipase compared to long chain triacylglycerides (Noguchi et al., 2002). Once they are cleaved from glycerol, due to their shorter chain length, MCFA are able to bypass micelle formation and be absorbed directly by the intestinal enterocytes. MCFA are also able to move across the mitochondrial membrane into the mitochondria without the help of binding proteins, which is where beta-oxidation occurs, and fatty acids are converted to acetyl-CoA (Papamandjaris et al., 1998). Acetyl-CoA will then enter the Krebs cycle and synthesize energy. Due to these factors, MCFA are a more rapid source of energy for the calf compared to longer chain FA.

It has also been reported that MCFA have antimicrobial properties (Hristov et al., 2004; Zentek et al., 2011). As described previously, MCFA have a hydrophilic/lipophilic structure, which is similar to the hydrophilic/lipophilic balance of bacterial cell membranes (Park et al., 2018). Due to this, MCFA are able to penetrate the cell membranes of bacteria, causing a breakage and leading to cell content leakage (Yoon et al., 2018). Research also suggests that MCFA interfere with the DNA replication of bacteria by intercalating the DNA, therefore reducing the replication and growth of bacteria (Hermans and De Laet, 2014).

Feeding FA to calves with the goal of improving their health status and performance is not a new concept. Hill et al. (2011) fed calves a blend of FA (butyrate, coconut oil, and flax oil) in their milk replacer and found that the FA treatment altered inflammatory and immune responses, increased antibody production, and improved performance in calves. After being challenged with a Salmonella typhimurium bacterin toxoid, TNF-α, a pro-inflammatory cytokine, was decreased and IL-4, which is involved in stimulating B-cells and T-cells, was increased in calves fed the FA treatment compared to the control calves. After being vaccinated with Pasteurella, hyperthermia, as measured by body temperature, and hypophagia, as measured by intake were reduced and TNF-

α mRNA decreased in calves that received the FA supplement. The results also showed that scours and medical treatments were reduced in the calves that received the FA supplement, as well as increased ADG, feed intake, and FE. The same research group that conducted this study has also reported increased growth when calves are fed FA (butyrate, coconut oil, flax oil, canola oil, and fish oil) in milk replacer, calf starter, and calf grower (Hill et al., 2007; Hill et al., 2009).

Other studies evaluating the effects of FA on calf health and growth reported that, linoleic and linolenic acids (Kadkhoday et al., 2017), a combination of butyrate, MCFA, and linoleic acid (Hill et al., 2011), and butyrate (Guilloteau et al., 2009) can improve nutrient digestion, growth, and immune responses in calves. A blend of fatty acids consisting primarily of butyric, lauric, myristic, and linolenic acid improved the digestibility of organic matter, DM, NDF, and ADF, as well as promoted body weight gain and hip width change over 112 d in calves on an accelerated milk feeding program (Hill et al., 2016). Quigley et al. (2019) found that in the first 56 d of life, this blend of FA increased ADG, body weight, FE, and hip width change and decreased treatment days. In the next 56 d of life, it increased ADG and hip width change. They observed increased digestibility of DM, OM, starch, NDF, ADF, CP, and fat when calves were fed a blend of FA and increased serum bactericidal activity before weaning.

Even though the impacts that FA have on the health status and performance of calves have been investigated, most of that research has focused on a combination or blend of FA. More research is needed that focuses specifically on MCFA, and how they solely might be impacting calf health and growth. Potential mechanisms have been explored to explain why MCFA have been shown to improve calf health and performance, but more research is warranted to fully understand this mechanism.

1.6 Conclusion

Calf mortality and morbidity continue to be areas for improvement within the U.S. dairy industry. That combined with the concern about antibiotic resistant bacteria has led to the need for solutions that improve calf mortality and morbidity and reduce reliance on antibiotics. Research has focused on strategies to improve the health status and performance of dairy calves through nutrition. This includes direct nutrition, feeding nutraceuticals directly to the calf, and indirect nutrition, maternal factors that impact colostrum composition. The immunoglobulin concentration in colostrum is essential for calf health and survival, but colostral lipids also play important roles

in the immune system. The lipidome of colostrum warrants further exploration to ensure calves receive the optimal lipid profile for health and growth, and to understand what factors influence colostral lipid profiles. Supplementing calves with SCFP and MCFA, which have immune system benefits, in milk replacer and starter, during a time when calves are vulnerable to disease, can also improve calf immune response and growth. The answer to reducing calf morbidity and mortality in the dairy industry, while also reducing reliance on antibiotics, is nutrition.

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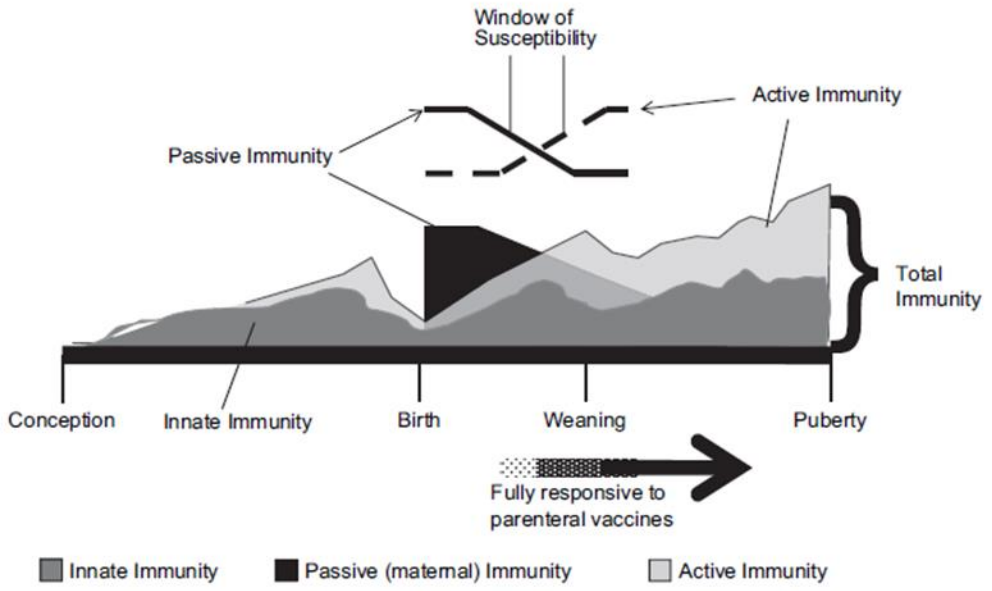
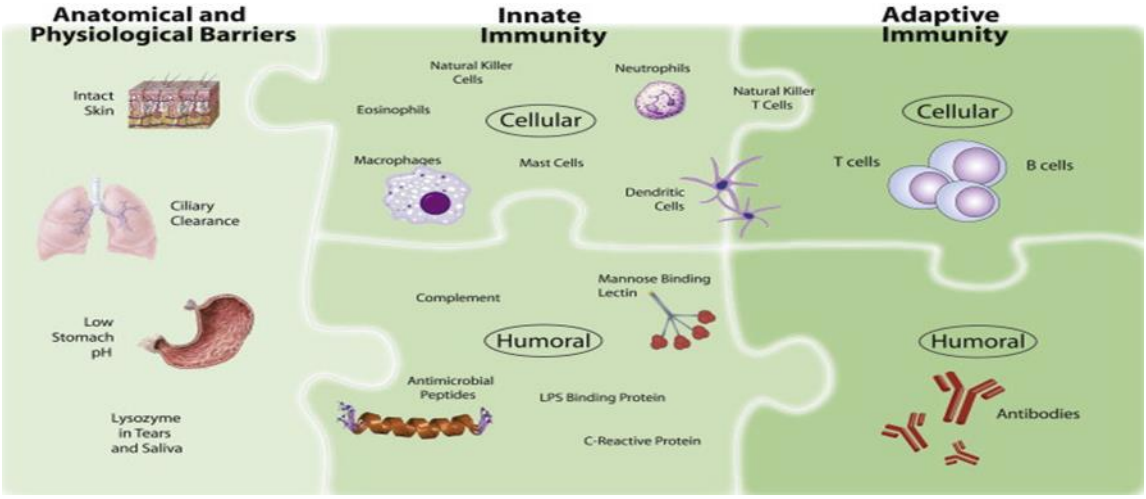


(Chase et al., 2008)

Figure 1.1. Visual representation of calf immunity from conception to puberty



(Turvey and Broide, 2010)

Figure 1.2 Three levels of the immune system.

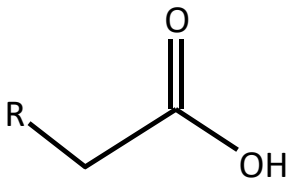


Figure 1.3 Fatty acid structure

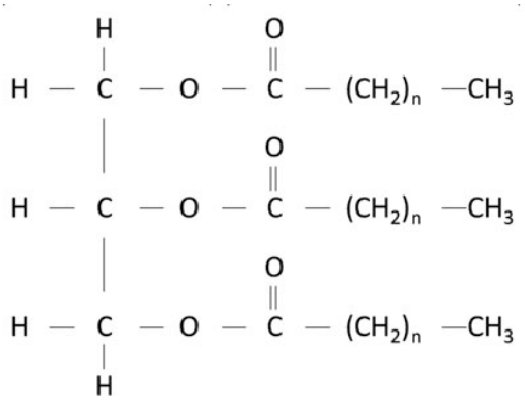


Figure 1.4 Triacylglycerol structure

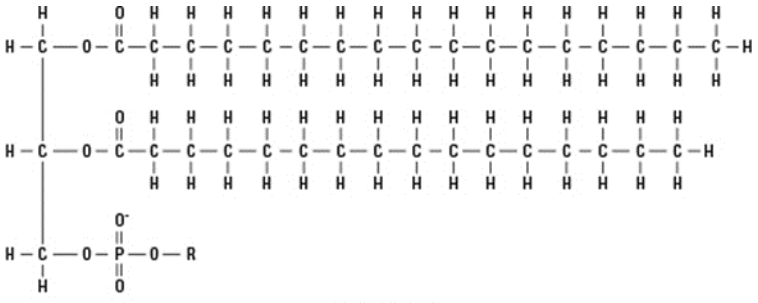


Figure 1.5 Glycerophospholipid structure

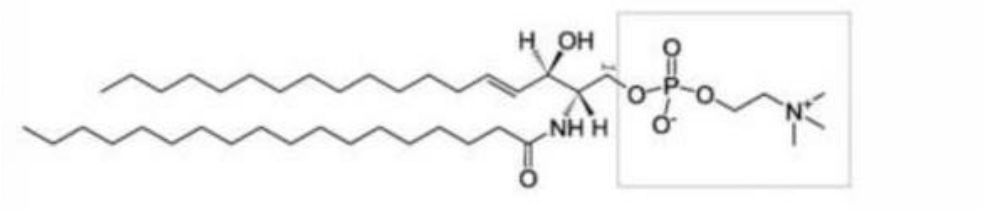


Figure 1.6 Sphingomyelin structure

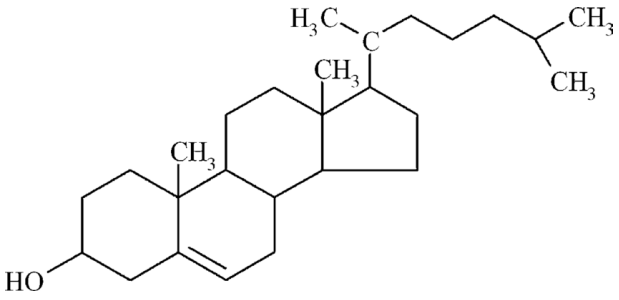
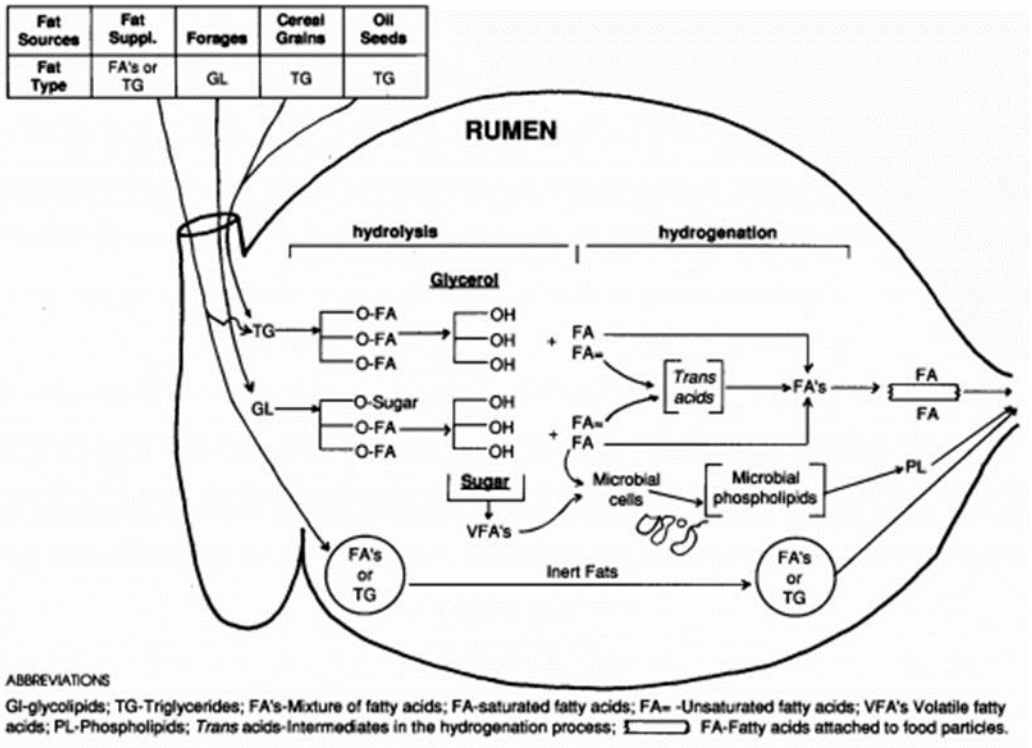
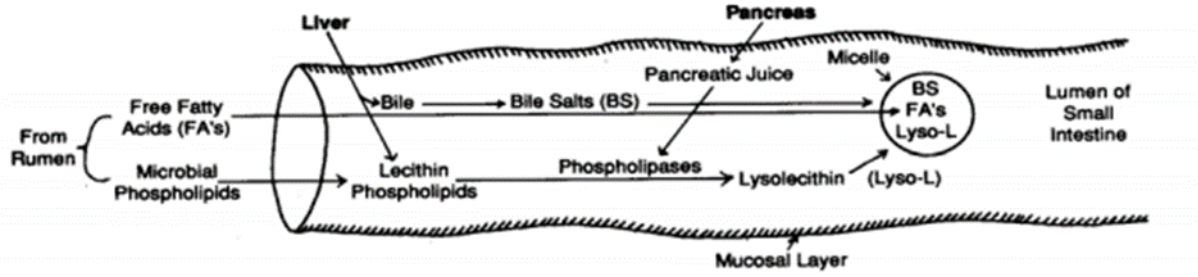


Figure 1.7 Cholesterol structure



(Lock et al., 2006)

Figure 1.8 Metabolism of lipids in the rumen



(Davis, 1990)

Figure 1.9 Formation of micelle in small intestine

CHAPTER 2. EFFECTS OF FEEDING *SACCHAROMYCES CEREVISIAE* FERMENTATION PRODUCTS ON THE HEALTH, GROWTH, AND FECAL MICROBIOME OF HOLSTEIN DAIRY CALVES THROUGH 4 MONTHS OF AGE

Klopp, R. N., Ruth Eunice Centeno-Martinez, Ilkyu Yoon, Timothy A. Johnson, Jacquelyn P. Boerman. Effects of feeding *Saccharomyces cerevisiae* fermentation products on the health and growth performance of Holstein dairy calves through four months of age. Submitted to *JDS Communications*.

2.1 Abstract

There is a necessity in the livestock industry to reduce antibiotic use, which leads to the need for alternatives to antibiotics that reduce illness and promote growth in dairy calves. The primary objective of this study was to evaluate the effect that feeding dairy calves *Saccharomyces cerevisiae* fermentation products (**SCFP**) has on average daily gain (**ADG**) and antibiotic use in dairy calves through four mo of age. The secondary objective of this study included measuring the effect of feeding calves SCFP on intake, feed efficiency (**FE**), health scores, and fecal microbiota until four mo of age. Holstein bull calves (n=60; 5 ± 3 d old) were blocked by body weight (**BW**) and serum total protein (**STP**) and assig ed to one of two treatments. The control treatment (**CON**) fed a 24% crude protein (**CP**):17% fat milk replacer (**MR**), calf starter, grower #1, and grower #2 with no SCFP added. The SCFP treatment fed a 24% CP:17% fat MR with 1 g/d of SmartCare® (Diamond V, Cedar Rapids, IA), calf starter with 0.8% NutriTek® (Diamond V, Cedar Rapids, IA), grower #1 with 0.44% NutriTek® from d 57- 84, and grower #2 with 0.275% NutriTek® from d 85-112. Calves were offered 2.84 L (12.5% solids) of MR twice daily (0630 and 1630 h) through d 51 of the study, from d 52-56 calves were fed MR once daily (0630 h) and weaned on d 57. From d 1-56, calves received *ad libitum* access to a texturized calf starter and water. On d 57, calves were switched to grower #1 and on d 84, calves were switched to grower #2, which contained lower CP and higher NDF. Individual calf BW, body condition score (**BCS**), hip height (**HH**), and hip width (**HW**) were measured biweekly from d 0-112. Feed intake was recorded daily and FE (gain/feed) and ADG were calculated. Daily fecal and respiratory scores were recorded for each calf through d 56 and all medical treatments were recorded for the duration of the study and grouped based on illness. Fecal samples were collected on d 0, 28, 56, 84, and 112 to characterize

microbiome composition. Data were analyzed as a completely randomized block design with repeated measures when applicable. No treatment effect was observed for BW, BCS, HH, or HW at d 0, d 56, or d 112 (all $P \geq 0.11$). A treatment tendency was observed for post-weaning ADG ($P = 0.07$) with SCFP calves tending to have greater ADG compared to CON calves. Calves receiving SCFP had improved FE compared to CON post-weaning ($P = 0.02$). A treatment effect was observed for respiratory illness ($P = 0.001$), with SCFP calves being treated less frequently than CON (0.31 vs. 0.98 treatments/calf). Calf fecal microbiome was affected by calf age ($P < 0.001$), SCFP tended to increase the evenness of the fecal microbiome ($P = 0.06$), and beta-diversity tended to be different between treatments at d 84 ($P = 0.07$) and was significantly different between treatments at d 112 ($P = 0.03$). This study suggests that feeding SCFP to calves could improve ADG and FE post-weaning as well as reduce the incidence of respiratory disease through 4 months of life.

2.2 Introduction

A goal of calf feeding systems is to provide calves with optimum nutrition to promote growth, health, and future milk production. Prior to weaning, calves in the U.S. have a high risk of morbidity. According to nationwide survey studies, conducted on U.S. dairy farms raising dairy heifers, digestive problems affect 20-25.3% of pre-weaned calves, while pneumonia affects 5.3-18.1%, (NAHMS, 2012; Walker et al., 2012). Based on the survey studies, of the pre-weaned calves affected by digestive illnesses, 71.8-83% were treated with antibiotics, and 90.2-100% of calves affected by pneumonia were treated with antibiotics (NAHMS, 2012; Walker et al., 2012). Post-weaning, respiratory disease affects 11.2% of heifers, where 82.1% of those affected receive antibiotics (NAHMS, 2012). Calf morbidity shifts energy from growth to the immune system, leading to reducing performance, it also increases the risk of calf mortality, both of which have a negative economic impact on producers (Stanton et al., 2012; Windeyer et al. 2014). Reducing antibiotic treatments, the incidence of illness, and improving growth in calves prior to weaning positively impacts first lactation milk production (Heinrichs and Heinrichs, 2011; Stanton et al., 2012; Gelsinger et al., 2016).

In the livestock industry, antibiotic use has more recently come under scrutiny because of the increasing prevalence of antibiotic resistant bacteria in both the animal and human medical fields (Langford et al., 2003; Loo et al., 2019). Due to the growing concern with antibiotic resistant

bacteria, the United States Food and Drug Administration (**FDA**) revised the veterinary feed directive (**VFD**) as a part of the Animal Drug Availability Act of 1996. Effective January 1, 2017, medically important antimicrobials included in feedstuffs were required to be authorized by a licensed veterinarian. Before the VFD, antibiotics were commonly included in feedstuffs as a preventative or prophylactic measure for respiratory disease and digestive issues. This has led to the need for alternatives to antibiotics that are able to improve calf health and productivity, therefore, reducing reliance on antibiotics.

NutriTek® (Diamond V, Cedar Rapids, IA) and SmartCare® (Diamond V, Cedar Rapids, IA) are natural products produced from anaerobic fermentation of *Saccharomyces cerevisiae*. Therefore, it is defined as *Saccharomyces cerevisiae* fermentation product (**SCFP**) as opposed to live yeast or yeast cell wall products. The SCFP contains combination of compounds (e.g., antioxidants, phytosterols, short chain fatty acids, organic acids, etc.) that work synergistically and naturally with the biology of the animal to provide health and production benefits. SmartCare® is designed to be supplemented in pre-weaning liquid calf diets to promote gut health and productivity and NutriTek® is used in all dairy animal rations to support health and immunity.

Research evaluating the effects of SCFP on calf health and growth would suggest that SCFP decreases the incidence of diarrhea (Brewer et al., 2014; Alugongo et al., 2017), mortality (Magalhães et al., 2008), and increases dry matter intake (**DMI**) and BW gain (Cole et al., 1992; Lesmeister et al., 2004). Feeding SCFP to calves improves rumen fermentation and helps to maintain a stable gut microbiome, which can be measured by VFA concentration, blood glucose concentrations, ruminal pH (Quigley et al., 1992), and papillae length (Brewer et al., 2014). The establishment of a beneficial gut microbiota, coupled with the reduction of pathogenic organisms can lead to increased propionate concentrations in the rumen and decreased incidence of subacute ruminal acidosis (**SARA**). Reducing SARA can minimize the establishment of pathogens that can cause scours and disease, (Krehbiel et al., 2003). The beneficial health effects that SCFP has demonstrated are more pronounced in calves that are stressed. For example, during reduced health status, heat stress, or weaning (Lesmeister et al., 2004; Brewer et al., 2014). Jensen et al. (2008) determined that SCFP can also benefit the immune system by activating the antioxidant system, in addition to improving rumen fermentation and maintaining a healthy balanced gut microbiome. Therefore, if feeding SCFP to calves improves calf growth and efficiency, while reducing morbidity and mortality, antibiotic use on dairy farms could be reduced. Reduced antibiotic use

would be an economic benefit to the producer. The long-term health and performance of calves may also be improved if there is less lung damage due to a reduction in respiratory infections.

The primary objective of this study is to evaluate the effects that SCFP, when supplemented in MR, calf starter, and calf grower, has on ADG and antibiotic use in dairy calves through four mo of age. The secondary objective of this study included the effects of SCFP on intake, feed efficiency, health scores, and fecal microbiota until four mo of age in dairy calves. The hypothesis for this study was that supplementing calves with SCFP would alter their gut microbiota, which was evaluated by analyzing fecal microbiome, leading to improved growth, feed efficiency, and reduced antibiotic treatment incidences.

2.3 Materials and Methods

2.3.1 Animals and Facility

All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee at Purdue University (Protocol #1808001783). Sixty Holstein bull calves, 5 ± 3 d of age (mean; ± SD), were received in two separate batches (n=30 calves per batch; 15 calves per treatment per batch) on 5/24/19 and 9/13/19 from a dairy farm 55 km from the Purdue University Animal Sciences Research and Education (**ASREC**) dairy farm. Calves were transported by a trailer (2.3 x 7.3 m; Wilson, Sioux City, IA) to ASREC, where they were placed in individual hutches (212 cm L x 114 cm W x 122 cm H; Calf-Tel, Germantown, WI) with a fenced-in, outside area (3.5 x 1.2 m). Individual hutches were bedded with wood shavings and re-bedded as needed. On d 59 of the study, calves were moved from individual hutches to group hutches (208 cm L x 259 cm W x 180 cm H; Calf-Tel, Germantown, WI) with a fenced-in outside area (5.2 x 2.6 m). Group hutches were bedded with wood shavings and straw, as needed, depending on the temperature and moisture (6 total group hutches, 4-5 calves each).

2.3.2 Treatments and Measurements

Prior to arrival (d -1), at the farm of origin, a 10 mL blood sample taken from each calf via the jugular vein. The blood sample was allowed to clot for 2 h and then centrifuged at 3,100 x g for 20 min to evaluate serum total protein (**STP**) for passive transfer of immunity using a refractometer (LW Scientific, Lawrenceville, GA). The same blood sample collected prior to

arrival was also used to measure packed-cell volume (**PCV**), which is an indicator of hydration status. Blood was transferred to a heparinized capillary tube, centrifuged at 12,300 x g for 5 min, and then a digital caliper was used to measure red blood cell (**RBC**) amount and total cell amount (PCV = RBC/total).

Calves were assigned to one of two treatments (n=60, 30 calves per treatment) upon arrival at the Purdue Dairy Farm. Calves on the control treatment (**CON**) were fed a 24% CP:17% fat milk replacer (**MR**), calf starter, grower #1, and grower #2 with no SCFP added. Calves on the SCFP treatment were fed 24% CP:17% fat MR with 1 g/d of SmartCare® (Diamond V, Cedar Rapids, IA), calf starter with 0.8% NutriTek® (Diamond V, Cedar Rapids, IA), grower #1 with 0.44% NutriTek® from d 57- 84, and grower #2 with 0.275% NutriTek® from d 85-112. Calves were received in two separate batches; each batch was blocked (3 blocks) by BW and STP. The average initial BW and STP for batch 1 calves was 45.2 ± 4.7 kg and 6.22 ± 0.55 mg/dL, respectively. While the average initial BW and STP was 44.8 ± 4.2 kg and 5.78 ± 0.74 mg/dL, respectively, for batch 2 calves. Calves from each batch (n= 30) were blocked into low BW (block 1, n=10), intermediate BW (block 2, n=10), and high BW (block 3, n=10) and then randomly assigned to treatment (CON vs. SCFP) within each block (Figure 2.1).

Calves were offered 2.84 L (12.5% solids) of MR twice daily (0630 and 1630 h) through d 51 of the study, d 52-56 calves were fed MR once daily (0630 h) and weaned on d 57. Refusals were recorded daily and calves with more than a 0.95 L refusal of MR were fed the remainder of the milk using an esophageal tube feeder (Nasco, Fort Atkinson, WI). This was to ensure calves were receiving enough nutrients, energy, and hydration to fight off infections and for growth. All calves received the same MR powder (Table 2.1). The commercial MR which was labeled to have at least 24% CP and 17% fat, contained 25.24% CP and 17.81% fat. For the SCFP calves, 15 g of SmartCare® was added to 150 mL of MR, mixed thoroughly, and each SCFP bottle received 10 mL of the mixed SmartCare solution.

From d 1-56, calves received *ad libitum* access to a texturized calf starter and water. The nutrient composition of the calf starters for CON and SCFP calves are shown in Table 2.1. On average, the CON starter contained 23.7% CP and 3.6% fat, while the SCFP starter contained 21.7% CP and 3.4% fat. Individual starter intake was recorded daily. Starting on d 57, calves were switched to calf grower #1 (CON, no SCFP added; SCFP, 0.44% NutriTek® added), the nutrient analysis is described in Table 2.1. The CON grower #1 contained 20.5% CP and 3.7% fat, while

the SCFP grower #1 contained 20.5% CP and 3.9% fat. On d 85, calves were switched to calf grower #2 (CON, no SCFP added; SCFP, 0.275% NutriTek® added; Table 2.1). The CON grower #2 contained 18.3% CP and 4.1% fat, while the SCFP grower #2 contained 18.4% CP and 3.8% fat. During the post-weaning period (d 57-112), calves received two different growers (grower #1 from d 57-84 and grower #2 from d 85-112) in order to meet the nutrient requirements of the animals as they grew and developed. Compared to grower #1, grower #2 contained lower protein and higher fiber. Individual calf grower intake was recorded on d 57-58 while calves remained in individual hutches, and group grower intake was recorded from d 59 until the end of the study on d 112.

Every other week a sample of each feedstuff being fed was collected and frozen at -20°C until analysis. Feed was composited and analysis was performed by Cumberland Valley Analytical Services (Waynesboro, PA). Feeds were analyzed according to AOAC International (2000) for DM (930.15), ash (942.05), CP (990.03), fat (954.02 for MR and 2003.05 for calf starters and growers), ADF (973.18), and NDF (Van Soest et al., 1991).

Individual calf BW (Tru-Test, Mineral Wells, TX; accuracy ± 1%), BCS, hip height (**HH**), and hip width (**HW**) were measured biweekly on d 0 (arrival), 14, 28, 42, 56, 70, 84, 98, 112. Lung consolidation was evaluated on d 0, 28, 56, and 112 for each calf using a portable ultrasonography machine (IBEX PRO, Loveland, CO) and a linear transducer (L6.2, Loveland, CO; Rabeling et al., 1998; Jung and Bostedt, 2004). Each lung was scored on a scale of 1-4 with a score of 1 indicating a normal lung with no consolidation (firming of the lungs) or comet-tail artifacts (pleural roughening), a score of 2 indicates the presence of comet-tail artifacts without consolidation, a score of 3 indicates consolidation affecting just 1 lobe, and a score of 4 indicates consolidation affecting 2 or more lobes (modified from (Ollivett and Buczinski, 2016). Feed efficiency (**FE**; gain/feed) was calculated pre-weaning by taking the BW change from d 0 to d 56 and dividing it by the total intake from d 0 to d 56 of each calf. Post-weaning FE was calculated by taking the taking the BW change from d 56 to d 112 divided by the total intake from d 56 to d 112 of each pen.

Daily fecal and respiratory scores were recorded for each calf through d 56. Fecal scores were measured on a scale of 1-5, with 1 being firm/solid and 5 being white/clear liquid modified from Kertz and Chester-Jones (2004). Overall respiratory status was evaluated on a scale of 0-3, with 0 being no sign of respiratory illness and 3 being multiple signs of respiratory illness including

coughing, labored breathing, fever, drooping ears, eye discharge, and/or nasal discharge, modified from the Wisconsin calf respiratory scoring chart (McGuirk and Peek, 2014). Medical treatments were also recorded for each calf throughout the entire study (d 0-112) and grouped based on illness (fecal, respiratory, and other).

Fecal samples were collected from each calf on d 0, 28, 56, 84, and 112 via rectal stimulation and stored at -20°C until processing. Samples were then thawed, and 0.25 g of each sample was weighed for DNA extraction. The DNA from the calf fecal samples was extracted using the MagAttract PowerMicrobiome DNA/RNA EP Kit following the manufacturer's protocol, which consists of a magnetic bead-based nucleic acid isolation (Qiagen, Germantown, MD, USA). The DNA was purified further using the ZR-96 genomic DNA Clean & Concentrator – 5 kit (ZymoResearch, CA, USA). The purified DNA quality was measured using Nanodrop 2000/2000c Spectrophotometer and the concentration was measured using the Quanti-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

The 16S rRNA gene amplicon library was constructed using a barcode indexed amplification product from the V4 region of the 16S rRNA gene (as described by (Kozich et al., 2013). The PCR amplification was performed with AccuPrime™ Pfx SuperMix (Thermo Fisher Scientific, Waltham, MA, USA) following Kozich et al. (2013) protocol. PCR-grade water was used as negative control and a mock community (20 Strain Even Mix 138 Genomic Material; ATCC® MSA-1002™) as positive control followed by PCR quality check via gel electrophoresis. Amplified DNA was normalized using SequalPrep Normalization Kit and pooled into a single library. For the pool, 5 µL of the fecal amplified DNA was mixed at equal molecule ratios and sequenced via Illumina MiSeq Sequencer (2x250 paired-end) at the Purdue Genomic Core Facility. Raw sequences were deposited in the National Center for Biotechnology Information (NCBI) sequence read archive (SRA) database under Bioproject PRJNA699317, BioSamples SAMN17773447 - SAMN17773722 (Supplementary Table 2.1).

The raw sequencing data were analyzed using the Quantitative Insight Into Microbial Ecology (**QIIME2**) v.2020.2. The QIIME2 generates a demultiplex file with the single-end sequences and identifies how many sequences were obtained per sample. In the study, using the DADA2 (Callahan et al., 2016), the forward sequences were trimmed at 12 and 251, and the reverse sequences were trimmed at 12 and 233, maintaining a quality score >30%. A total of 30,252,835 sequences were identified before the denoising step (DADA2), and after denoising,

24,394,047 sequences remained in the analysis. All the sequences were clustered into Operational Taxonomic Units (**ASV**) with 100% similarity.

Next, alpha-diversity was estimated in QIIME2 by calculating Observed ASV as an indicator of richness and Pielou index (Pielou, 1966; DeSantis et al., 2006) as a measure of evenness. Alpha-diversity estimates the richness, phylogenetic diversity, and evenness of the microbiome within the samples (Whittaker, 1960). Richness is an indicator of how many different species are present in the sample whereas, evenness is an indicator of the abundance distribution of species within each sample. If the evenness value is close to 1, it means that all species found in the sample have the same relative abundance, but if the value is close to 0, the microbiota of the sample is dominated by a few members. To generate fecal alpha-diversity, the ASV table was rarefied to 19,681 sequences per sample.

The beta-diversity which measures the similarity or dissimilarity of microbial community structure or distance between two groups was determined using the Weighted UniFrac (Lozupone and Knight, 2005) method and plotted as principal coordinate analysis (PCoA) using RStudio (v1.3.1093). The taxonomy was assigned using Greengenes 13_8, 515F/806 region database.

A subset of calves from batch 2 (10 calves per treatment), were enrolled in a lipopolysaccharide challenge, approximately one week prior to weaning. Lipopolysaccharide (LPS) challenge data will be presented in a separate manuscript.

2.3.3 Treatment Protocol

Calves were treated for respiratory illness after they showed two or more physical symptoms, including eye or nasal discharge, rapid or raspy breathing, droopy ears, coughing, fever, and/or refused MR. Calves were treated with Nuflor® (Merck Animal Health, Kenilworth, NJ) or Draxxin® (Zoetis US, Parsippany, NJ) and if the issue persisted after three days they were treated with the other antibiotic drug not used initially. The first respiratory antibiotic used to treat calves was based on success with prior cases and consultation with a veterinarian. Calves were treated for diarrhea with sulfamethoxazole after given a fecal score of ≥ 4. Calves with diarrhea and dehydration also received electrolytes as needed, for example, electrolytes were administered if a calf was dehydrated or had a fecal score ≥ 4 for multiple days after receiving sulfamethoxazole. Calves receiving electrolytes were offered 1.89 L at 1200 h, if they refused electrolytes an esophageal tube feeder was used to administer the remainder of electrolytes. Other medications

included broad-spectrum antibiotics like Baytril® (Bayer, Whippany, NJ) and Polyflex (Boehringer Ingelheim, Ridgefield, CT) and anti-inflammatory agent Dexamethasone (Vet One, Boise, ID). These were administered after veterinary recommendation when the exact illness was unknown or the calf had an illness not classified as fecal or respiratory-related, i.e. a joint infection.

2.3.4 Statistical Analyses

A power analysis was performed to calculate the sample size for the primary outcome variable, ADG. Based on data from Harris et al. (2017), with 95% confidence, and 80% power, 19 animals per treatment group were needed to detect differences. To account for potential calf mortality during the trial, 30 animals per treatment group were enrolled. Data were analyzed as a completely randomized block design using the Mixed Procedure and GLM Procedure of SAS v.9.4 with repeated measures, when applicable, using a first order autoregressive structure AR(1) based on minimizing AIC and BIC. Growth and performance measurements, including BW, BCS, HH, HW, ADG, intake, and FE were analyzed for normality using the Shapiro-Wilk test and data were normally distributed ($W > 0.85$). The fixed effects included treatment (T_i; CON and SCFP), timepoint (P_j; d 0, 14, 28, 42, 56, 70, 84, 98, and 112), batch (Ba_k; 1 or 2 of 30 calves each), block (Bl_l; 1, 2, or 3 within each batch based on initial BW and STP), and the interactions between treatment and timepoint ($TP_{(ij)}$), batch ($TBa_{(ik)}$), and block ($TBl_{(il)}$). The random effect of calf nested within treatment ($C_{m(i)}$) was also included in the model. The model with repeated measures was represented as:

$$Y_{ijklm} = \mu + T_i + P_j + Ba_k + Bl_l + TP_{(ij)} + TBa_{(ik)} + TBl_{(il)} + C_{m(i)} + e_{ijklm},$$

where Y_{ijklm} is the response variable, μ is the overall mean, and e_{ijklm} is the error. Continuous response variables with repeated measures included BW, BCS, HH, HW, ADG, intake, and fecal alpha-diversity. Categorical response variable with repeated measures included lung scores. The model without repeated measures was represented as:

$$Y_{iklm} = \mu + T_i + Ba_k + Bl_l + TBa_{(ik)} + TBl_{(il)} + C_{m(i)} + e_{iklm}.$$

Continuous response variables without repeated measures included STP, intake, and FE, and categorical response variables included antibiotic use, fecal scores, and respiratory scores.

Performance data including pre-weaning and post-weaning BW, BCS, HH, HW, ADG and pre-weaning intake and FE were analyzed with calf as the experimental unit (n=60). Post-weaning intake and FE were calculated by pen (n=12). Health data including lung scores, antibiotic incidences, and fecal and respiratory scores were analyzed with calf as the experimental unit. All calf fecal alpha-diversity data was analyzed with calf as the experimental unit. A P-value ≤ 0.05 was determined to be significant and a P-value > 0.05 and < 0.10 was determined to be a tendency. A Kaplan-Meier survival analysis and an odds ratio test were performed on respiratory treatment data to determine when calves had the greatest risk of respiratory treatment and if study treatment impacted the risk for respiratory treatment.

The calf fecal beta-diversity was analyzed with a Permutational Multivariate Analysis of Variance Test (PERMANOVA; $P \leq 0.05$) with 999 permutations, using the function 'adonis' from the vegan package (Oksanen et al., 2019). For the analysis of the treatments (SCFP and CON), batch (1 and 2), block (1, 2, and 3) and its interactions, a PERMANOVA test was performed separately for each day (28 d, 56 d, 84 d, and 112 d). The authors chose to run the analysis separately for each day because time has a large effect on the microbiota composition. For time effect, a PERMANOVA test was performed with the calf ID specified as the blocking factor using the argument "strata". The effect size (R^2) for each of the factors was calculated using the 'adonis' function. Pairwise comparison analysis ($P \leq 0.05$) of the different timepoints was evaluated with the function pairwise.adonis (Martinez and Monteux, 2017). Additionally, a dispersion test was performed to calculate the distance of each sample to the centroid of the factors. The dispersion was calculated using the function betadisper from the vegan package, followed by a permutation test of multivariate homogeneity of group dispersion using the function permutest from the same vegan package (Oksanen et al., 2019). In order to identify differentially abundant taxa, the DESeq statistical test in RStudio was utilized (Anders and Huber, 2010). All statistical analysis of the calf fecal microbiome involving was completed in R (v1.2.1335) and codes are available at https://github.com/EuniceCenteno/CalfFecalMicrobiome.

2.4 Results

2.4.1 Growth and ADG

Table 2.2 presents calf STP, initial (d 0), weaning (d 56), and final (d 112) body measurements and pre- and post-weaning ADG of the calves throughout the study. As mentioned above, some calves were enrolled in an LPS challenge, prior to weaning, in this study. Post-weaning growth data was analyzed with LPS calves removed; however, minimal growth differences were observed so LPS claves were included in the analysis. No treatment effect was observed for BW, BCS, HH, or HW at d 0, 56, or 112 (all $P \geq 0.11$). A treatment tendency was observed for post-weaning ADG ($P = 0.07$) with SCFP calves having greater ADG compared to CON calves (1.21 vs 1.13 kg/d, respectively). There was no treatment effect for ADG prior to weaning ($P = 0.95$).

Various batch and block effects were observed for STP, BW, BCS, HH, and HW (Table 2.2). A treatment by batch interaction was observed for HW on d 56 ($P = 0.04$), with SCFP calves having increased HW in batch 2, but CON calves having increased HW in batch 1. A treatment by batch tendency was observed for HH on d 112 ($P = 0.09$), with SCFP calves having increased HH in batch 2, but CON calves having increased HH in batch 1. A treatment by block tendency was seen for BCS on d 56 ($P = 0.08$), with SCFP calves having greater BCS in block 1, but CON calves had greater BCS in blocks 2 and 3. Figure 2.2 shows the biweekly change in BW for the duration of the trial (d 0 to 112), while Figure 2.3 shows the biweekly ADG from d 0 to 112.

2.4.2 Intake and Feed Efficiency

Table 2.3 presents individual calf intake and FE before weaning (d 0-56) as well as group pen intake and FE post-weaning (d 57-112). The SCFP calves had improved FE post-weaning compared to CON ($P = 0.02$). Even though this difference was significant, numerically it was minute. A batch effect was observed for biweekly starter intake, total starter intake, total individual intake, and FE before and after weaning ($P \leq 0.005$). Batch 2 had greater biweekly starter intake, total starter intake, total individual intake, and improved FE after weaning compared to batch 1, which had improved FE before weaning. A block effect was observed for FE after weaning ($P = 0.003$), where block 2 was more efficient compared to block 1 and 3. A treatment by batch interaction tendency was observed for group intake ($P = 0.07$) and a treatment by block interaction

was observed for FE from d 57 to 112 (P = 0.04). Group intake tended to be greater for CON calves in batch 1 but for SCFP calves in batch 2 and FE was greater for SCFP calves in block 1 and 3 but for CON calves in block 2.

2.4.3 Health

Medication administration based on illness, average daily fecal and respiratory scores, and lung scores (right and left) are reported in Table 2.4. A treatment effect was observed for respiratory illness (P = 0.001), with SCFP calves being treated less frequently than CON. A batch effect was seen for the right and left lung as well as for respiratory score ($P \leq 0.01$), all were increased in batch 1 compared to batch 2. A block effect was observed for respiratory treatments (P = 0.05), with block 1 calves having the most incidence of respiratory treatments. A treatment by batch interaction tendency was seen for fecal score (P = 0.09). Due to the significant treatment effect seen for respiratory illness, a risk analysis test was run to determine when calves were the least healthy and had the greatest risk of respiratory treatment (Figure 2.4). As health status probability decreases, respiratory treatment increases. An odds ratio test was also run, which showed that SCFP calves were more likely to be healthy or not be treated for a respiratory illness (11 fewer calves treated in SCFP treatment; P = 0.006). Of the 60 calves enrolled in this study, 52 made it through the entire study (13% mortality rate). Of the eight calves that died during the study, four were from the CON treatment and four were from the SCFP treatment. Three calves from batch 1 and five from batch 2 did not complete the study. All three calves from batch 1 died prior to weaning. Two calves from batch 2 died prior to weaning, one died during the LPS challenge, and 2 died post-weaning. In regard to morbidity, 42% of calves were treated for scours/fecal concerns and 42% were treated for respiratory disease. Of all the treatments given for the duration of this study, 69% were administered during the first four wks, 12% during the second four wks, and 19% were administered post-weaning.

2.4.4 Fecal Microbiome

A total of 6,212 ASV were observed in the study. Nevertheless, the microbial richness predicted by the Observed ASV index was not affected by diet treatment (P = 0.17). On the contrary, Observed ASV (P < 0.0001; Figure 2.5A) increased with time, with every day (0, 28, 56,

84, and 112 d) being different from each other. Additionally, batch had an effect on the Observed ASV number (P = 0.001), where batch 2 was greater compared to batch 1. The microbial community evenness measured with Pielou Index (Figure 2.5B) determined that there was an increase over time (P < 0.0001), that batch affected (P = 0.04; batch 2 > batch 1) the microbial evenness, and treatment tended to affect the microbial evenness (P = 0.06), which tended to be greater in SCFP calves compared to CON. No effects were seen for block and there were no interactions for richness or evenness (all P > 0.19).

Beta-diversity of the calf fecal microbial community was not affected by treatment at the timepoints 28, 56, and 84 d, but on 112 d the community composition was affected (P = 0.03). The change in the average group microbiome composition due to diet treatment on day 112 was a minor effect, explaining only about 3% of the variability (R^2) in the data. Batch affected the calf fecal microbial community structure (weighted UniFrac) at the timepoints 28, 56, 84, and 112 d (all P = 0.001). The dispersion of the samples according to batch was different on 56 and 112 d (all P < 0.006). The block effect was significant on day 84 and 112 (both P < 0.01). Time affected the calf fecal community structure (weighted UniFrac; P = 0.001; Figure 2.6), with every day being significantly different from each other (all P = 0.001). In addition, dispersion among samples of the same treatment group was greater in the early timepoints compared to later timepoints (Figure 2.7). This indicated that the composition was changed at different timepoints, and there was less variability between animals at later timepoints.

The calf fecal bacterial community was mainly composed of (without separating them into treatment, time, batch, or block) *Firmicutes* phyla (41.50%), followed by the phyla *Bacteroidetes* (37.39 %) and *Actinobacteria* (7.47%). At a family taxonomical level, the calf fecal microbiota was mainly composed of *Ruminococcaceae* (15.29%), *Prevotellaceae* (15.04%), *Lachnospiraceae* (11.44%), and *Bacteroidaceae* (10.17%). At the genus taxonomical level, the most abundant genera in the calf fecal microbiota were *Prevotella* (18.13%), *Bacteroides* (10.17%), and unclassified *Ruminococcaceae* (8.86%). Supplementary Figure 2.1 shows the relative abundance of the top 15 phyla found in the calf fecal microbiome, separated by timepoint and treatment. On d 0, the three most abundant phyla are *Actinobacteria, Firmicutes*, and *Proteobacteria*. With time, *Actinobacteria* and *Proteobacteria* were reduced, while *Bacteroidetes* increased. After d 0, *Bacteroidetes* and *Firmicutes* became the most abundant phylum. Treatment did not appear to have

a significant influence on phylum. In addition, there were no clear patterns to explain differentially abundant ASV (DESeq) due to treatment effect, and those results are not reported here.

As stated above, during this trial a subset of calves experienced an LPS challenge (details in separate manuscript), and its impact on fecal microbiome was evaluated. Fecal alpha-diversity was not different between calves that were enrolled in the LPS challenge and those that were not (Supplementary Figure 2.2) based on Observed ASV as an indicator of richness ($P = 0.30$) and Pielou index as a measure of evenness ($P = 0.29$). Fecal beta-diversity was also measured between calves that were challenged and those that were not (Supplementary Figure 2.3) and the LPS challenge did altered beta-diversity ($P = 0.002$).

2.5 Discussion

Improving calf growth and efficiency leads to increased productivity and profitability. In this study, there were no treatment differences for weaning (d 56) BW, final (d 112) BW, or preweaning ADG (0-56 d). This is similar to another trial, which did not observe treatment effects on BW or ADG prior to weaning (Alugongo et al., 2017). That study fed calves SmartCare® in MR at the same rate as the current study, but instead of feeding NutriTek®, like the current study, they fed Original XPC® (Diamond V, Cedar Rapids, IA) in the calf starter. Other studies, however, reported that SCFP increased the growth percentage of calves challenged with *Salmonella* two-weeks after being supplemented with SCFP (Brewer et al., 2014) and final BW (d 42) of calves that were not challenged (Lesmeister et al., 2004). These disagreements in the literature could be due to differences in MR feeding rate, starter consumption, the SCFP supplement used, or differences in the health status of calves.

A trend for increased ADG in the SCFP group post-weaning was observed, which agrees with previous research that found a significantly greater post-weaning ADG (Lesmeister et al., 2004). This could imply that SCFP helps to improve the growth of calves after experiencing a stress event (weaning and LPS challenge), which is in agreement with prior research that observed improved weight gain after a *Salmonella* challenge (Brewer et al., 2014). SCFP supplementation has been shown to increase rumen development, through longer papillae length and greater papillae width (Lesmeister et al., 2004; Brewer et al., 2014), so this increase in ADG could be explained by improved rumen development and absorption capability in the SCFP calves (Xiao et al., 2016).

Calf HH, HW, and BCS were not different between treatments at weaning (d 56) or the end of the trial (d 112). Another study did not observe changes in wither height, length, HW, and heart girth (Xiao et al., 2016). Alugongo et al. (2017) also reported no structural differences between treatments. None of the previous studies measured BCS. Lesmeister et al. (2004) did see increased HH and HW in SCFP calves which they believed was due to the increased intake they observed in SCFP calves, which was not observed in the current study.

Consistent with BW, HH, HW, BCS, and pre-weaning ADG, feeding SCFP to calves did not affect individual intake, group intake, or FE prior to weaning. One study was in agreement with the current study and did not observe treatment differences between individual starter intakes (Alugongo et al., 2017), however, Lesmeister et al. (2004) observed that SCFP calves had a greater starter intake. The first study mentioned, however, reported low overall intake due to an increased MR feeding rate which has been shown to reduce starter intake (Khan et al., 2008). The second study that reported starter intake differences, weaned calves at approximately 35 days of age and only evaluated calves through 42 days of age. These differences could be why research reports conflicting results. No other study was found that supplemented SCFP to calves and observed intake in a group setting post-weaning through 112 days of age. Consistent with the current study, previous research also reported no treatment effects of SCFP on pre-weaning FE (Lesmeister et al., 2004; Alugongo et al., 2017). However, a treatment effect on FE post-weaning was observed, with SCFP calves having improved FE. This observed treatment effect was significant even though numerically SCFP calves only had a slight increase in FE. This is consistent with the increased ADG post-weaning in SCFP calves that was observed. Due to no prior studies evaluating FE in a group setting after weaning, this finding warrants further study to validate. However, an improved FE after weaning could imply that SCFP is able to mitigate the negative effects caused by stress through improved rumen development and absorption efficiency (Xiao et al., 2016).

Batch 2 had greater biweekly starter intake, total starter intake, total individual intake, and improved FE after weaning compared to batch 1. This indicates that the second batch of thirty calves consumed more feed and had an improved FE post-weaning compared to the first batch of calves. This difference could be due to seasonal differences in temperature. The first batch of the study was conducted during the summer months and the second batch was conducted in the fall.

Based on the findings of this study and the literature, it appears that many factors can affect how SCFP supplementation impacts the calf, such as MR intake, starter consumption, morbidity,

stress, and management. If the direct connection between SCFP and improved growth and health is increased rumen development, research should focus on looking at the optimal feeding regime that promotes starter intake without compromising growth and health.

Calf health is an essential factor in ensuring a calf meets its genetic potential. Poor health can compromise growth, future productivity, and increase treatment costs. In this study, no treatment effects were observed for number of medical treatments based on fecal issues or other illnesses (not fecal or respiratory related), daily fecal score, or daily respiratory score. The most commonly reported health benefit of SCFP is a reduction of diarrhea and improved fecal scores (Lesmeister et al., 2004; Magalhães et al., 2008).

However, a significant treatment effect was observed for respiratory medication treatments. Calves that were supplemented with SCFP were treated less frequently for respiratory diseases compared to CON calves, indicating they experienced fewer incidences of respiratory illness. This was also seen with SCFP calves having a greater probability of being healthy compared to CON calves. Not many prior studies feeding SCFP to calves looked into respiratory illness. Recently, Mahmoud et al. (2020) evaluated immune parameters, respiratory disease related clinical signs, and gross lung pathology in control and SCFP supplemented calves that had been challenged with bovine respiratory syncytial virus. The SCFP calves had reduced cases of secondary infection, respiratory clinical disease, and lung pathology following the viral challenge. It is possible that SCFP supplementation enhances the innate immune function in the blood of calves while also regulating the immune reaction in the lungs to reduce damage or consolidation and expedite recovery. Again, illness rate inconsistencies could be due to environmental and seasonal differences, the type of SCFP used, as well as the level of pathogenicity.

Prior research indicates that SCFP supplemented to calves improves DMI, BW gain, and health (Cole et al., 1992; Lesmeister et al., 2004), which is believed to be the result of improved rumen fermentation, gut microbiota stability, and gut morphology stimulation (Brewer et al., 2014). In this study, fecal samples, which are both noninvasive to collect and easily repeatable, were analyzed to represent the intestinal microbiome (Santiago et al., 2014) in dairy calves. The current study clearly indicates that with time, the fecal microbiome of calves' increases in both richness and evenness. This suggests that as a calf matures their gut microbiota becomes more diverse. This increase in bacterial diversity with age is a common phenomenon observed in a variety of species from humans (Koenig et al., 2011; Yatsunenko et al., 2012), to turkeys (Johnson et al., 2019) and

cattle (Li et al., 2012; Jami et al., 2013; Rey et al., 2014; Meale et al., 2016). In the current study, treatment did not appear to play a role in the richness of the fecal microbiome, however, SCFP calves did tend to have increased species evenness, implying that when calves are fed SCFP, it leads to a more even distribution of species.

Beta-diversity was used to measure the dissimilarity in the composition and abundance of community members between samples. With time, inter-animal variation in fecal microbiome composition is reduced, as seen by tighter sample clustering at d 56, 84, and 112 compared to d 0 and 28. Other studies have reported that the fecal microbiota in calves post-weaning is more similar to each other compared to pre-weaning (Meale et al., 2016; 2017). No clear distinct clustering based on treatment at any timepoint was observed, indicating that treatment did not play a role in how similar or dissimilar one sample is from another.

When looking at the relative abundance of the fecal microbiota based on phylum, a similar pattern based on time was observed for richness, evenness, and similarity. Samples collected on d 0 and 28 have a different taxonomical composition compared to those at d 56, 84, and 112 which are more similar to each other. No effect of SCFP on fecal microbiota in calves, based on relative abundance at the phylum level was observed. These results indicate that time has a major impact on the fecal microbiome in calves as seen based on species richness, evenness, similarity, and relative abundance. The major impact of time is reasonable to believe because at birth the calf's gastrointestinal tract is colonized by microbes from the dam and environment, helping to further the development of the gut (Bryant et al., 1958; Minato et al., 1992). This development and colonization eventually lead to the stabilization of the rumen and intestinal environments (Jami et al., 2013), which is what was observed in the current study.

A limitation of this study includes a 13% mortality rate, with 52 out of the 60 calves enrolled making it through the entire study. Also, 42% of calves in the current study were treated for respiratory disease and 42% for scours/fecal concerns. This high incidence of treatment could alter the effects of SCFP by limiting energy for growth and efficiency. This study also transported calves from a commercial dairy farm (55 km; 45 min) and exposed calves to a new environment upon arrival, both likely played a role in the increased incidence of calf morbidity and mortality. Transportation is stressful to calves, leading to increased plasma cortisol and NEFA concentrations. Prior research suggests that the crucial factors causing this stress response is calf handling and transportation in general, not the duration of the journey (Mormede et al., 1982; Sartorelli et al.,

1992). The transportation of adult cattle, which is more heavily researched compared to calves, indicates that transportation activates the release of cytokines, stress hormones, and acute phase proteins (Arthington et al., 2003) and it can impact innate immune cell function (Hulbert et al., 2011).

Further research into SCFP includes evaluating the long-term health and growth impacts that it could have on dairy calves. Also, further research is needed to confirm if SCFP is mitigating the negative effects seen after a stress event in calves, like weaning or an illness. Specifically looking at the rumen microbiome in calves fed SCFP is worth exploring to determine if rumen microbiota have a clearer relationship with improved health and productivity in calves compared to fecal microbiota.

2.6 Conclusion

In conclusion, SCFP improved average daily gain and feed efficiency in dairy calves post-weaning, indicating that SCFP may be minimizing the negative implications observed in calves after a stress event. The SCFP could also reduce the incidence of respiratory disease among 1 to 112-day old dairy calves, therefore, reducing treatments and antibiotic use. Even though this study illustrated that SCFP supplemented to calves can improve the evenness of the fecal microbiome and that it alters beta-diversity post-weaning, more research is needed to fully determine if these changes in fecal microbiome are directly improving the productivity and health of calves.

2.7 Acknowledgements

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Table 2.1. Chemical composition of milk replacer (MR), starter, and grower for control (CON) and *Saccharomyces cerevisiae* fermentation products (SCFP) experimental treatment diets[1].

Item	MR	CON Starter	SCFP Starter[2]	CON Grower #1[5]	SCFP Grower #1[3,5]	CON Grower #2[6]	SCFP Grower #2[4,6]
Dry Matter, %	96.6±0.1	86.6±0.3	86.6±0.5	88.8±0.3	88.5±0.4	89.0±0.7	89.4±0.7
CP, % DM	25.2±0.3	23.7±2.2	21.7±1.2	20.5±0.3	20.5±0.3	18.3±0.2	18.4±0.1
Fat, % DM	17.8±0.2	3.6±0.2	3.4±0.6	3.7±0.1	3.9±0.2	4.1±1.1	3.8±0.6
ADF, % DM	.	10.7±0.7	10.3±0.5	11.4±0.8	10.9±0.8	21.0±1.7	22.6±1.0
aNDF, % DM	.	18.4±0.5	19.0±1.1	30.8±1.2	29.5±1.1	40.3±2.8	42.3±1.4
Ash, % DM	8.7±0.1	7.8±0.8	7.3±0.5	7.9±0.2	7.7±0.2	5.7±0.7	6.2±0.7

[1]CON (MR, calf starter, and calf growers with no SCFP added) or SCFP (MR with 1 g/d of SmartCare® (Diamond V, Cedar Rapids, IA), calf starter with NutriTek® (Diamond V, Cedar Rapids, IA), and calf growers with NutriTek®).
[2]Contained 0.8% NutriTek®
[3]Contained 0.44% NutriTek®
[4]Contained 0.275% NutriTek®
[5]Fed from d 57- 84
[6]Fed from d 85- 112

Table 2.2. Serum total protein (STP), initial (0 d), weaning (56 d), and final (112 d) body measurements, as well as pre- and post-weaning average daily gain (ADG) of control (CON) and *Saccharomyces cerevisiae* fermentation products (SCFP) calves.

	Treatments[1]			*P*-values		
Items	CON	SCFP	SEM	Treatment	Batch	Block
STP, mg/dL	5.95	6.04	0.12	0.55	0.007	0.19
Body Weight, kg						
0 d	45.2	45.0	0.4	0.81	0.72	<0.001
56 d	85.4	84.5	1.5	0.67	0.39	0.04
112 d	148.5	152.5	2.7	0.30	0.99	0.06
BCS[2]						
0 d	2.22	2.22	0.01	0.80	0.99	0.001
56 d	2.88	2.84	0.03	0.31[3]	0.041	0.12
112 d	3.46	3.45	0.03	0.79	<0.001	0.07
Hip Height, cm						
0 d	82.7	83.1	0.4	0.49	0.56	<0.001
56 d	95.3	95.7	0.5	0.52	0.60	<0.001
112 d	106.7	107.9	0.5	0.11[4]	0.046	0.001
Hip Width, cm						
0 d	16.8	16.6	0.1	0.34	0.96	<0.001
56 d	21.4	21.3	0.2	0.62[5]	0.67	0.02
112 d	26.9	27.3	0.2	0.21	0.67	0.32
ADG, kg/day						
0-56 d	0.71	0.71	0.03	0.95	0.45	0.78
57-112 d	1.13	1.21	0.03	0.07	0.44	0.03

[1]CON (24% CP: 17% fat MR, calf starter, and calf grower with no SCFP added) or SCFP (24% CP: 17% fat MR with SmartCare® (Diamond V, Cedar Rapids, IA), calf starter with NutriTek® (Diamond V, Cedar Rapids, IA), and calf grower with NutriTek®.

[2]BCS was measured on a scale of 1-5.

[3]There was a Treatment x Block tendency ($P = 0.08$).

[4]There was a Treatment x Batch tendency ($P = 0.09$).

[5]There was a Treatment x Batch interaction ($P = 0.04$).

Table 2.3. Intake and feed efficiency (FE) of control (CON) and *Saccharomyces cerevisiae* fermentation products (SCFP) calves from d 0 to 112.

	Treatments[1]			*P*-values			
Items	CON	SCFP	SEM	Treatment	Timepoint	Batch	Block
Starter Intake, kg				0.79	<0.0001	0.003	0.62
1-14 d	0.60	0.76	0.71				
15-28 d	4.38	4.95	0.71				
29-42 d	11.72	12.10	0.71				
43-56 d	18.32	17.96	0.71				
Total Milk Replacer Intake, kg	42.05	42.05	0.02	0.95	-	0.14	0.30
Total Starter Intake, kg	35.06	35.15	2.17	0.98	-	0.0002	0.52
Total Individual Intake[2], kg	77.11	77.20	2.17	0.98	-	0.0002	0.52
FE[3] (d 1-56), kg/kg	0.52	0.52	0.01	0.87	-	<0.0001	0.04
Group Intake[4], kg/calf	220.83	228.30	5.59	0.30[5]	-	0.20	0.74
FE[3] (d 57-112), kg/kg	0.29	0.30	0.002	0.02[6]	-	0.005	0.003

[1]CON (24% CP: 17% fat MR, calf starter, and calf grower with no SCFP added) or SCFP (24% CP: 17% fat MR with SmartCare® (Diamond V, Cedar Rapids, IA), calf starter with NutriTek® (Diamond V, Cedar Rapids, IA), and calf grower with NutriTek®.

[2]Total individual intake = MR intake + starter intake

[3]FE = BW gain / feed intake

[4]Group intake = individual pen intake / # of calves in that pen

[5]Treatment × Batch tendency ($P = 0.08$).

[6]Treatment × Block interaction ($P = 0.04$).

Table 2.4. Health data of control (CON) and *Saccharomyces cerevisiae* fermentation products (SCFP) calves from d 0 to 112.

	Treatments[1]			*P*-values			
Items	CON	SCFP	SEM	Treatment	Timepoint	Batch	Block
Medication[2]							
Fecal	0.52	0.46	0.12	0.71	-	0.86	0.24
Respiratory	0.98	0.31	0.14	0.001	-	0.38	0.05
Other	0.17	0.04	0.07	0.20	-	0.57	0.73
Fecal Score[3]	2.12	2.15	0.02	0.38	-	0.36	0.70
Respiratory Score[4]	0.14	0.12	0.02	0.38	-	<0.0001	0.70
Right Lung[5]				0.61	<0.0001	0.0007	0.64
0 d	1.50	1.57	0.07				
28 d	1.98	1.97	0.08				
56 d	2.11	1.97	0.08				
112 d	1.76	1.78	0.08				
Left Lung[5]				0.51	<0.0001	0.01	0.23
0 d	1.67	1.47	0.08				
28 d	1.96	1.95	0.09				
56 d	2.16	2.07	0.09				
112 d	1.80	1.93	0.09				

[1]CON (24% CP: 17% fat MR, calf starter, and calf grower with no SCFP added) or SCFP (24% CP: 17% fat MR with SmartCare® (Diamond V, Cedar Rapids, IA), calf starter with NutriTek® (Diamond V, Cedar Rapids, IA), and calf grower with NutriTek®.

[2]Medication counts were determined based on the number of medication incidences by study treatment / # of calves on that study treatment

[3]Fecal scores were assigned on a scale of 1-5, only recorded until d 56 (average daily score)

[4]Respiratory scores were assigned on a scale of 0-3, only recorded until d 56 (average daily score)

[5]Lung scores were assigned on a scale of 1-5

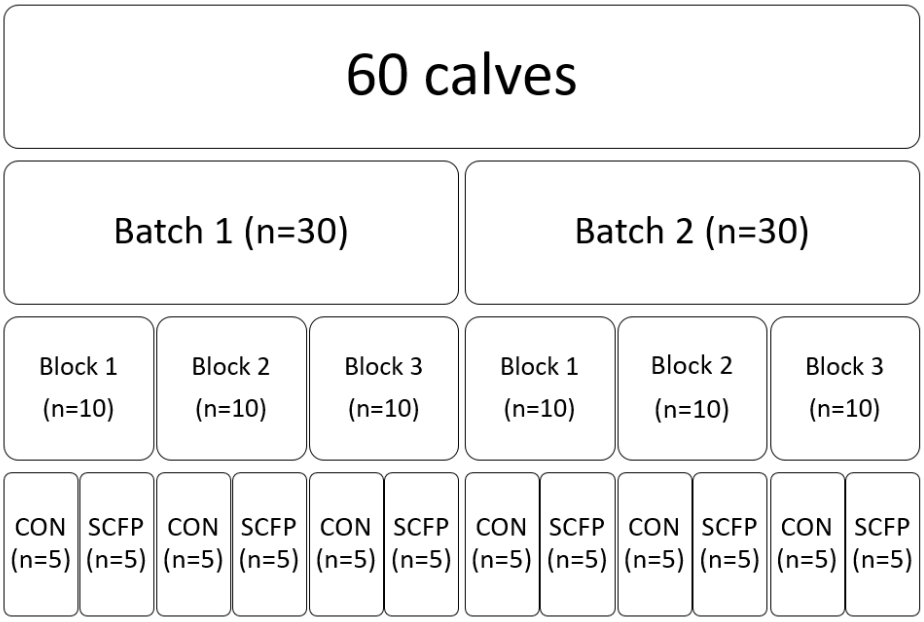

Figure 2.1. Diagram illustrating how calves (n=60) were received in two batches (1 or 2), blocked (1, 2, or 3) by BW and serum total protein, and then assigned to treatment (CON vs. SCFP).

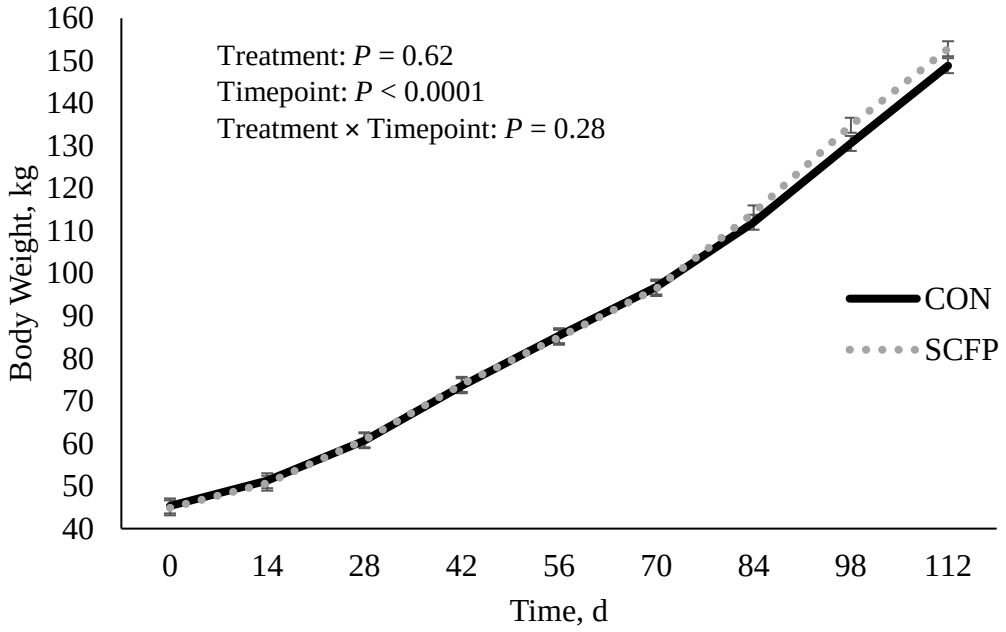

Figure 2.2. Biweekly calf body weight by treatment from d 0 to 112. Calves were assigned to one of two treatments, CON (24% CP: 17% fat MR, calf starter, and calf grower with no SCFP added) or SCFP (24% CP: 17% fat MR with SmartCare® (Diamond V, Cedar Rapids, IA), calf starter with NutriTek® (Diamond V, Cedar Rapids, IA), and calf grower with NutriTek®.

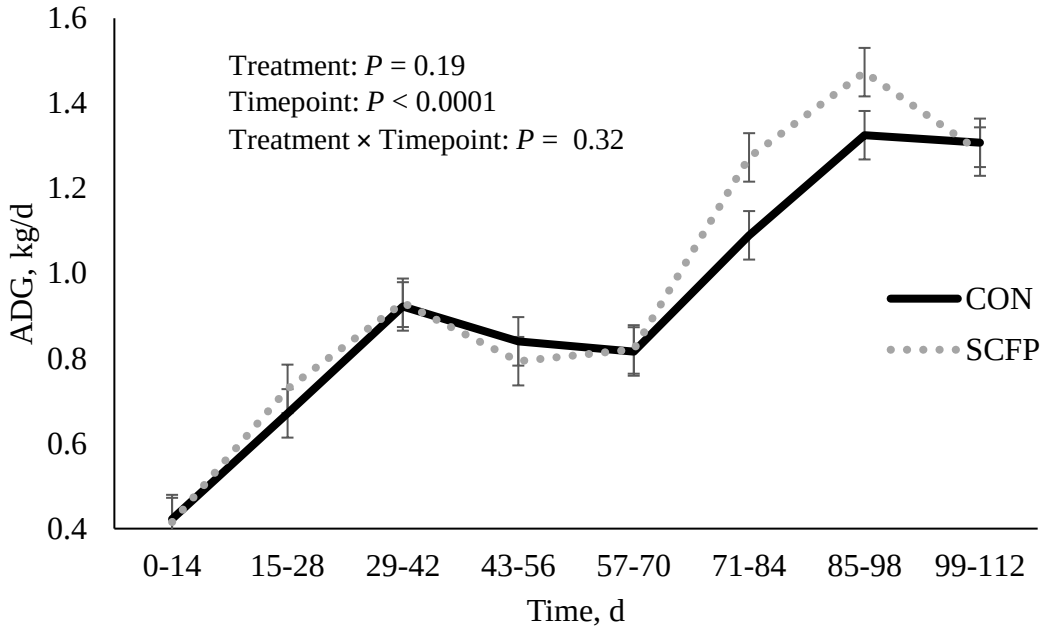

Figure 2.3. Biweekly calf ADG by treatment from d 0 to 112. Calves were assigned to one of two treatments, CON (24% CP: 17% fat MR, calf starter, and calf grower with no SCFP added) or SCFP (24% CP: 17% fat MR with SmartCare® (Diamond V, Cedar Rapids, IA), calf starter, and calf grower with NutriTek® (Diamond V, Cedar Rapids, IA).

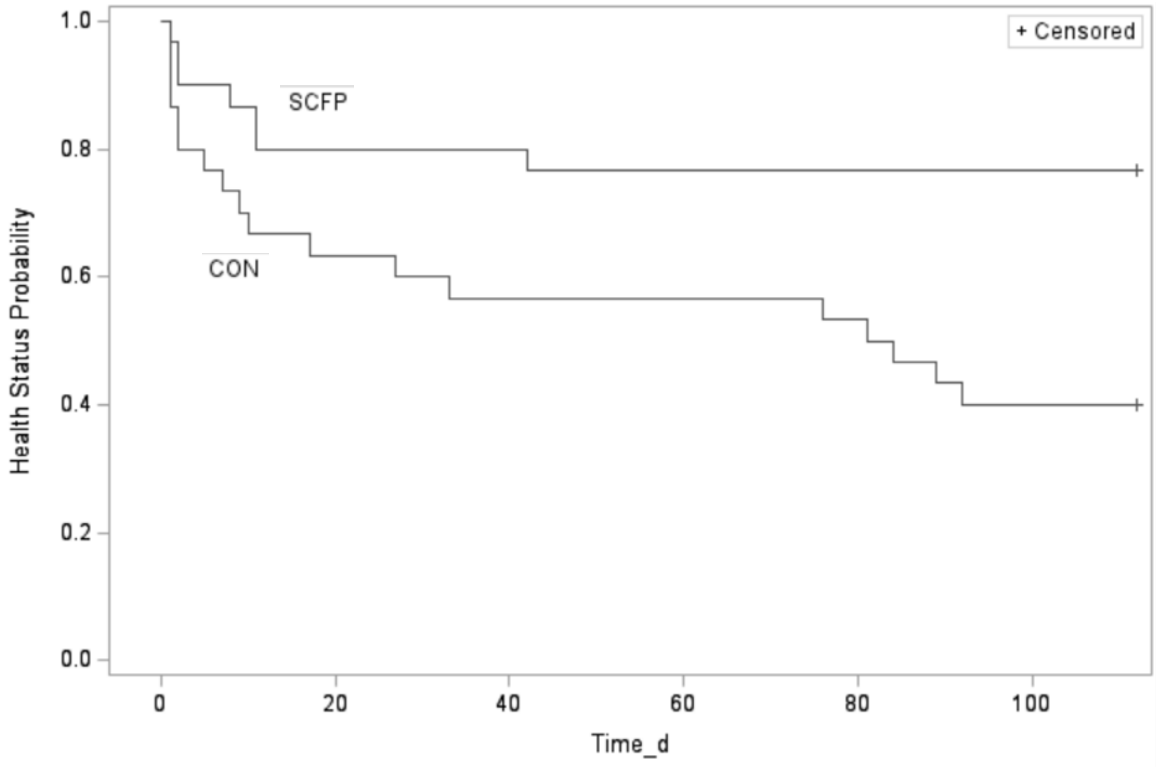

Figure 2.4. Health status probability based on respiratory illness by treatment from d 0 to 112.

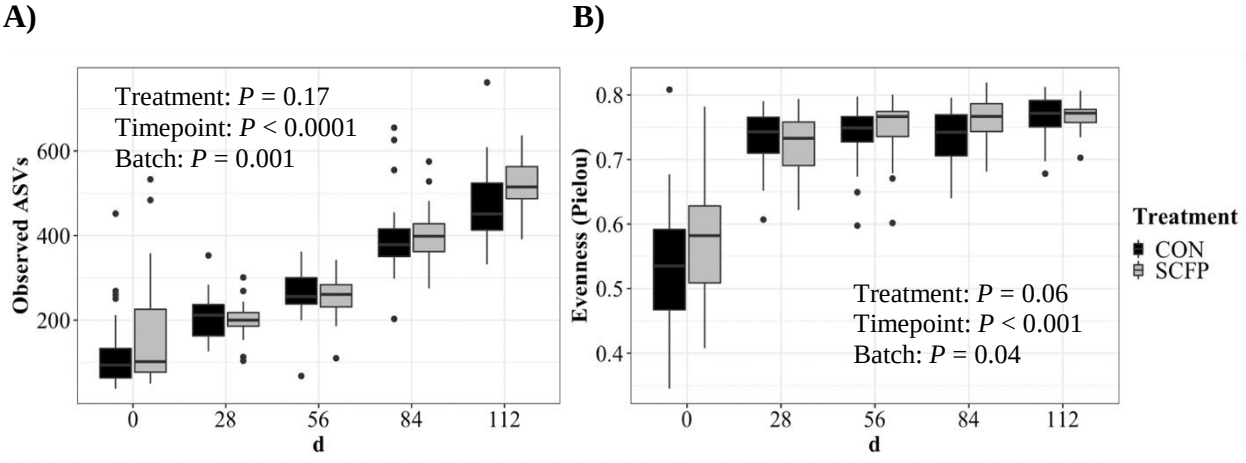

Figure 2.5. Calf fecal alpha-diversity measured by Observed ASV Index (A) and Pielou Index which measures evenness (B) at each timepoint (0 d, 28 d, 56 d, 84 d, and 112 d) based in treatment (CON and SCFP).

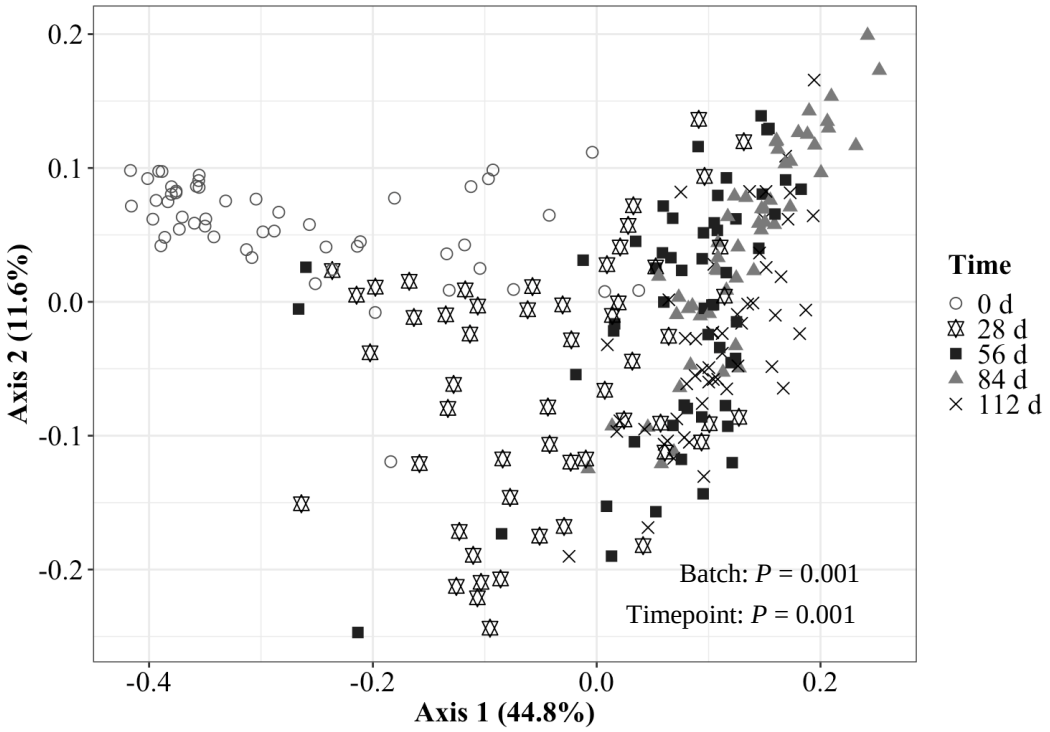

Figure 2.6. Principal Coordinates Analysis plot illustrating the beta-diversity of the calf fecal microbiota at each timepoint (0 d, 28 d, 56 d, 84 d, and 112 d) estimated by the Weighted UniFrac distances.

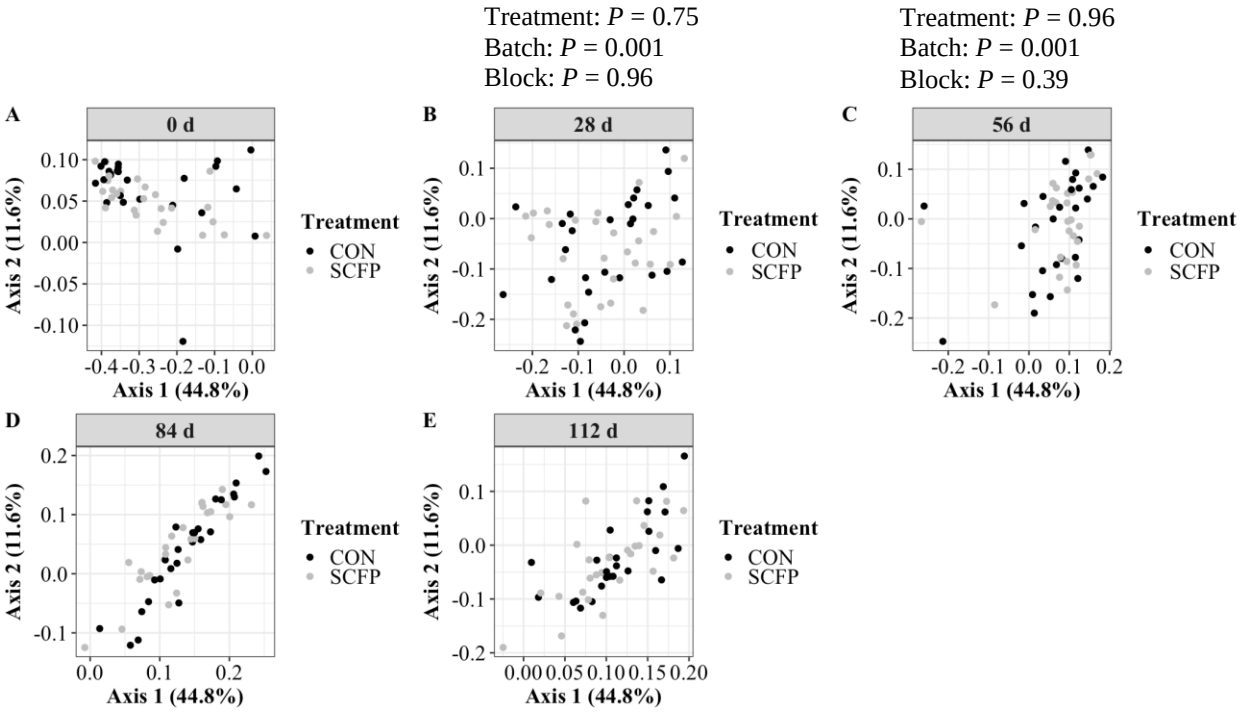

Figure 2.7. Principal Coordinates Analysis plots illustrating the beta-diversity of the calf fecal microbiota under the effect of the dietary treatments (CON and SCFP) at each timepoint (0 d (A), 28 d (B), 56 d (C), 84 d (D), and 112 d (E) estimated by the Weighted UniFrac distances.

CHAPTER 3. EFFECTS OF FEEDING *SACCHAROMYCES CEREVISIAE* FERMENTATION PRODUCTS ON THE HEALTH OF HOLSTEIN DAIRY CALVES FOLLOWING A LIPOPOLYSACCHARIDE CHALLENGE

Klopp, R. N., Ilkyu Yoon, Susan Eicher, Jacquelyn P. Boerman. Effects of feeding *Saccharomyces cerevisiae* fermentation products on the health of Holstein dairy calves following a lipopolysaccharide challenge. *J. Dairy. Sci.* Accepted.

3.1 Abstract

Before weaning, dairy calves are at high risk for illness, especially respiratory and digestive diseases, which reduces average daily gain (**ADG**), age at first calving, and first lactation milk production. While these illnesses are commonly treated with antibiotics, there is an effort to reduce antibiotic use, due to the concern for antibiotic-resistance bacteria. The objective was to evaluate the effects of *Saccharomyces cerevisiae* fermentation products (**SCFP**) on the immune status of calves, following a lipopolysaccharide (**LPS**) challenge that occurred just prior to weaning. Thirty Holstein bull calves were blocked based on initial body weight (**BW**) and then assigned to one of two study treatments. The control (**CON**) was fed a 24% crude protein (**CP**):17% fat milk replacer (**MR**) and calf starter with no SCFP added. The SCFP treatment was fed the same 24% CP:17% fat MR with 1 g/d of SmartCare® (Diamond V, Cedar Rapids, IA) and calf starter with 0.8% NutriTek® (Diamond V, Cedar Rapids, IA). SmartCare® and NutriTek® are both products produced from the anaerobic fermentation of *Saccharomyces cerevisiae*. Calves were offered 2.84 L (12.5% solids) of MR twice daily at 0630 and 1630 h through d 51, from d 52-56 calves were fed MR once daily at 0630 h, and then weaned on d 57. Calves also received *ad libitum* access to a texturized calf starter and water. On d 50, a subset of calves (n=20, 10 calves per treatment), were enrolled in an LPS challenge. At -1.5, -0.5, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, and 24 h relative to dosing with LPS, 20 mL of blood was collected, and rectal temperature and respiration rate were measured on each calf. Blood serum samples were analyzed for interleukin 6 (**IL-6**), tumor necrosis factor-alpha (**TNF-α**), interferon-gamma **(IFN-γ**), haptoglobin **(Hp**), serum amyloid-A (**SAA**), fibrinogen, non-esterified fatty acid (**NEFA**), cortisol, and glucose. This study observed increased concentrations of TNF-α at 1 h and 1.5 h and glucose at 0.5 h after dosing with LPS in SCFP calves compared to CON. Calves supplemented with SCFP also had an increase in

respiration rate 0.5 h and 1.5 h after dosing with LPS, and reduced feed intake the day of the challenge compared to CON calves. These results would suggest that when dairy calves are supplemented with SCFP, they elicit an increased acute immune response, as observed by increased TNF-α, glucose, and respiration rate just after dosing with LPS compared to CON calves.

3.2 Introduction

Dairy calves in the U.S. have a high risk for morbidity prior to weaning with 1 in 5 experiencing a respiratory disease and 1 in 4 a digestive illness, according to a nationwide survey of dairy farms (NAHMS, 2012). The most common way to treat these illnesses is with antibiotics. Of calves that are diagnosed with a respiratory disease, 9 in 10 are treated with antibiotics and 7 in 10 calves diagnosed with digestive illnesses are treated with antibiotics (NAHMS, 2012). In animal agriculture, the use of antibiotics has come under scrutiny because of the increasing prevalence of antibiotic resistance of both human and animal pathogens (Langford et al., 2003; Loo et al., 2019), indicating that there is a need for strategies to both improve calf health and reduce dependency on antibiotics.

Many different feed additives have been evaluated in dairy calves to reduce morbidity and improve health and growth, including *Saccharomyces cerevisiae* fermentation products (**SCFP**). When *Saccharomyces cerevisiae* yeast is anaerobically fermented, amino acids, lipids, nucleotides, B vitamins, and organic acids are produced (Deters et al., 2018) and these are referred to as SCFP. Therefore, the effect of SCFP does not rely on the viability of live yeast. Studies suggest that SCFP may be enhancing health status through anti-inflammatory and anti-oxidant activities (Jensen et al., 2008b), by increased rumen development and reduced colonization of pathogenic bacteria (Brewer et al., 2014), priming the innate response prior to an immune challenge (Burdick Sanchez et al., 2020), reducing the incidence of scouring (Alugongo et al., 2017; Harris et al., 2017), as well as increasing innate immune system cytokine production by peripheral blood mononuclear cells (Mahmoud et al., 2020).

The impact that SCFP has on dry matter intake (**DMI**) and ADG has been reported in multiple studies. Research have reported positive effects on DMI (Lesmeister et al., 2004; Harris et al., 2017) as well as ADG (Lesmeister et al., 2004; Harris et al., 2015) when calves are fed SCFP. In regard to the impact SCFP has on calf health, the most common health benefits are improved fecal score and reduced diarrhea (Magalhães et al., 2008; Alugongo et al., 2017). The rumen

environment in calves can be a major indicator of health status. A reduction in ruminal microbial diversity within young calves is linked to increased incidence of diarrhea (Xiao et al., 2016), SCFP may be helping to improve microbial diversity and therefore reducing diarrhea. Additionally, *in vitro*, SCFP are capable of inhibiting pathogenic bacteria while promoting commensal bacteria (Jensen et al., 2008a), potentially indicating another mode of action for how SCFP reduces the incidence of diarrhea in calves.

Even though the impacts that SCFP has on calf health and performance are widely researched, there are still more questions to explore regarding the specific mechanism behind the effects of SCFP on calf immunity. To evaluate this, calves' innate immune systems were tested using an LPS challenge model. The objective of this study was to evaluate the effects of SCFP on the immune status (temperature, respiration rate, metabolite, cytokine, and acute-phase protein analysis) of calves, following an LPS challenge that occurred prior to weaning. The hypothesis for this study was that all calves following the LPS dosing would experience an activated innate immune response as evidenced by increased concentrations of cytokines and acute-phase proteins in circulation, as well as an increase in temperature and respiration rate. However, the SCFP calves would have an elevated and rapid innate immune response, increased cytokine, and heat production, compared to CON calves. This acute immune response could be neutralizing the foreign invader more rapidly, leaving more energy available for growth and less negative effects from pathogens, which could explain the increased growth performance and health status observed in prior research when calves are fed SCFP.

3.3 Materials and Methods

3.3.1 Animals and Facility

All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee (Protocol #1808001783) at Purdue University (West Lafayette, IN). Thirty Holstein bull calves, approximately 5±3 (mean; SD) days of age, were received on 9/13/19 from a dairy farm 55 km from the Purdue University Animal Sciences Research and Education Center (**ASREC**) Dairy Farm. Calves were transported to ASREC by trailer (2.3 x 7.3 m; Wilson, Sioux City, IA). Calves were housed in individual hutches (Calf-Tel, Germantown, WI) with a fenced-

in, outside area (3.5 x 1.2 m). Individual hutches were bedded with wood shavings and re-bedded, as needed.

3.3.2 Study Treatments

Upon arrival to the ASREC Purdue Dairy Farm, calves were assigned to one of two treatments (n=15 each; Table 3.1). The control (**CON**) treatment fed a 24% CP:17% fat milk replacer (**MR**) and a texturized calf starter with no SCFP added. The SCFP treatment fed a 24% CP:17% fat MR with 1 g/d of SmartCare® (Diamond V, Cedar Rapids, IA) and calf starter with 0.8% NutriTek® (Diamond V, Cedar Rapids, IA). Calves were blocked into low BW (block 1, n=10), intermediate BW (block 2, n=10), and high BW (block 3, n=10) and then randomly assigned to treatment (CON vs. SCFP) within each block. Packed-cell volume (**PCV**) was also measured upon arrival to determine the hydration status of each calf with all calves above 21% for PCV.

3.3.3 Feeding Regimen

Calves received 2.84 L of MR (12.5% solids) twice daily at 0630 and 1630 h until d 51 of the study. From d 52 to 56 calves were fed 2.84 L of MR once daily at 0630 h and were weaned on d 57. If a calf refused more than 0.95 L of MR, they were fed the remainder of the milk using an esophageal tube feeder (Nasco, Fort Atkinson, WI), this was to ensure sick calves that would not drink received sufficient nutrients and maintained hydration status. Refusals of MR less than 0.95 L were reported as a refusal. Calves also received *ad libitum* access to a texturized calf starter from d 1-56. Growth, intake, feed efficiency, medical treatments, fecal scores, and respiratory scores data was collected as part of a larger study and will be reported elsewhere.

3.3.4 Lung Scanning

On d 0, 28, and 49 (prior to LPS challenge), lung consolidation was evaluated for each calf using a portable ultrasonography machine (IBEX PRO; E.I. Medical, Loveland, CO) and a linear transducer (L6.2; E.I. Medical, Loveland, CO). Calves were restrained using a halter. The left and right lungs were scored separately on a scale of 1-4. A score of 1 indicates a normal lung with no lung consolidation (firming of the lungs) and no comet-tail artifacts (pleural roughening). A score of 2 indicates the presence of comet-tails but no lung consolidation. A score of 3 indicates lung

consolidation that affects only 1 lobe. A score of 4 indicates lung consolidation that affects 2 or more lobes (modified from (Ollivett and Buczinski, 2016).

3.3.5 Lipopolysaccharide Challenge

A subset of calves (20 total, 10 calves per treatment), were enrolled in an LPS challenge on d 50. Calves were enrolled in the challenge if they had not been previously treated for a respiratory illness, had no lung consolidation, and were consuming greater than 900 g of starter daily. On d 49 (pre-challenge) and d 52 (post-challenge), 3 mL of blood was collected from the jugular vein of calves using an EDTA vacutainer tube to quantify the cell types in blood, also known as complete blood counts, using a hematology analyzer (Genesis, Oxford Science, Oxford, CT). On d 49, calves were moved from their individual hutches to individual stalls in a tie-stall barn at the Purdue Dairy Farm and restrained using halters. Calves were weighed (Tru-Test, Mineral Wells, TX) to calculate LPS dose, scanned again for lung consolidation (IBEX PRO, Loveland, CO), and catheterized in the jugular vein using a 16 Ga x 7.5 cm extended use MILACATH® (MILA International Inc., Florence, Kentucky).

At 0830 h on d 50 of the study, two of the twenty calves received 0.125 µg/kg of BW of LPS from *Escherichia coli* (O111:B4; Sigma L4391, Sigma Aldrich, St. Louis, MO) intravenously. At 0930 h, the remaining eighteen calves received the same dose of LPS. The LPS administration was staggered to ensure the dose was effective and to minimize any potential negative effects of the LPS prior to dosing all twenty calves. Epinephrine, flunixin meglumine (Banamine®, Merck Animal Health, Kenilworth, NJ), and Dexamethasone were available for medical intervention in case of an emergency. Blood was collected at -1.5, -0.5, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, and 24 h relative to dosing with LPS. At each blood collection, catheters were first flushed with 5 mL of saline (0.9% NaCl), then 20 mL of blood was collected, catheters were flushed again with 5 mL of saline, followed by 3 mL of heparinized saline (2 mL of heparin 1,000 IU/mL in 1 L of saline). At the same time points that blood was collected, rectal temperature was measured using a digital thermometer (Vicks SpeedRead, Helen of Troy Ltd., El Paso, TX) and respiration rate was determined by counting the number of breaths per minute. Blood was allowed to clot and then centrifuged at 3,100 x g for 20 min at 4°C. Serum was transferred to microcentrifuge tubes and frozen to -20°C until further analysis.

Serum samples were sent to the University of Minnesota Cytokine Reference Laboratory (Minneapolis, MN) and were analyzed for interleukin 6 (**IL-6**), tumor necrosis factor-alpha (**TNF-α**), interferon-gamma **(IFN-γ**), haptoglobin **(Hp**), serum amyloid-A (**SAA**), and fibrinogen. Additional serum samples were analyzed using commercial kits for non-esterified fatty acid (**NEFA**; Wako HR series NEFA-HR(2); FUJIFILM Wako Diagnostics U.S.A Corporation, Mountain View, CA), cortisol (EKC31009; Biomatik Corp., Cambridge, Ontario), and glucose (P7119; Sigma Aldrich, St. Louis, MO). Inter assay coefficients of variation were 5.45%, 4.65%, 13.11% for NEFA, glucose, and cortisol, respectively. Intra assay coefficients of variation were 3.65%, 2.67%, 4.24% for NEFA, glucose and cortisol, respectively.

3.3.6 Statistical Analyses

A power analysis was performed to calculate the sample size for a primary outcome variable, TNF-α concentrations, following an LPS challenge in calves. Based on data from Benjamin et al. (2016), with 95% confidence, and 80% power, 9 animals per treatment group were needed to detect differences. To account for potential calf mortality during the LPS challenge, 10 animals per treatment group were enrolled in the LPS challenge. Data were analyzed as a completely randomized design using the Mixed Procedure of SAS v.9.4 with repeated measures using a first order autoregressive structure AR(1) based on minimizing AICC and BIC. To identify serum analysis outliers, a Cook's distance cutoff of 0.2 was set. One data point was removed from the NEFA analysis and three data points were removed from the cortisol analysis. Measurements for serum analytes, rectal temperature, and respiration rate at all timepoints were analyzed for normality using a Shapiro-Wilk test and data were normally distributed. One calf on the SCFP treatment died during the LPS challenge, that calf's data was removed from analysis. The experimental unit was individual calf (n=19). The fixed effects included treatment (T_i; CON (n=10) and SCFP (n=9)), timepoint (P_j; -1.5, -0.5, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, and 24 h relative to LPS dosing for rectal temperature, respiratory rate, metabolites, cytokines, and acute-phase proteins; 1 and 2 for complete blood count analysis before and after LPS challenge; -1, 0, 1, 2, 3, 4 for intake in relation to LPS challenge), and the interactions between treatment and timepoint ($TP_{(ij)}$). The random effect was calf nested within treatment ($C_{k(i)}$). The model was represented as:

$$Y_{ijk} = \mu + T_i + P_j + TP_{(ij)} + C_{k(i)} + e_{ijk},$$

where Y_{ijk} is the response variable, μ is the overall mean, and e_{ijk} is the error. Continuous response variables included rectal temperature, respiratory rate, intake in relation to LPS challenge, complete blood count analysis, NEFA, TNF-α, IL-6, IFN-γ, Hp, SAA, fibrinogen, cortisol, and glucose. A *P*-value ≤ 0.05 was determined to be statistically significant and a *P*-value > 0.05 and ≤ 0.10 was determined to be a statistical tendency. When TP(ij) was statistically significant or a tendency (P ≤ 0.10), the slice option was used to evaluate the acute (0.5 to 4 h post LPS dosing) treatment effects within timepoint. A Bonferroni adjustment was applied to the slice option to decrease the probability of type 1 error.

3.4 Results

One calf died during the LPS challenge, even after emergency medical intervention, that calf was a part of the SCFP treatment and passed away 2 h after dosing.

3.4.1 Rectal Temperature, Respiratory Rate, and Intake

A timepoint effect was observed for rectal temperature (*P* < 0.0001), which increased after dosing with LPS, peaked around 3.5 h after dosing, and then continued to decrease until 24 h after dosing (Figure 3.1). A tendency for a treatment by timepoint interaction was observed for respiration rate (*P* = 0.07; Figure 3.2). At 0.5 h, SCFP calves had an increased respiration rate (103 vs. 80 breaths/min ± 5.4 (SEM; *P* = 0.02) compared to CON calves.

There were no starter intake differences between treatments the day prior to the LPS challenge, with SCFP calves consuming 1,093 g and CON calves consuming 1,267 g (*P* = 0.54). On the day of the LPS challenge, SCFP calves consumed 761 g/calf less starter compared to the CON calves (*P* = 0.002; Table 3.2). There were no intake differences based on treatment after the day of the LPS challenge (*P* > 0.29).

3.4.2 Complete Blood Count Pre-Challenge and Post-Challenge

When comparing blood counts pre-challenge (d 49) and post-challenge (d 52), there was a timepoint effect for white blood cells (WBC; 10^3/µL), lymphocytes (10^3/µL), eosinophils (10^3/µL), neutrophil/lymphocyte ratio, basophils (10^3/µL), and red blood cells (RBC; 10^6/µL; all *P* < 0.01; Table 3.3). Compared to prior to the LPS challenge, WBC, lymphocytes, and RBC increased after

the LPS challenge (all $P < 0.001$). Eosinophils, basophils, and neutrophil/lymphocyte ratio decreased after the LPS challenge (all $P < 0.05$). There were no treatment or treatment by timepoint interactions for complete blood counts ($P \geq 0.20$).

3.4.3 Blood Parameters during LPS Challenge

A treatment by timepoint interaction was observed for TNF-α (Figure 3.3; $P = 0.005$) and glucose (Figure 3.4; $P = 0.02$). At 1 and 1.5 h after dosing with LPS, SCFP calves had increased concentrations of TNF-α in circulation compared to CON calves ($P \leq 0.02$). At 0.5 h after dosing with LPS, SCFP calves had increased concentrations of serum glucose compared to CON calves ($P = 0.02$). A treatment by timepoint tendency was observed for SAA, where SCFP calves had greater SAA concentrations at various timepoints after dosing with LPS ($P = 0.10$; Table 3.4). No treatment by timepoint interactions were observed for NEFA, IL-6, IFN-γ, Hp, fibrinogen, and cortisol ($P > 0.10$). No treatment differences were observed for NEFA, TNF-α, IL-6, IFN-γ, Hp, fibrinogen, cortisol, or glucose based on overall treatment averages ($P > 0.10$). However, a treatment tendency was observed for SAA ($P = 0.10$). There was a timepoint effect for NEFA, TNF-α, IL-6, IFN-γ, SAA, fibrinogen, cortisol, and glucose ($P < 0.05$).

3.5 Discussion

The overall findings from this study were that SCFP calves had a greater acute immune response after dosing with LPS, however, this increased response only lasted approximately 2 h. This was evident by increased respiration rate, TNF-α, and glucose within the first 2 h after dosing with LPS and reduced intake the day of the challenge.

An LPS challenge is designed to mimic a bacterial infection, causing the innate immune system to be activated. However, during an LPS challenge the animal is not actually exposed to bacteria, just LPS, a component in the cell wall of gram-negative bacteria, therefore the immune response is acute and short-lived. When the immune system is activated, a variety of inflammatory mediators (cytokines and acute-phase proteins) are stimulated, which can result in an increase in body temperature (Dinarello, 1996), which explains why an increase in rectal temperature shortly after dosing with LPS was observed in all calves in this study. Temperature increased quickly and peaked 3.5 h after dosing with LPS and then returned to values similar to pre-challenge 7 h after

dosing. A study looking at the acute immune response of pure-bred Angus steers after an LPS challenge, observed a similar rectal temperature response where values increased shortly after dosing with LPS, peaked 4.5 h, and at 8 h after dosing were still elevated compared to pre-challenge values (Carroll et al., 2009). This same increased temperature response shortly after dosing with LPS was observed in other studies as well (Steiger et al., 1999; Waldron et al., 2003). The current study, however, did not observe a treatment by timepoint interaction for temperature, dissimilar to a previous studies evaluating SCFP. Burdick Sanchez et al. (2020) fed SCFP to weaned beef steers (274 ± 1.9 kg BW) and observed a treatment by time interaction for rectal temperature. They observed increased rectal temperature with SCFP fed calves at 1 h after dosing with LPS (0.25 µg/kg BW). They observed treatment difference in rectal temperature again at 6-11 h, 13 h, 15-20 h, and 22-24 h post challenge although the temperature during these time points were equal to or lower than basal temperature prior to the challenge.

During periods of stress, like an immune challenge, the adrenal gland will release adrenaline and increase respiration rates. In cattle, the major anaphylactic shock organ is the lung (Eyre et al., 1973). The current study observed an increase in respiratory rate for all calves, which peaked 0.5 h after dosing with LPS and returned to pre-challenge values 3.5 h after. Carroll et al. (2009) also observed similar respiration rate responses in Angus steers that had a sharp increase 0.5 h after dosing with LPS which then returned to pre-challenge rates at 2.5 h. The current study also observed that SCFP calves had an increase in respiration rate compared to CON calves, 0.5 and 1.5 h after dosing with LPS. This suggest that SCFP fed calves responded to the LPS challenge quickly and at a greater rate compared to CON calves. However, the increased response was not prolonged as no differences in respiration rate were observed for any time points after 3.5 h following LPS dosing. Burdick Sanchez et al. (2020) did not report respiration rates, however, they did report sickness behavior scores, which is based on both behavior and respiration rate, with 1 being normal behavior and 4 being laying on side with labored breathing and frothing at the mouth. They reported that SCFP calves tended to have lower sickness behavior scores compared to CON calves at 1-2 h and 3.5 h after dosing with LPS, they did mention that overall sickness behavior scores were relatively low in both treatments. The discrepancy about how SCFP affects respiration rates between Burdick Sanchez et al. (2020) and the current study could be due to the age, breed, diet, LPS dose, and length of treatment of the calves. In the current study, calves were

younger and smaller when they experienced the LPS challenge compared to the calves in Burdick Sanchez et al. (2020).

Even though the current study did not observe increased rectal temperature in SCFP calves following an LPS challenge, this response has been observed prior (Burdick Sanchez et al., 2020). The current study did however observe an increased respiration rate in SCFP calves compared to CON. An increase in respiration rate at the same time as increased temperature, is reasonable because it has been reported that the two are correlated (Scharf et al., 2010). These responses following an LPS challenge would suggest an increased acute immune response. An increase in body temperature has been shown to reduce the growth of bacteria and increase animal survivability, as increased body temperature reduces the ability of pathogenic bacteria to grow (Kluger and Rothenburg, 1979; Kluger et al., 1998). The lack of a treatment effect or a treatment by timepoint interaction for rectal temperature in the current study may be due to the timing and quantity of timepoints analyzed and the briefness of an acute immune response. However, it is worth noting that a prolonged increase in body temperatures can have a negative impact on energy stores in the body and could be detrimental to survivability (Carroll and Forsberg, 2007).

On the day of the LPS challenge, SCFP calves consumed less starter compared to the CON calves. This difference in intake, based on treatment, was not observed prior to the challenge (d 49) or in the days after the challenge (d 51-54). Anorexia (decreased appetite) is a defense mechanism used by hosts to reduce feed intake and therefore the entry of the pathogen across the lumen into circulation (Exton, 1997). To the best of the author's knowledge, no other study reported reduced intake in SCFP calves compared to CON during an LPS challenge. However, other studies have reported reduced intakes in general in cattle during an LPS challenge (Werling et al., 1996; Steiger et al., 1999; Waldron et al., 2003). Also, acute immune response induced stress can often lead to a reduction in feed intake (Carroll and Forsberg, 2007). In this study, the observed response was a sharp increase in the stress hormone cortisol in circulation 0.5 h after dosing with LPS which progressively decreased over the next 24 h.

White blood cells are the immune cells of the body and include eosinophils, basophils, monocytes, lymphocytes, and neutrophils. Red blood cells help to regulate the homeostasis of chemokines (Anderson et al., 2018), which are chemoattractant cytokines that attract immune cells to the site of infection (Delves and Roitt, 2000). Therefore, observing an increase in immune cells and RBC in circulation after an immune challenge is typical as the body calls upon them to prompt

an effective immune response. Research in weaned pigs using the LPS challenge model observed a steady increase in WBC from 2 to 24 h after dosing with LPS (Burdick Sanchez et al., 2018). Another study reported WBC counts in non-lactating dairy cows exposed to different amounts of LPS from 0 to 144 h after dosing (Jacobsen et al., 2005). They observed elevated WBC from 24 to 96 h after dosing when cows received 100 or 1000 ng of LPS/ kg of BW, which aligns with the results of increased WBC at 48 h after LPS dosing in this study.

When the immune system is activated, TNF-α, a pro-inflammatory cytokine is secreted. Therefore, it is expected to see an increase in circulating TNF-α during an LPS challenge (Carroll et al., 2009; Benjamin et al., 2016; Burdick Sanchez et al., 2020). The cytokine TNF-α signals inflammation which leads to heat production by impacting the thermoregulatory centers in the brain (Dinarello, 1996). This study observed a treatment by timepoint interaction for TNF- α, where SCFP calves had increased TNF-α concentrations soon after dosing with LPS. Although we did not see a treatment or treatment by timepoint effect for rectal temperature, a timepoint effect was observed for rectal temperature, which increased in calves soon after dosing with LPS. Burdick Sanchez (2020) reported increased body temperature in SCFP calves but decreased TNF-α and were therefore unclear what was causing the increased body temperature. However, their TNF-α concentrations were relatively low compared to this study and what others have reported (Waldron et al., 2003; Carroll et al., 2009; Benjamin et al., 2016). This variability in TNF- α concentrations is likely due to differences in the dose of LPS used across studies as well as the health status and age of animals. Even though IL-6 concentrations were not statistically different between treatment, likely due to the large variation observed, SCFP calves did have numerically increased IL-6 compared to CON. Increases in IL-6 combined with elevated TNF- α, SAA, and respiration rate in SCFP calves supports that SCFP calves had a different innate immune response following the LPS challenge than CON calves.

Shortly after dosing, an increase in circulating glucose concentrations in SCFP calves was observed. Glucose is the main energy source for the immune system (Wolowczuk et al., 2008), so this increase in glucose could be the body's response to an activated immune system and ensuring enough energy to elicit an immune response to the foreign invader (Lang et al., 1985). This study did observe a treatment by timepoint interaction for glucose, SCFP calves had numerically increased glucose concentrations for most of the post-challenge period. This numerical increase in glucose observed in the SCFP calves compared to the CON, coupled with an increase in respiration

rate and TNF-α, as well as reduced intake, all suggest an increased acute immune response by the SCFP calves in response to the LPS challenge. Burdick Sanchez et al. (2020) also reported greater glucose in SCFP claves at various timepoints during the post-challenge period, which was believed to be a glucose sparing effect by the SCFP calves, but an area that requires further research.

Further research is needed to understand the mechanism behind how SCFP influences the innate immune system. While not specifically addressed here, another area where knowledge is limited is at what point an increase in the responsiveness of the immune system becomes detrimental to the growth and survivability of the animal, by diverting excessive amounts of energy to the immune system.

3.6 Conclusion

In summary, during an LPS challenge, calves supplemented with SCFP experienced a greater innate immune response. This was evident by an increase in circulating TNF-α, which promotes inflammation and protects the body from foreign invaders. The stress of an activated immune system led to increased respiration rates and a reduction in feed intakes. Calves on the SCFP treatment also had increased circulating glucose directly following the LPS challenge. These results suggest that supplementing calves with SCFP increases the acute immune response of calves. More research is needed to determine if SCFP improves health outcomes in animals challenged with specific pathogens that elicit an immune response.

3.7 Acknowledgements

Conflict of interest: IY is employed of Diamond V, Cedar Rapids, Iowa. This research was funded by Diamond V® (Cedar Rapids, IA). The authors acknowledge the Purdue Animal Sciences graduate students N. Briggs, T. Hurst, and C. McCabe for their help and dedication throughout the study. The authors would also like to thank Purdue Animal Sciences undergraduates, K. Dager, K. Harmes, M. Keirn, E. Ludwick, K. Teeple, and E. Thrasher, for their contributions to the project and to M. Grott and the remainder of the staff at the Animal Sciences Research and Education Center for making this work possible.

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Table 3.1. Chemical composition of milk replacer (MR) and calf starter for control (CON; n=15) and *Saccharomyces cerevisiae* fermentation products (SCFP; n=15) experimental treatment diets[1].

Item	MR	CON Starter	SCFP Starter[2]
Dry Matter, %	96.6±0.1	86.6±0.3	86.6±0.5
CP, % DM	25.2±0.3	23.7±2.2	21.7±1.2
Fat, % DM	17.8±0.2	3.6±0.2	3.4±0.6
ADF, % DM	.	10.7±0.7	10.3±0.5
aNDF, % DM	.	18.4±0.5	19.0±1.1
Ash, % DM	8.7±0.1	7.8±0.8	7.3±0.5

[1]CON (MR and calf starter with no SCFP added) or SCFP (MR with 1g/d of SmartCare® (Diamond V, Cedar Rapids, IA) and calf starter with NutriTek® (Diamond V, Cedar Rapids, IA).
[2]Contained 0.8% NutriTek®

Table 3.2. Starter intake of control (CON; n=10) and *Saccharomyces cerevisiae* fermentation products (SCFP; n=9) calves in relation to the lipopolysaccharide (LPS) challenge (d 50).

Intake in relation to LPS challenge[2] (g)	Treatments[1]		SEM	*P*-value
	CON	SCFP		
d-1	1,267	1,093	198	0.54
d0	1,328	567	148	0.002
d1	1,469	1,346	109	0.42
d2	1,884	1,891	89	0.95
d3	1,866	1,819	90	0.71
d4	1,890	1,871	94	0.89

[1]CON (24% CP: 17% fat milk replacer and calf starter with no SCFP added) or SCFP (24% CP: 17% fat milk replacer with 1 g per day of SmartCare® (Diamond V, Cedar Rapids, IA) and calf starter with 0.8% NutriTek® (Diamond V, Cedar Rapids, IA).
[2]Calves received 0.125 µg/kg of BW of lipopolysaccharide from *Escherichia coli* (O111:B4; Sigma L4391, Sigma Aldrich, St. Louis, MO) intravenously.

Table 3.3. Complete blood count of control (CON; n=10) and *Saccharomyces cerevisiae* fermentation products (SCFP; n=9) calves pre- (d 49) and post- (d 52) lipopolysaccharide (LPS) challenge (d 50).[1]

	Treatments[2]			*P*-value		
Items	CON	SCFP	SEM	Treatment	Timepoint	Treatment × Timepoint
White Blood Cells (10^3/µL)				0.95	<0.001	0.42
Pre-LPS	6.97	7.27	0.48			
Post-LPS	8.62	8.39	0.50			
Neutrophils (10^3/µL)				0.63	0.92	0.33
Pre-LPS	3.53	3.96	0.31			
Post-LPS	3.75	3.68	0.32			
Lymphocytes (10^3/µL)				0.50	<0.001	0.68
Pre-LPS	2.64	2.52	0.25			
Post-LPS	4.26	3.97	0.26			
Neutrophils: Lymphocytes				0.24	<0.001	0.20
Pre-LPS	1.34	1.64	0.11			
Post-LPS	0.94	0.94	0.12			
Monocytes (10^3/µL)				0.84	0.93	0.79
Pre-LPS	0.59	0.59	0.06			
Post-LPS	0.57	0.60	0.06			
Eosinophils (10^3/µL)				0.96	<0.001	0.74
Pre-LPS	0.18	0.17	0.02			
Post-LPS	0.03	0.03	0.02			
Basophils (10^3/µL)				0.33	<0.001	0.41
Pre-LPS	0.03	0.04	0.00			
Post-LPS	0.01	0.01	0.01			
Red Blood Cells (10^6/µL)				0.81	0.01	0.88
Pre-LPS	7.69	7.66	0.20			
Post-LPS	8.23	8.15	0.21			

[1]Calves received 0.125 µg/kg of BW of lipopolysaccharide from *Escherichia coli* (O111:B4; Sigma L4391, Sigma Aldrich, St. Louis, MO) intravenously.

[2]CON (24% CP: 17% fat milk replacer and calf starter with no SCFP added) or SCFP (24% CP: 17% fat milk replacer with 1 g per day of SmartCare® (Diamond V, Cedar Rapids, IA) and calf starter with 0.8% NutriTek® (Diamond V, Cedar Rapids, IA).

Table 3.4. Serum analysis of metabolites, cytokines, and acute-phase proteins of control (CON; n=10) and *Saccharomyces cerevisiae* fermentation products (SCFP; n=9) calves during the lipopolysaccharide (LPS) challenge (d 50).[1]

	Treatments[2]			*P*-value		
Items	CON	SCFP	SEM	Treatment	Timepoint	Treatment × Timepoint
Non-esterified fatty acid (mEq/L)	0.221	0.242	0.016	0.36	<0.001	0.40
Tumor necrosis factor-α (pg/mL)	440.9	701.7	148.8	0.22	<0.001	0.005
Interleukin-6 (pg/mL)	154.7	308.7	97.52	0.27	<0.001	0.26
Interferon-γ (pg/mL)	3.691	4.286	1.550	0.79	<0.001	0.51
Haptoglobin (μg/mL)	2,401	2,564	315.9	0.72	0.10	0.62
Serum amyloid-A (pg/mL)	2,990	3,864	355.2	0.10	0.03	0.10
Fibrinogen (mg/mL)	11.34	13.59	1.065	0.15	0.01	0.16
Cortisol (ng/mL)	232.9	275.2	17.93	0.11	<0.001	0.71
Glucose (mg/dL)	89.2	106.5	12.1	0.33	<0.001	0.02

[1]Calves received 0.125 μg/kg of BW of lipopolysaccharide from *Escherichia coli* (O111:B4; Sigma L4391, Sigma Aldrich, St. Louis, MO) intravenously.

[2]CON (24% CP: 17% fat milk replacer and calf starter with no SCFP added) or SCFP (24% CP: 17% fat milk replacer with 1 g per day of SmartCare® (Diamond V, Cedar Rapids, IA) and calf starter with 0.8% NutriTek® (Diamond V, Cedar Rapids, IA).

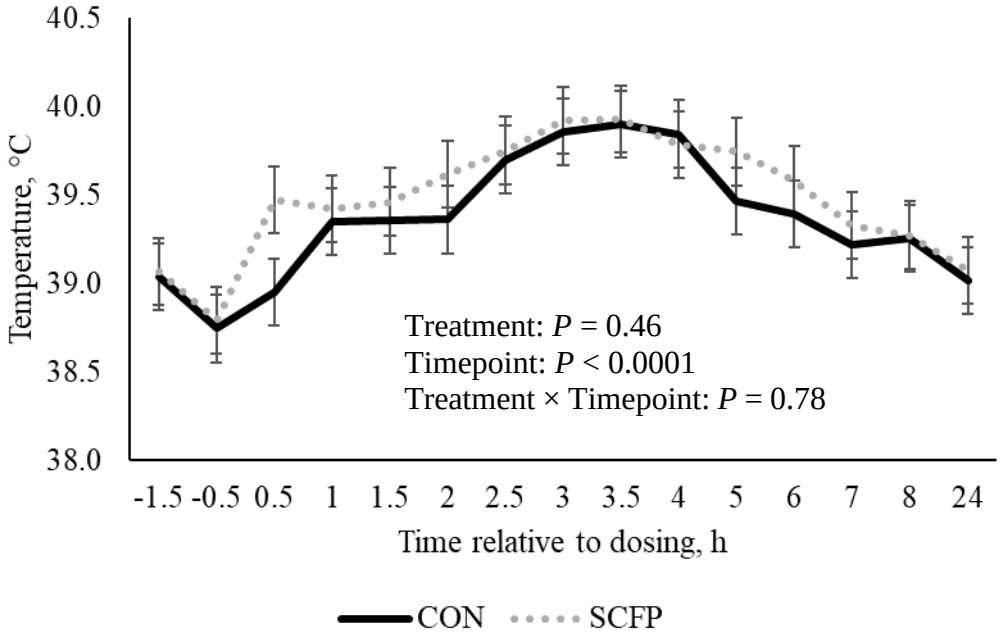

Figure 3.1. Rectal temperature by treatment in relation to lipopolysaccharide challenge dosing. Calves were assigned to one of two treatments, CON (24% CP: 17% fat milk replacer and calf starter with no SCFP added; n=10) or SCFP (24% CP: 17% fat milk replacer with 1 g per day of SmartCare® (Diamond V, Cedar Rapids, IA) and calf starter with 0.8% NutriTek® (Diamond V, Cedar Rapids, IA); n=9). Error bars represent SEM.

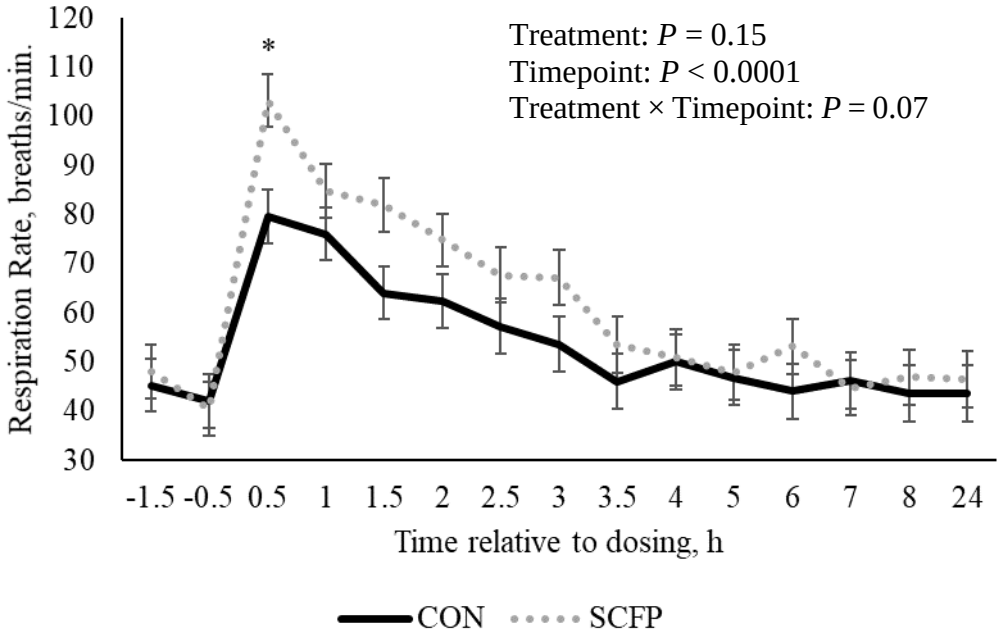

Figure 3.2. Respiration rate by treatment in relation to lipopolysaccharide challenge dosing. Calves were assigned to one of two treatments, CON (24% CP: 17% fat milk replacer and calf starter with no SCFP added; n=10) or SCFP (24% CP: 17% fat milk replacer with 1 g per day of SmartCare® (Diamond V, Cedar Rapids, IA) and calf starter with 0.8% NutriTek® (Diamond V, Cedar Rapids, IA); n=9). * Indicates a statistically significant difference ($P \leq 0.05$) between treatments. Error bars represent SEM.

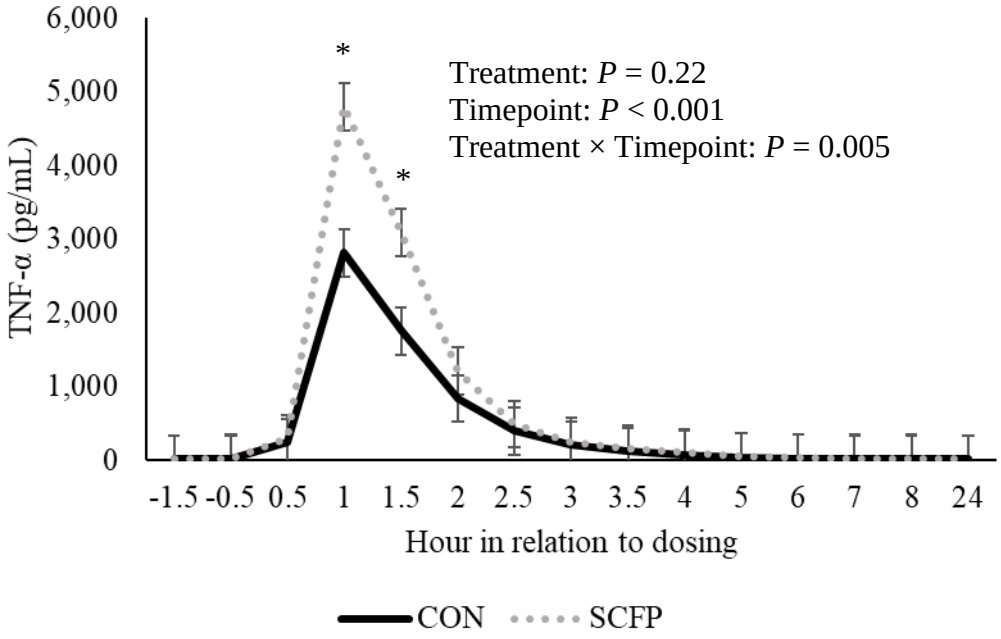

Figure 3.3. TNF-α concentrations by treatment in relation to lipopolysaccharide dosing. Calves were assigned to one of two treatments, CON (24% CP: 17% fat milk replacer and calf starter with no SCFP added; n=10) or SCFP (24% CP: 17% fat milk replacer with 1 g per day of SmartCare® (Diamond V, Cedar Rapids, IA) and calf starter with 0.8% NutriTek® (Diamond V, Cedar Rapids, IA); n=9). * Indicates a statistically significant difference ($P \leq 0.05$) between treatments. Error bars represent SEM.

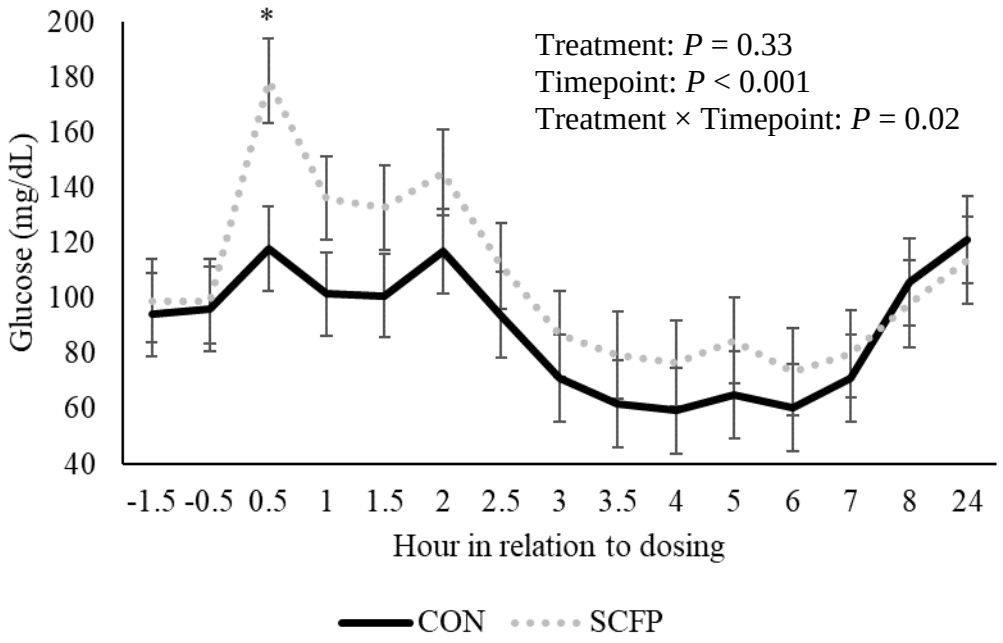

Figure 3.4. Glucose concentrations by treatment in relation to lipopolysaccharide dosing. Calves were assigned to one of two treatments, CON (24% CP: 17% fat milk replacer and calf starter with no SCFP added; n=10) or SCFP (24% CP: 17% fat milk replacer with 1 g per day of SmartCare® (Diamond V, Cedar Rapids, IA) and calf starter with 0.8% NutriTek® (Diamond V, Cedar Rapids, IA); n=9). * Indicates a statistically significant difference ($P \leq 0.05$) between treatments. Error bars represent SEM.

CHAPTER 4. EFFECTS OF FEEDING MEDIUM CHAIN FATTY ACIDS OR A BLEND OF FATTY ACIDS ON THE GROWTH, HEALTH, ENERGY STATUS, AND ADAPTIVE IMMUNE RESPONSE OF HOLSTEIN DAIRY CALVES

Klopp, R. N., J. F. Hernandez Franco, H. HogenEsch, T. S. Dennis, K. E. Cowles and J. P. Boerman. Effects of feeding medium chain fatty acids on the growth, health, energy status, and adaptive immune response of Holstein dairy calves. Manuscript in preparation for *J. Dairy. Sci.*

4.1 Abstract

There is a necessity in the dairy industry to reduce calf morbidity and mortality, as well as reduce reliance on antibiotics to treat sick calves, due to the growing concern regarding antibiotic resistance bacteria. The primary objective of this study was to evaluate the effect that feeding dairy calves medium chain fatty acids (**MCFA**) has on growth performance and health through 60 d of age. The secondary objective of this study included measuring the effect of feeding calves MCFA on energy balance around weaning and the adaptive immune response following a vaccine challenge at 3 wk of age. Fifty Holstein bull calves (5 ± 1.6 d of age) were randomly assigned to one of three treatments. Control (**CON)** calves were fed MR with no C8:0/C10:0 oil added, MCFA calves were fed MR with 0.5% C8:0-C10:0 oil added, and NeoTec plus medium chain fatty acid (**NT+MCFA)** calves were fed MR with NeoTec5g™ (Provimi, Brookville, OH) and 0.5% C8:0-C10:0 oil added. Calves were offered 0.37 kg of MR twice daily from d 1 to 7, 0.50 kg of MR twice daily from d 8 to 42, 0.50 kg of MR once daily from d 43 to 49 and were weaned on d 50. Body weight (**BW**) and average daily gain (**ADG**) were measured weekly. Feed efficiency (**FE**; gain/feed) was calculated based on total BW gain and total intake for the duration of the study. Change in body condition score (**BCS**), hip width (**HW**), hip height (**HH**), heart girth (**HG**), and paunch girth (**PG**) were calculated based on d 1 and d 57 measurements. Fecal scores were recorded daily, scouring days were calculated as the number of d with a fecal score > 2, and all medical treatments were documented for the duration of the trial. On d 42, 49, and 56, a serum sample was collected from each calf and used to measure NEFA, BHBA, insulin, and glucose concentrations, in order to evaluate energy status around weaning. A subset of 11 calves from each treatment were enrolled in a vaccine challenge. On d 16/17, deep nasopharyngeal swabs and fecal

samples were collected to determine viral and bacterial shedding and calves were vaccinated intramuscularly with 1 ml of endotoxin-free ovalbumin (**OVA**) mixed with aluminum hydroxide (**AH**) adjuvant. On d 37/38 fecal samples and deep nasopharyngeal swabs were collected again, blood samples were collected and used to analyze OVA-specific immunoglobulin G1 (**IgG1**) and immunoglobulin G2 (**IgG2**), and calves were vaccinated a second time. On d 51/52, fecal samples, deep nasopharyngeal swabs, and blood samples were collected again but in addition, another blood sample was collected and used to measure interferon-γ (**IFN-γ**) and interleukin 4 (**IL-4**) secreted from peripheral blood mononuclear cells (**PBMCs**) treated with OVA or phytohemagglutinin (**PHA**). Data were analyzed as a completely randomized block design with repeated measures when applicable. No treatment effects were observed for BW, ADG, FE, BCS change, HW change, HH change, HG change, PG change, scouring days, or BRD treatments (all $P \geq 0.25$). A tendency for greater daily fecal score was observed for MCFA calves compared to CON ($P = 0.08$). At d 42, NEFA was greater in CON calves compared to MCFA ($P = 0.001$) and greater in NT+MCFA calves compared to MCFA ($P = 0.05$). At d 49, NEFA was greater in NT+MCFA calves compared to CON ($P = 0.02$) and tended to be greater in NT+MCFA calves compared to MCFA ($P = 0.06$). On d 37/38 and 51/52, anti-OVA IgG1 concentrations for CON, MCFA, and NT+MCFA calves were greater than pre-vaccination samples ($P \leq 0.0001$). On d 37/38, anti-OVA IgG2 concentrations for CON calves were increased compared with pre-vaccination samples ($P \leq 0.01$). On d 51/52, anti-OVA IgG2 concentrations for both CON and MCFA calves were greater than pre-vaccination samples ($P \leq 0.01$). There were no significant treatment differences for IFN-γ or IL-4 secretion ($P > 0.05$) upon stimulation with OVA or PHA. This study suggests that feeding MCFA and NT+MCFA impacts the energy balance of calves around weaning and vaccinating dairy calves with ovalbumin combined with an AH adjuvant at 3 week of age is an effective way to evaluate the adaptive immune responses.

4.2 Introduction

In the dairy industry, pre-weaned heifers are highly susceptible to both disease and death. The most recent estimates of pre-weaned calf morbidity and mortality in the United States were conducted as part of the USDA National Animal Health Monitoring System's (**NAHMS**) Dairy 2014 study. Of the 2,545 calves from 104 farms in 13 states enrolled in that nationwide survey, morbidity affected 33.8% of calves and mortality affected 5.0% (USDA, 2016). These estimates

were down from the USDA NAHMS Dairy 2007 study, which reported calf morbidity at 38.5% and mortality at 7.8% (USDA, 2010). However, the most recent numbers are still higher than the target rates for preweaned heifer morbidity and mortality, which are 25 and 5%, respectively (Dairy Calf and Heifer Association, 2016) indicating there are still opportunities for improvement regarding calf health and management. In addition, concerns regarding antibiotic resistance in animal and human medical fields have led to a ban on the feeding of antibiotics to animals to promote growth in many countries (Casewell et al., 2003; Qiao et al., 2018) or requiring medically important antimicrobials to be authorized by a licensed veterinarian (US Food and Drug Administration, 1996). This has led to the need for alternative strategies to reduce calf morbidity and mortality to decrease reliance on antibiotics.

Feeding fatty acids (**FA**) like butyrate (C4:0), medium chain fatty acids (**MCFA**; C6:0 to C12:0), and linolenic acid (C18:3) to calves has been shown to improve calf performance, health, and immune status. For example, supplementing milk replacer (**MR**) and calf starter with a blend of FA (butyrate, MCFA, and linolenic acid) increased average daily gain (**ADG**), increased feed efficiency (**FE**), and decreased scouring in dairy calves (Hill et al., 2009; Hill et al., 2011a; Hill et al., 2011b). Although some studies have evaluated the impacts of FA specifically on the immune system and observed increased serum titers after vaccination (Hill et al., 2011a; Hill et al., 2011b; Esselburn et al., 2013), more research is needed to fully understand how FA improve the health status of calves. Both C8:0 (caprylic acid) and C10:0 (capric acid) have been reported to have antimicrobial properties *in vitro* (Hristov et al., 2004; Zentek et al., 2011), however, their effect in calves is limited. Mills et al. (2010) fed calves a milk replacer high in C8:0 and C10:0, but observed less ADG compared to control calves, and no effects on total empty body composition.

The primary objective of this study was to evaluate the effects of feeding pre-weaned dairy calves MCFA (C8:0 and C10:0) or a blend of butyrate, MCFA, and linolenic acid for 56 d on the adaptive immune response following a vaccine challenge. The secondary objective was to investigate whether feeding pre-weaned calves C8:0 and C10:0 or a blend of butyrate, MCFA, and linolenic acid for 56 d enhances ADG, FE, fecal scores, and medical treatments. The hypothesis of this study was that calves receiving C8:0 and C10:0 would have an improved adaptive immune response as assessed by antibody and cytokine production, leading to improved growth and health status compared to calves not supplemented with MCFA. Additionally, those calves receiving a

blend of fatty acids would have a further improvement in adaptive immune responses, increased growth performance, and reduced health treatments.

4.3 Materials and methods

4.3.1 Animals and Facility

All animals were cared for as described in the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (FASS, 2020). Fifty Holstein bull calves (5 ± 1.6 d of age; mean ± SD) with an initial BW of 42.3 kg (SD = 3.7 kg), were received from a single commercial calf raising facility. At their birth farms, calves received 3 L of pooled colostrum within two h of birth and another 3 L, 12 h later. Thereafter, calves received 2 L of pasteurized whole milk twice daily until they were transported approximately 3.5 h to the Provimi Nurture Research Center. The first PM (d 0) and the following AM (d 1) feeding, calves were fed 0.37 kg of milk replacer (**MR**; 24% CP: 19% fat on DM basis; 13% solids). Calves were housed in individual pens (1.2 m × 2.4 m) located inside the research facility with natural ventilation, curtain sides, and no added heat. Pens had a rock tile-drained base and were bedded with wheat straw. This research trial took place from October 2020 to December 2020. The average temperature in the nursery was 5.7 ºC (range from -8 to 26ºC) with an average humidity of 71% (range from 13 to 93%).

4.3.2 Treatments

Calves were randomly assigned to one of three treatments; control (**CON)** calves were fed MR with no C8:0/C10:0 oil added, MCFA calves were fed MR with 0.5% C8:0-C10:0 oil added, or NeoTec plus medium chain fatty acid (**NT+MCFA)** where calves were fed MR with NeoTec5g™ (Provimi, Brookville, OH) and 0.5% C8:0-C10:0 oil added. Starting at the d 1 PM feeding, calves were offered 0.37 kg of MR twice daily (0630 and 1530 h) from d 1 to 7, 0.50 kg of MR twice daily from d 8 to 42, and 0.50 kg of MR once daily (0630 h) from d 43 to 49. Calves were weaned on d 50. Calves received *ad libitum* access to a texturized calf starter and water for the duration of the study (d 1- 57). Starter and MR refusals were recorded daily and once per week a sample of each MR and starter was collected for analysis for the duration of the study. Feed analysis was performed by Cumberland Valley Analytical Services (Waynesboro, PA) and

reported in Table 4.1. Feeds were analyzed according to AOAC International (2016) for DM (oven method 930.15), ash (oven method 942.05), CP (Kjeldahl method 988.05), and fat ether extract (alkaline treatment with Roese-Gottlieb method 932.06 for milk replacer powder and diethyl ether extraction method 2003.05 for starters). Starters were also analyzed for ADF with ash (Robertson and Van Soest, 1981), NDF with ash (Van Soest et al., 1991), starch (α-amylase method; Hall, 2009), and sugar (colormetric method; DuBois et al., 1956).

4.3.3 Serum Total Protein, Performance, and Health Measurements

The day after arrival (d 1), a 10 mL blood sample from each calf was collected by jugular venipuncture into evacuated tubes without anticoagulant. Blood samples were allowed to clot for 1 h and then serum was harvested by centrifugation at 3,000 × *g* for 15 min at room temperature (VWR, Batavia, IL) and used to estimate serum total protein (**STP**; 6.0 ± 0.61 g/dL) using an optical refractometer (MISCO, Solon, OH). Calf BW was measured on d 1 and every 7 d thereafter and was used to calculate average daily gain (**ADG**). Feed efficiency (**FE;** gain/feed) was also calculated based on total BW gain and total intake for the duration of the 57-d study. Body condition score (**BCS**), hip width (**HW**), and hip height (**HH**) were measured biweekly starting on d 1. Heart girth (**HG**) and paunch girth (**PG**) were measured at the start (d 1) and the end of the study (d 57). Change in BCS, HW, HH, HG, and PG were calculated based on d 1 and d 57 measurements. Calf fecal scores were recorded daily on a scale of 1 to 5 (1 = firm, normal, 2 = less firm, normal, 3 = thick, batter-like, 4 = thin, batter-like, 5 = watery); modified from Kertz and Chester-Jones (2004). Scouring days were calculated as the number of d with a fecal score > 2. All medical treatments were documented for the duration of the trial. Medical days are the number of days that a calf received an antibiotic treatment. Bovine respiratory disease complex (**BRD**) treatments are the number of incidences that a calf was treated for BRD with *tulathromycin injection* (DRAXXIN®, Zoetis, Parsippany, NJ), *florfenicol and flunixin meglumine* (Resflor Gold®, Merck Animal Health, Kenilworth, NJ), or *ceftiofur hydrochloride* (EXCENEL® RTU EZ, Zoetis, Parsippany, NJ). Treatments were in accordance with veterinarian oversight and standard operating procedures.

4.3.4 Blood Metabolite Analysis

On d 42, 49, and 56, a blood sample (10 mL evacuated tubes without anticoagulant) was collected by jugular venipuncture from each calf between 1100 and 1200 h. Blood samples were centrifuged at 3,000 × *g* for 15 min at room temperature (VWR, Batavia, IL). Serum was harvested and four aliquots of serum were frozen at -20°C until further analysis. Serum concentrations of NEFA were analyzed in duplicate using the Wako HR Series NEFA-HR(2) enzymatic colorimetric method assay protocol (FUJIFILM Wako Diagnostics U.S.A. Corp., Mountain View, CA) and serum concentrations of BHBA were measured using the manufacturer's protocol for the Bovine Beta-Hydroxybutyric Acid ELISA kit (MyBioSource, San Diego, CA). Insulin concentrations were analyzed using the Bovine Insulin ELISA kit following the manufacturer's protocol (ALPCO, Salem, NH) and glucose concentrations were determined using the manufacturer's instructions for the PGO Enzyme colorimetric method assay (Sigma-Aldrich, St. Louis, MO). Intraplate coefficients of variation were 2.60%, 4.38%, 3.00%, and 2.10% for NEFA, BHBA, insulin, and glucose, respectively. Interplate coefficients of variation were 4.46%, 12.03%, 8.83%, and 4.15% for NEFA, BHBA, insulin, and glucose, respectively.

4.3.5 Vaccine Challenge and Analysis

On d 16/17, a subset of 11 calves from each treatment were enrolled in a vaccine challenge. Calves were selected one wk prior to the start of the vaccine challenge based on 4 criteria: antibiotic treatments (CON = 0.2 vs. MCFA = 0.0 vs. NT+MCFA = 0.0), STP value (CON = 6.1 g/dL vs. MCFA = 5.8 g/dL vs. NT+MCFA = 6.4 g/dL), last MR refusal before challenge (CON = 11 d vs. MCFA = 11.5 d vs. NT+MCFA = 11.1 d), and starter consumption (CON = 218 g vs. MCFA = 147 g vs. NT+MCFA = 157 g). In order to minimize potential confounding issues of vaccination and processing time, vaccinations and sample collection were divided over 2 d, where five calves per treatment were vaccinated and sampled d 16 and the remaining six calves per treatment were vaccinated and sampled d 17.

On d 16/17, deep nasopharyngeal swabs and fecal samples were collected to determine viral and bacterial shedding just before calves were vaccinated. Deep nasopharyngeal samples were collected using a double guarded culture swab (33-inch length); swabs were stored in 5 ml of 1× PBS at 4°C until analyzed by the Indiana Animal Disease Diagnostic Laboratory (**ADDL**;

West Lafayette, IN) for a bovine respiratory virus screen PCR, which analyzed samples for bovine corona virus, bovine respiratory syncytial virus, bovine viral diarrhea virus, infectious bovine rhinotracheitis virus, and parainfluenza-3 virus. Fecal samples were collected via rectal stimulation and stored at 4°C until processing by ADDL for a bovine neonatal diarrhea screen PCR, which analyzed samples for bovine corona virus, rota virus A, *E. Coli* K99, *Cryptosporidium*, and *Salmonella*.

Vaccines were prepared by mixing endotoxin-free ovalbumin (**OVA**; InvivoGen, San Diego, CA) and aluminum hydroxide (**AH**) adjuvant (Rehydragel HPA; Chemtrade, Berkeley Heights, NJ) in 1 ml of sterile Tris-buffered saline (pH 7.4) for 1 h at room temperature for a final concentration of 200 μg OVA and 1 mg of Al^{3+} per ml. On d 16/17, calves were injected intramuscularly with 1 ml of the vaccine, with a second booster dose administered on d 37/38 of the study. Thermochron temperature loggers (OnSolution Pty Ltd, Baulkham Hills, Australia) were attached to the underside of the tail of each calf enrolled in the vaccine challenge using Vetrap™ Bandaging Tape (3M, St. Paul, MN) 24 h prior to vaccination through 24 h post vaccination to record body temperature of the calves.

On d 37/38 and 51/52, fecal samples, deep nasopharyngeal swabs, and blood samples were collected. Blood samples (10 mL evacuated tubes without anticoagulant) collected on d 37/38 (21 d post initial injection) and d 51/52 (14 d after the booster injection) were used to analyze OVA-specific immunoglobulin G1 (**IgG1**) and immunoglobulin G2 (**IgG2**) by ELISA as previously described (HogenEsch et al., 1996; Hernandez-Franco et al., 2021). Briefly, 96-well plates were coated with 100 μl/well of OVA (1 μg/ml) overnight at 4°C. The plates were then washed and blocked with 1% BSA in tris-buffered saline with 0.1% Tween 20 (**TBST**) for 1 h at 37°C. Subsequently, the plates were washed and 100 μl serum diluted 1:100 in 1% BSA in TBST was added to the wells in duplicate for 1 h at 37°C. The plates were washed and 100 μl of HRP-conjugated sheep anti-bovine IgG1 and IgG2 (Invitrogen, Carlsbad, CA) was added to each well for 1 h at 37°C. After a final well wash, 100 μl/well TMB substrate (Neogen, Lexington, KY) was added and allowed to react at room temperature in the dark for 5 min. After stopping the reaction with 50 μl/well of 2 M sulfuric acid, the absorbance at 450 nm (OD 450) was measured in a microplate reader (BioTek Instruments, Winooski, VT) to quantify IgG1 and IgG2.

In addition, on d 51/52 (14 d after booster injection), another blood sample (10 mL) was collected from each vaccinated calf in sodium heparin tubes. A total of 8 ml of diluted 1:1 blood

with HBSS (Corning Inc., Corning, NY) was layered over 3 ml of Histopaque-1083 (Sigma-Aldrich, St. Louis, MO) in 15 ml tubes. Density gradient centrifugation was performed at 670 × g for 30 min at 25°C (Jouan Inc., Saint-Herblain, France). Isolated peripheral blood mononuclear cells (**PBMCs**) were washed with RPMI 1640 (Corning Inc., Corning, NY) and centrifuged at 210 × g for 5 min at 25°C. The supernatant was discarded, and cells were resuspended in 2 ml complete RPMI 1640 (RPMI 1640 supplemented with 50 μM 2-ME, 2 mM L-glutamine, 25 mM HEPES, 0.25 μg/ml amphotericin B, 100 U/ml penicillin, and 100 μg/ml streptomycin) containing 10% FBS. The isolated cells were seeded in 1 ml of complete medium at a concentration of 2×10^6 cells/ml and treated with 10 μg/ml OVA, 10 μg/ml phytohemagglutinin (**PHA**, Sigma-Aldrich) as a positive control, and medium only as negative control in 24-well plates for 48 h at 37°C with 5% CO_2. The plate was centrifuged at 210 × g for 5 min at 25°C and the supernatant was collected and used to determine the concentration of secreted interferon-γ (**IFN-γ**) and interleukin 4 (**IL-4**) from PBMCs by ELISA (Invitrogen, Carlsbad, CA).

4.3.6 Statistical Analyses

A power analysis was performed to calculate the sample size needed to detect differences in calf performance measures. With 95% confidence and 80% power, 15 animals per treatment were needed to detect 100 g difference in ADG, 10 animals per treatment were needed to detect 200 g/d difference in starter intake, and 9 animals per treatment were needed to detect 0.05 difference in gain/feed. To account for potential calf mortality during the trial, 16-17 animals per treatment were enrolled.

After arrival, calves were randomly assigned to treatments using initial BW, STP, and location within the barn to minimize variation. During the first week of the trial, one CON calf died and data from that calf was removed from the analysis. Performance, health, blood metabolite, and vaccine challenge antibody and cytokine data were analyzed as a completely randomized design using the MIXED Procedure of SAS v.9.4 with repeated measures when applicable, using Toeplitz (TOEP) as the covariance structure based on minimizing AIC and BIC. Performance, health, blood metabolite, and vaccine challenge data were analyzed for normality using the Shapiro-Wilk test and data were normally distributed ($W > 0.85$). Viral and bacterial shedding data were analyzed as count data using the GENMOD procedure of SAS v.9.4, contrasts (CON vs.

MCFA, CON vs. NT+MCFA, MCFA vs. NT+MCFA) were used to compare treatment differences. Calf within treatment was our experimental unit for all statistical analysis.

For performance, blood metabolite, and vaccine challenge data with repeated measures, the model was represented as:

$$Y_{ijk} = \mu + T_i + P_j + TP_{ij} + C_{k(i)} + e_{ijk},$$

Where Y_{ijk} is the response variable and μ is the overall mean. The fixed effects included treatment ($\mathbf{T_i}$; CON (n = 15 for performance and blood metabolites, n = 11 for vaccine challenge), MCFA (n = 17, n = 11, respectively), and NT+MCFA (n = 17, n = 11, respectively)), timepoint ($\mathbf{P_j}$; d 1, 8, 15, 22, 36, 43, 50, 57 for BW; wk 1, 2, 3, 4, 5, 6, 7, 8 for ADG; d 42, 49, 56 for NEFA, BHBA, insulin, and glucose; d 37/38, 51/52 for anti-OVA IgG1 and anti-OVA IgG2;), and the interaction between treatment and timepoint ($\mathbf{TP_{(ij)}}$). The random effect of calf nested within treatment ($\mathbf{C_{k(i)}}$) was also included in the model and $\mathbf{e_{ijk}}$ is the error.

For performance, health data, and vaccine challenge without repeated measures, the model was represented as:

$$Y_{ik} = \mu + T_i + C_{k(i)} + e_{ik},$$

Where Y_{ik} is the response variable and μ is the overall mean. The fixed effect was treatment ($\mathbf{T_i}$; CON (n = 15 for performance and blood metabolites, n = 11 for vaccine challenge), MCFA (n = 17, n = 11, respectively), and NT+MCFA (n = 17, n = 11, respectively)), the random effect was calf nested within treatment ($\mathbf{C_{k(i)}}$), and $\mathbf{e_{ik}}$ is the error.

For the temperature data, the model was represented as:

$$Y_{ijkl} = \mu + T_i + P_j + B_k + TP_{ij} + C_{l(i)} + e_{ijkl},$$

Where Y_{ijk} is the response variable and μ is the overall mean. The fixed effect was treatment ($\mathbf{T_i}$; CON (n = 11), MCFA (n = 11), and NT+MCFA (n = 11)), timepoint ($\mathbf{P_j}$; h 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 for temperature), batch ($\mathbf{B_k}$; 1 = d 16/17, 2 = d 37/38), and the interaction between treatment and timepoint ($\mathbf{TP_{(ij)}}$). The random effect of calf nested within treatment ($\mathbf{C_{k(i)}}$) was also included in the model and $\mathbf{e_{ijk}}$ is the error. For all data, a *P*-value ≤ 0.05 was determined to be statistically significant and a *P*-value > 0.05 and ≤ 0.10 was determined to be a statistical tendency.

Antibody and cytokine data are presented as means with standard errors of the means. The statistical significance of differences between groups was determined by one-way ANOVA test followed by a Tukey multiple comparisons test (GraphPad Prism version 9.0.0, San Diego, CA). Statistical significance was identified as *P*-values < 0.05; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

4.4 Results

4.4.1 Fatty Acid Intake

The MCFA calves had greater intakes of C8:0 compared to the CON calves (74.84 vs. 14.78 g; $P < 0.0001$) and greater intakes of C10:0 compared to the CON calves (77.40 vs. 29.07 g; $P < 0.0001$; Table 4.2). The NT+MCFA calves had greater intakes of C8:0 compared to the MCFA and CON calves (133.29 vs. 74.84 vs. 14.78 g, respectively; $P < 0.0001$) and greater intakes of C10:0 compared to the MCFA and CON calves (120.30 vs. 77.40 vs. 29.07 g, respectively; $P < 0.0001$). The NT+MCFA calves also had greater intakes of C4:0 and C18:3w3 compared to both MCFA and CON calves (44.92 vs. 21.24 vs. 22.86 g and 45.29 vs. 41.36 vs. 41.49 g, respectively; $P < 0.0001$). Total FA coming from MR was greatest in NT+MCFA calves (7.53 g), followed by CON (7.24 g), with MCFA calves being the lowest (7.14 g; $P \leq 0.02$). There was no difference in MR intake between CON, MCFA, and NT+MCFA calves ($P = 0.31$). Due to the presence of FA in calf starter, Table 4.3 shows the total combined (MR and calf starter) FA intake based on study treatment. Intake of C8:0 and C10:0 was greatest in NT+MCFA calves based on total combined FA intake followed by MCFA and last CON calves (133.69 vs. 75.49 vs. 19.32 g and 120.30 vs. 77.40 vs. 30.45 g, respectively; $P < 0.0001$). The NT+MCFA calves had greater total combined FA intake compared to CON and MCFA calves ($P \leq 0.03$). There were also no differences in starter intake and total intake (MR and starter) between CON, MCFA, and NT+MCFA calves ($P \geq 0.33$).

4.4.2 Growth, Feed Efficiency, and Health

A timepoint effect was observed for BW ($P < 0.0001$), which steadily increased throughout the duration of the study (Figure 4.1). A timepoint effect was also observed for ADG ($P < 0.0001$), which increased from wk 1 to wk 6, decreased at wk 7, and then increased again during wk 8

(Figure 4.2). No treatment or treatment by timepoint interaction was observed for BW or ADG ($P \geq 0.52$). No treatment differences were observed for FE, BCS change, HW change, HH change, HG change, and PG change (Table 4.4; $P > 0.29$). A tendency for greater daily fecal score was observed for MCFA calves compared to CON ($P = 0.08$; Table 4.5), however, there were no differences in scouring days between CON, MCFA, and NT+MCFA calves ($P \geq 0.42$). Medical days tended to be greater for CON calves compared to both MCFA and NT+MCFA calves ($P \leq 0.10$). No differences were observed for BRD treatments between CON, MCFA, and NT+MCFA calves ($P \geq 0.35$)

4.4.3 Blood Metabolite Analysis

The STP of NT+MCFA calves was greater than MCFA calves (Table 4.5; $P = 0.05$). Table 4.6 shows NEFA, BHBA, insulin, and glucose concentrations at d 42, 49, and 56 based on study treatment. At d 42, NEFA was greater in CON calves compared to MCFA (0.29 vs. 0.18 mmol/L, respectively; $P = 0.001$) and greater in NT+MCFA calves compared to MCFA (0.24 vs. 0.18 mmol/L, respectively; $P = 0.05$). At d 49, NEFA was greater in NT+MCFA calves compared to CON (0.25 vs. 0.17 mmol/L, respectively; $P = 0.02$) and tended to be greater in NT+MCFA calves compared to MCFA (0.25 vs. 0.19 mmol/L, respectively; $P = 0.06$). A timepoint effect was observed for NEFA ($P < 0.0001$) and a treatment by timepoint interaction was observed ($P = 0.03$) with NEFA decreasing from d 42 to 56 in CON calves but increasing from d 42 to 49 in MCFA and NT+MCFA calves, and then decreasing from d 49 to 56.

At d 42, BHBA was greater in MCFA calves compared to NT+MCFA (603.4 vs. 542.9 nmol/mL, respectively; $P = 0.02$). A timepoint effect was observed for BHBA ($P = 0.0008$), but no treatment by timepoint interaction was observed ($P = 0.18$). At d 49, insulin tended to be greater in CON calves compared to NT+MCFA (1.66 vs. 1.11 ng/mL, respectively; $P = 0.06$). A timepoint effect was observed for insulin ($P < 0.0001$), but no treatment by timepoint interaction was observed ($P = 0.83$). No treatment differences were observed for glucose at any timepoint ($P \geq 0.21$) and no treatment by timepoint interaction was observed for glucose ($P \geq 0.81$). However, a timepoint effect was observed for glucose ($P < 0.0001$), with glucose decreasing from d 42 to 56.

4.4.4 Viral and Bacterial Shedding

On d 37/38 (21 d after initial vaccination; post-vac), more MCFA calves had *Salmonella* detected in feces compared to CON calves (0.27 vs. 0.00; $P = 0.04$; Table 4.7A). No treatment differences were observed for proportion of calves with Bovine Corona Virus, Rota Virus A, or Cryptosporidium in feces ($P \geq 0.20$). Also, no treatment effects were observed for the proportion of calves with Bovine Corona Virus, Bovine Viral Diarrhea, or Parainfluenza-3 from the deep nasopharyngeal swabs ($P \geq 0.24$; Table 4.7B).

4.4.5 Vaccination Challenge: Antibody, Cytokine, and Temperature Analysis

On d 37/38 (21 d post initial vaccination) and on d 51/52 (14 d post booster vaccination), anti-OVA IgG1 concentrations for CON, MCFA, and NT+MCFA calves were greater than pre-vaccination samples ($P \leq 0.0001$; Figure 4.3A and B). A treatment by timepoint interaction was also observed ($P \leq 0.0001$) as anti-OVA IgG1 concentrations of CON, MCFA, and NT+MCFA calves increased from d 37/38 to d 51/52. On d 37/38, serum anti-OVA IgG2 concentrations for CON calves were increased compared with pre-vaccination samples ($P \leq 0.01$; Figure 4.3C). On d 51/52, anti-OVA IgG2 concentrations for both CON and MCFA calves were greater than baseline concentrations ($P \leq 0.01$; Figure 4.3D). Like with anti-OVA IgG1, anti-OVA IgG2 concentrations for CON, MCFA, and NT+MCFA calves increased between d 37/38 to d 51/52 ($P \leq 0.01$). The overall concentration of anti-OVA IgG2 was lower than anti-OVA IgG1. No differences were observed between treatments for IFN-γ secretion by PBMCs upon stimulation with OVA or PHA ($P > 0.05$; Figure 4.4A and B), and no differences were observed between treatments IL-4 secretion by PBMCs upon stimulation with OVA or PHA ($P > 0.05$; Figure 4.4C and D).

During the initial vaccination (d 16/17), calf temperature change from baseline, for the first 12 h, observed no treatment difference ($P = 0.43$) and no treatment by timepoint interaction ($P = 0.27$). However, a timepoint effect ($P < 0.0001$; Figure 4.5) was observed. A batch effect was also observed ($P < 0.0001$), where batch 1 calves (vaccinated on d 16) had increased temperature change from baseline compared to batch 2 calves (vaccinated on d 17). During the booster vaccination (d 37/38), calf temperature change from baseline observed no treatment difference ($P = 0.38$) and no treatment by timepoint interaction ($P = 0.92$). A timepoint effect ($P < 0.0001$;

Figure 4.6) was observed, with temperature fluctuating from h 0 to 12. A batch effect was also observed ($P = 0.007$), where batch 1 calves (vaccinated on d 37) had reduced temperature change from baseline compared to batch 2 calves (vaccinated on d 38).

4.5 Discussion

Based on the FA profile analysis for each treatment, MCFA calves received 60.06 g more of caprylic acid and 48.33 g more of capric acid via MR, compared to CON calves. The NT+MCFA calves received more butyrate, caprylic acid, capric acid, and linolenic acid via MR than CON calves. Previous research that has evaluated the impacts of FA profile on calf performance reported increased ADG and FE (Hill et al., 2009; Hill et al., 2011a; Hill et al., 2011b; Esselburn et al., 2013; Quigley et al., 2019). However, the current study did not see any differences in growth performance between treatments, which is in agreeance with one study that fed calves a MR high in C8:0 and C10:0 and observed lower ADG compared to control calves (Mills et al., 2010). Prior studies also observed reduced scouring when calves were supplemented with FA (Hill et al., 2011a; Hill et al., 2011b; Esselburn et al., 2013), which was not observed in the current study. One reason for these discrepancies could be differences in FA intake between studies. Prior studies have supplemented calves with FA in both MR and calf starter (Hill et al., 2011b) or only in starter (Hill et al., 2009; Quigley et al., 2019), while this study only supplemented FA in the MR. Also, previous studies have supplemented calves using a slightly different FA profile compared to the current study (Hill et al., 2011a; Esselburn et al., 2013). These differences in feeds would affect the total amount of specific FA calves are consuming.

The relative energy status of calves around weaning was evaluated using insulin, glucose, NEFA, and BHBA concentrations. The concentration of NEFA in circulation is reflective of both the relative amount of adipose tissue mobilization (McNamara, 1991) and diet (Zhang et al., 2010). At d 42, CON calves had increased NEFA concentrations compared to MCFA calves, suggesting that CON calves may be mobilizing more adipose tissue to meet energy demands. At d 42, NT+MCFA calves also had increased NEFA concentrations compared to MCFA calves. This indicates that when calves were still receiving MR twice daily, MCFA calves may be mobilizing less adipose tissue to keep up with energy demands compared to both CON and NT+MCFA calves. In calves, MCFA are relatively rapid sources of energy, because MCFA can be absorbed directly in the abomasum and small intestine and travel to the liver without needing to be incorporated into

chylomicrons like long chain fatty acids (Hocquette and Bauchart, 1999). This could potentially explain why MCFA calves mobilized less adipose tissue to meet energy demands compared to CON calves because they increased concentration of a rapid source of energy. It is worth noting that these blood samples were taken about 4 h post AM feeding and therefore are reflective of a time in the day when we would expect calves to be in a fasting state. Also, MCFA MR contained slightly less fat (% of DM) compared to CON and NT+MCFA, which could be influencing the lower serum NEFA in MCFA calves. A prior study conducted using Jersey calves observed that fasting serum NEFA concentrations decreased as dietary MCFA increased (Swank et al., 2013), which is in agreeance with the current study. Concentrations of NEFA also decreased from d 42 to 56. As calves are weaned their energy source changes from glucose and FA in MR to organic acids from the fermentation of calf starter (Chilliard, 1993), which leads to decreased NEFA and has been reported previously (Kmicikewycz et al., 2013).

Serum BHBA concentrations increased from d 42 to 56 in all calves. In calves, BHBA is an indicator of rumen development, which is produced from butyrate as a result of carbohydrate fermentation in the rumen (Quigley et al., 1991), and increased BHBA concentrations in calves during weaning has been reported previously (Bush, 1988; Muya et al., 2015). At d 42, BHBA concentrations were greater in MCFA calves compared to NT+MCFA calves, since BHBA is an indicator of rumen development, the MCFA treatment might be aiding rumen development in calves prior to weaning.

After a calf consumes a meal, during the absorptive phase circulating glucose concentrations increase leading to an increase in insulin production. An increase in insulin results in greater uptake of glucose by the liver for glycogen synthesis and by adipose tissue for lipid synthesis. Hours after a calf consumes a meal, during the post-absorptive phase the uptake of nutrients will diminish, resulting in reduced insulin production, causing glycogen and lipid stores to be mobilized to meet energy needs (Bauman and Currie, 1980). In the current study, at d 49, insulin tended to be greater in CON calves compared to NT+MCFA calves, however, glucose concentration did not differ. This suggests that NT+MCFA is either improving insulin sensitivity, meaning less insulin is needed for glucose uptake or it could mean NT+MCFA changed calves' pattern of eating and therefore influenced glucose to insulin ratios. Both insulin and glucose decreased in all calves from d 42 to 56. A decrease in glucose as calves are weaned and age has been reported previously (Quigley et al., 1991). The differences in insulin, BHBA, and NEFA

concentrations between treatments indicates that calf metabolism and energy balance is influenced by FA profile.

A vaccine challenge is a research model used to evaluate an animal's adaptive immune response and is a common model used in dairy calves (Hill et al., 2011b; Kim et al., 2011; Hill et al., 2012; Esselburn et al., 2013). In the current study, calves were vaccinated with an egg white protein (OVA) combined with an AH adjuvant. The benefit of vaccinating with OVA is to ensure the calf does not have any acquired immunity from natural exposure prior to the vaccine challenge (Husband and Lascelles, 1975). Antibody concentrations at 21 d post initial vaccination and 14 d post booster vaccination were not different based on study treatments. Upon stimulation with OVA and PHA, IFN-γ and IL-4 secretion by PBMCs were also not different between treatments. The authors believed that feeding calves MCFA would improve the adaptive immune response by increasing antibody and cytokine production. As mentioned previously, prior studies supplemented calves with FA in both MR and calf starter, and other studies used slightly different FA profiles compared to the current study. It is possible that calves in the current study did not receive enough MCFA to elicit an increased immune response and improved growth performance.

It is worth noting that anti-OVA IgG1 and anti-OVA IgG2 concentrations post initial vaccination were greater than baseline values and anti-OVA IgG1 and anti-OVA IgG2 concentrations post booster vaccination was greater than concentrations post initial vaccination, indicating that the vaccination was successful and there was a significant booster effect. The immunization induces a greater increase of anti-OVA IgG1 than anti-OVA IgG2. In cattle, the type 2 cytokine IL-4 has been shown to preferentially induce IgG1 production over IgG2, whereas the type 1 cytokine IFN-γ has been shown to preferentially induce IgG2 production over IgG1 (Estes et al., 1994; Estes et al., 1995). The greater production of IgG1 following vaccination is consistent with the role of aluminum adjuvants which drive a Th2-biased immune response in mice and humans (HogenEsch, 2013). However, only low secretion of both IL-4 and IFN-γ was observed following *ex vivo* stimulation of PBMCs with OVA.

4.6 Conclusion

In conclusion, fatty acid profile influenced calf metabolites associated with energy status around weaning, NEFA concentrations were greater in CON and NT+MCFA calves compared to MCFA calves prior to weaning. The current study however did not observe any performance or

health differences when feeding calves MCFA or NT+MCFA. Even though fatty acid profile did not affect antibody or cytokine production following a vaccine challenge, vaccinating calves with ovalbumin combined with an aluminum hydroxide adjuvant is an effective way to evaluate the adaptive immune response in calves. Next steps include feeding calves MCFA or NT+MCFA in calf starter in addition to milk replacer and evaluating the impacts on calf performance and the adaptive immune response, as well as evaluating the effects MCFA or NT+MCFA have on the innate immune system.

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Table 4.1. Chemical composition of experimental treatments (CON vs. MCFA vs. NT+NCFA).

	Treatments[1]		
Item	CON	MCFA	NT+MCFA
Milk Replacer (MR; n = 21)			
DM, % as fed	96.30 ± 0.26	95.97 ± 0.23	95.78 ± 0.22
Ash, % of DM	6.78 ± 0.12	6.72 ± 0.17	6.69 ± 0.16
CP, % of DM	24.17 ± 0.53	24.86 ± 0.44	24.47 ± 0.51
Fat, % of DM	19.30 ± 0.78	18.29 ± 0.52	18.96 ± 0.95
Calf Starter (n = 24)			
DM, % as fed	88.64 ± 0.44	88.80 ± 0.43	88.78 ± 0.54
Ash, % of DM	6.65 ± 0.45	6.85 ± 0.39	6.92 ± 0.49
CP, % of DM	19.46 ± 0.60	19.16 ± 0.50	19.73 ± 0.31
Fat, % of DM	3.74 ± 0.20	3.64 ± 0.16	3.66 ± 0.17
ADF, % of DM	7.19 ± 0.64	7.71 ± 0.25	7.73 ± 0.41
NDF, % of DM	14.56 ± 0.68	15.21 ± 0.42	15.51 ± 0.54
Starch, % of DM	43.18 ± 1.61	43.16 ± 1.30	41.93 ± 0.95
Sugar, % of DM	5.20 ± 1.11	5.05 ± 1.26	4.94 ± 0.99

[1]Treatments: CON = no C8:0-C10:0 oil added to MR; MCFA = 0.5% C8:0-C10:0 oil added to MR; NT+MCFA = 0.5% C8:0-C10:0 oil and NeoTec5g (Provimi, Brookville, OH) added to MR.

Table 4.2. Individual and total fatty acid intake from milk replacer (MR; n = 21) and MR intake of calves from d 1 to 57 based on study treatments (CON vs. MCFA vs. NT+MCFA).

	Treatments[1]				*P*-values		
Item	CON	MCFA	NT+MCFA	SEM	CON vs. MCFA	CON vs. NT+MCFA	MCFA vs. NT+MCFA
Fatty acid							
4:0, g	22.86	21.24	44.92	0.13	<0.0001	<0.0001	<0.0001
6:0, g	16.60	16.55	16.47	0.07	0.57	0.16	0.38
8:0, g	14.78	74.84	133.29	0.35	<0.0001	<0.0001	<0.0001
10:0, g	29.07	77.40	120.3	0.33	<0.0001	<0.0001	<0.0001
12:0, g	37.34	33.09	32.94	0.14	<0.0001	<0.0001	0.40
14:0, g	190.86	177.87	181.16	0.73	<0.0001	<0.0001	0.0014
14:1, g	12.45	8.27	12.35	0.04	<0.0001	0.12	<0.0001
15:0, g	16.60	16.55	16.47	0.07	0.57	0.16	0.38
16:0, g	1,875.44	1,815.90	1,860.99	7.36	<0.0001	0.16	<0.0001
16:1, g	161.82	157.18	168.81	0.65	<0.0001	<0.0001	<0.0001
17:0, g	29.04	28.96	28.82	0.11	0.57	0.16	0.38
18:0, g	941.87	914.15	951.08	3.72	<0.0001	0.08	<0.0001
18:1 *cis*-9, g	2,676.24	2,614.23	2,705.02	10.59	<0.0001	0.05	<0.0001
18:1 *trans*-11, g	132.77	132.37	139.99	0.54	0.58	<0.0001	<0.0001
18:2n-6, g	883.78	864.52	918.14	3.53	0.0002	<0.0001	<0.0001
18:3n-3, g	41.49	41.36	45.29	0.17	0.59	<0.0001	<0.0001
20:0, g	16.60	16.55	16.47	0.07	0.57	0.16	0.38
20:1n-9, g	45.64	45.50	49.41	0.19	0.59	<0.0001	<0.0001
20:2n-6, g	37.34	37.23	41.17	0.15	0.59	<0.0001	<0.0001
20:3n-6, g	8.30	8.27	8.23	0.03	0.58	0.16	0.38
20:3n-3, g	4.15	4.14	4.12	0.02	0.58	0.16	0.38
20:4n-6, g	16.60	16.55	16.47	0.07	0.58	0.16	0.38
22:0, g	12.45	8.27	8.23	0.04	<0.0001	<0.0001	0.47
22:5n-3, g	4.15	4.14	4.12	0.02	0.58	0.16	0.38
24:0, g	8.30	8.27	8.23	0.03	0.58	0.16	0.38
Total FA, kg	7.24	7.14	7.53	0.03	0.02	<0.0001	<0.0001
MR Intake, kg	43.22	43.13	42.98	0.17	0.71	0.31	0.50

[1]Treatments: CON = no C8:0-C10:0 oil added to MR; MCFA = 0.5% C8:0-C10:0 oil added to MR; NT+MCFA = 0.5% C8:0-C10:0 oil and NeoTec5g (Provimi, Brookville, OH) added to MR.

Table 4.3. Individual and total fatty acid intake from milk replacer and calf starter combined, starter intake, and total intake of calves from d 1 to 57 based on study treatments (CON vs. MCFA vs. NT+MCFA).

	Treatments[1]				*P*-value		
Item	CON	MCFA	NT+MCFA	SEM	CON vs. MCFA	CON vs. NT+MCFA	MCFA vs. NT+MCFA
Fatty acid							
4:0, g	23.89	21.63	49.85	0.33	<0.0001	<0.0001	<0.0001
6:0, g	18.38	18.15	17.98	0.20	0.40	0.15	0.52
8:0, g	19.32	75.49	133.69	0.44	<0.0001	<0.0001	<0.0001
10:0, g	30.45	77.40	120.30	0.34	<0.0001	<0.0001	<0.0001
12:0, g	37.91	33.52	32.94	0.16	<0.0001	<0.0001	0.007
14:0, g	192.96	179.64	182.75	0.83	<0.0001	<0.0001	0.007
14:1, g	12.45	8.27	12.35	0.04	<0.0001	0.12	<0.0001
15:0, g	17.18	17.12	16.98	0.10	0.67	0.16	0.31
16:0, g	2012.02	1970.86	2003	19.14	0.12	0.73	0.21
16:1, g	163.65	158.69	170.11	0.73	<0.0001	<0.0001	<0.0001
17:0, g	30.08	29.93	29.64	0.18	0.56	0.09	0.23
18:0, g	963.45	935.98	971.43	5.08	0.0003	0.26	<0.0001
18:1 *cis*-9, g	2876.88	2854.83	2922.05	28.79	0.58	0.26	0.09
18:1 *trans*-11, g	142.01	143.18	149.6	1.34	0.53	0.0002	0.0008
18:2n-6, g	1226.41	1301.55	1302.95	41.40	0.19	0.18	0.98
18:3n-3, g	61.74	65.86	68.25	2.36	0.21	0.05	0.45
20:0, g	18.83	19.25	18.90	0.29	0.29	0.86	0.36
20:1n-9, g	48.90	49.49	52.97	0.48	0.37	<0.0001	<0.0001
20:2n-6, g	37.34	37.23	41.17	0.15	0.58	<0.0001	<0.0001
20:3n-6, g	8.30	8.27	8.23	0.03	0.58	0.16	0.38
20:3n-3, g	4.15	4.14	4.12	0.02	0.58	0.16	0.38
20:4n-6, g	16.60	16.55	16.47	0.07	0.57	0.16	0.38
22:0, g	14.21	10.31	10.13	0.21	<0.0001	<0.0001	0.53
22:5n-3, g	4.15	4.14	6.74	0.14	0.95	<0.0001	<0.0001
24:0, g	10.76	11.12	10.86	0.28	0.36	0.81	0.49
Total FA, kg	7.99	8.05	8.35	0.10	0.66	0.01	0.03
Starter Intake, kg	25.49	29.19	26.64	2.72	0.33	0.76	0.49
Total Intake[2], kg	68.71	72.32	69.62	2.81	0.35	0.81	0.47

[1]Treatments: CON = no C8:0-C10:0 oil added to MR; MCFA = 0.5% C8:0-C10:0 oil added to MR; NT+MCFA = 0.5% C8:0-C10:0 oil and NeoTec5g (Provimi, Brookville, OH) added to MR.
[2]Total intake = MR intake + starter intake

Table 4.4. Feed efficiency (FE), body condition score (BCS), hip width (HW), hip height (HH), heart girth (HG), and paunch girth (PG) change of calves from d 1 to 57 based on study treatments (CON vs. MCFA vs. NT+MCFA).

	Treatments[1]				*P*-values		
Item	CON	MCFA	NT+MCFA	SEM	CON vs. MCFA	CON vs. NT+MCFA	MCFA vs. NT+MCFA
FE[2], kg/kg	0.53	0.55	0.54	0.02	0.39	0.57	0.76
BCS Change[3]	0.13	0.07	0.10	0.04	0.29	0.59	0.59
HW Change, cm	3.92	4.13	3.91	0.21	0.45	0.99	0.43
HH Change, cm	12.13	13.32	12.71	0.81	0.29	0.61	0.57
HG Change, cm	20.00	21.18	19.88	0.96	0.38	0.93	0.32
PG Change, cm	31.20	32.71	31.65	1.31	0.41	0.80	0.55

[1]Treatment: CON = no C8:0-C10:0 oil added to MR; MCFA = 0.5% C8:0-C10:0 oil added to MR; NT+MCFA = 0.5% C8:0-C10:0 oil and NeoTec5g (Provimi, Brookville, OH) added to MR.
[2]FE = body weight gain / total intake
[3]Body condition score was based on a scoring system from 1 to 5 using 0.25-unit increments, with 1 being emaciated to 5 being obese.

Table 4.5. Serum total protein (STP), daily fecal scores, scouring days, medical days, and bovine respiratory disease (BRD) treatments of calves from d 1 to 57 based on study treatments (CON vs. MCFA vs. NT+MCFA).

	Treatments[1]				*P*-value		
Item	CON	MCFA	NT+MCFA	SEM	CON vs. MCFA	CON vs. NT+MCFA	MCFA vs. NT+MCFA
STP, g/dL	6.05	5.81	6.22	0.15	0.26	0.44	0.05
Daily Fecal Score[2]	2.18	2.25	2.23	0.03	0.08	0.25	0.53
Scouring Days[3]	19.60	20.53	21.06	1.30	0.60	0.42	0.76
Medical Days[4]	1.80	0.71	0.65	0.48	0.10	0.09	0.93
BRD Treatments[5]	0.53	0.29	0.29	0.18	0.35	0.35	1.00

[1]Treatments: CON = no C8:0-C10:0 oil added to MR; MCFA = 0.5% C8:0-C10:0 oil added to MR; NT+MCFA = 0.5% C8:0-C10:0 oil and NeoTec5g (Provimi, Brookville, OH) added to MR.
[2]Daily fecal scores were assigned on a scale of 1-5 (1 = firm, normal, 2 = less firm, normal, 3 = thick, batterlike, 4 = thin, batter-like, 5 = watery).
[3]Scouring days were calculated as the number of d with a fecal score > 2.
[4]Medical days were calculated based on the number of days a calf received an antibiotic treatment.
[5]BRD treatments are the number of incidences a calf was treated for BRD.

Table 4.6. Non-esterified fatty acid (NEFA), beta-hydroxybutyric acid (BHBA), insulin, and glucose concentrations at d 42, 49, and 56 based on study treatments (CON vs. MCFA vs. NT+MCFA).

		Treatments[1]				*P*-values				
Item	Timepoint	CON	MCFA	NT+MCFA	SEM	CON vs. MCFA	CON vs. NT+MCFA	MCFA vs. NT+MCFA	Timepoint	TRT × Timepoint
NEFA (mmol/L)	d 42	0.29	0.18	0.24	0.02	0.001	0.12	0.05	< 0.0001	0.03
	d 49	0.17	0.19	0.25	0.02	0.53	0.02	0.06		
	d 56	0.13	0.10	0.13	0.02	0.44	1.00	0.42		
BHBA (nmol/mL)	d 42	557.9	603.4	542.9	19.0	0.08	0.57	0.02	0.0008	0.18
	d 49	590.5	574.0	579.2	19.0	0.53	0.67	0.84		
	d 56	634.0	616.8	602.9	19.0	0.51	0.24	0.58		
Insulin (ng/mL)	d 42	1.39	1.35	1.01	0.21	0.88	0.19	0.23	< 0.0001	0.83
	d 49	1.66	1.49	1.11	0.21	0.55	0.06	0.18		
	d 56	0.40	0.29	0.19	0.23	0.74	0.51	0.73		
Glucose (mg/mL)	d 42	1.16	1.15	1.14	0.05	0.92	0.77	0.85	< 0.0001	0.81
	d 49	1.13	1.21	1.20	0.05	0.21	0.30	0.84		
	d 56	0.68	0.70	0.67	0.05	0.80	0.77	0.57		

[1]Treatments (TRT): CON = no C8:0-C10:0 oil added to MR; MCFA = 0.5% C8:0-C10:0 oil added to MR; NT+MCFA = 0.5% C8:0-C10:0 oil and NeoTec5g (Provimi, Brookville, OH) added to MR.

Table 4.7. Viral and bacterial shedding count data (fraction of animals that were positive) from fecal samples (A) and deep nasopharyngeal swabs (B) on d 16 or 17 (prior to vaccination; Pre-Vac), d 37 or 38 (21 d post initial vaccination; Post-Vac), and d 51 or 52 (14 d post booster vaccination; Post-Boost) based on study treatments (CON vs. MCFA vs. NT+MCFA).

A)

		Treatment[1]			*P*-value		
Item[2]	Timepoint	CON	MCFA	NT+MCFA	CON vs. MCFA	CON vs. NT+MCFA	MCFA vs. NT+MCFA
Corona Virus	Pre-Vac	0.64	0.82	0.82	0.62	0.62	1.00
	Post-Vac	0.36	0.27	0.36	0.71	1.00	0.71
	Post-Boost	0.27	0.18	0.09	0.65	0.31	0.56
Rota Virus A	Pre-Vac	0.55	0.64	0.45	0.78	0.76	0.56
	Post-Vac	0.27	0.36	0.64	0.71	0.20	0.36
	Post-Boost	0.45	0.36	0.45	0.74	1.00	0.74
Cryptosporidiosis	Pre-Vac	0.27	0.36	0.45	0.71	0.48	0.74
Salmonella	Pre-Vac	0.82	0.55	0.73	0.44	0.81	0.59
	Post-Vac	0.00	0.27	0.09	0.04	0.24	0.31

B)

		Treatment[1]			*P*-value		
Item[3]	Timepoint	CON	MCFA	NT+MCFA	CON vs. MCFA	CON vs. NT+MCFA	MCFA vs. NT+MCFA
Corona Virus	Pre-Vac	0.91	0.73	0.73	0.64	0.64	1.00
	Post-Vac	0.55	0.45	0.55	0.76	1.00	0.76
	Post-Boost	0.09	0.18	0.27	0.56	0.31	0.65
Viral Diarrhea	Post-Vac	0.00	0.00	0.09	1.00	0.24	0.24
Parainfluenza 3	Post-Boost	0.00	0.09	0.00	0.24	1.00	0.24

[1]Treatments (TRT): CON = no C8:0-C10:0 oil added to MR; MCFA = 0.5% C8:0-C10:0 oil added to MR; NT+MCFA = 0.5% C8:0-C10:0 oil and NeoTec5g (Provimi, Brookville, OH) added to MR.

[2]Fecal samples were analyzed for Bovine Corona Virus, Rota Virus A, E. Coli K99, Cryptosporidium, and Salmonella. Certain pathogens were not present at every timepoint and were therefore not included in the above tables.

[3]Deep nasopharyngeal swabs samples were analyzed for Bovine Corona Virus, Bovine Respiratory Syncytial Virus, Bovine Viral Diarrhea, Infectious Bovine Rhinotracheitis, and Parainfluenza-3. Certain pathogens were not present at every timepoint and were therefore not included in the above tables.

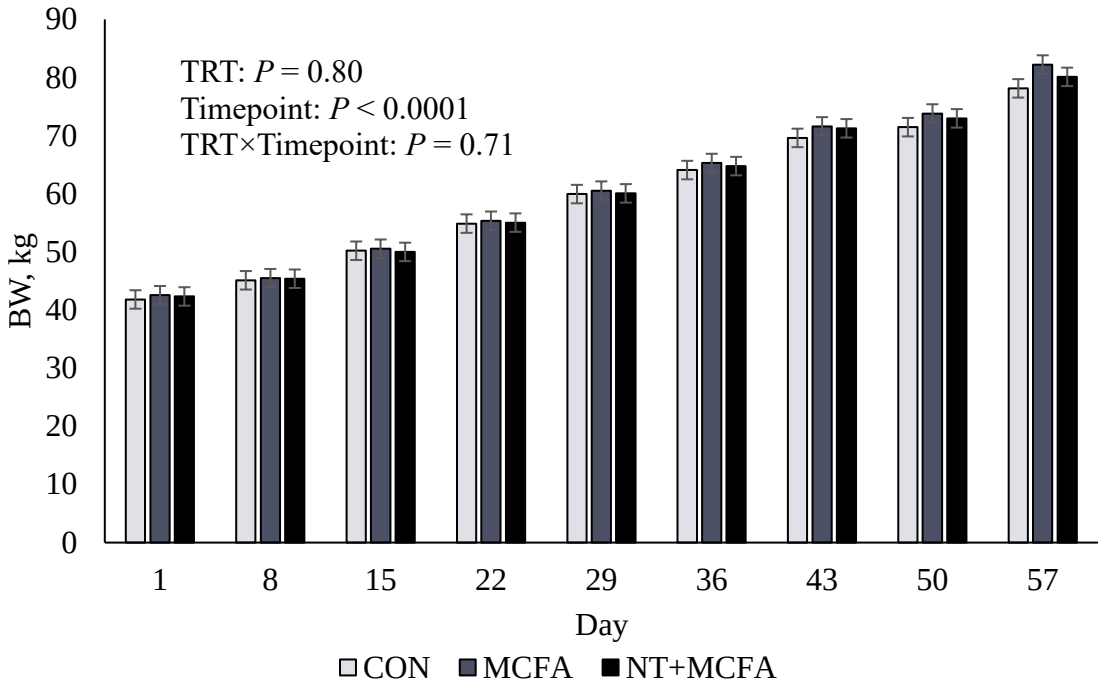

Figure 4.1. Weekly calf body weight by treatment from d 1 to 57. Calves were assigned to one of three treatments; control (CON) where calves were fed milk replacer (MR) with no C8:0-C10:0 oil added, medium chain fatty acid (MCFA) where calves were fed MR with 0.5% C8:0-C10:0 oil added, or NeoTec with medium chain fatty acid (NT+MCFA) where calves were fed MR with NeoTec5g™ (Provimi, Brookville, OH) and 0.5% C8:0-C10:0 oil added.

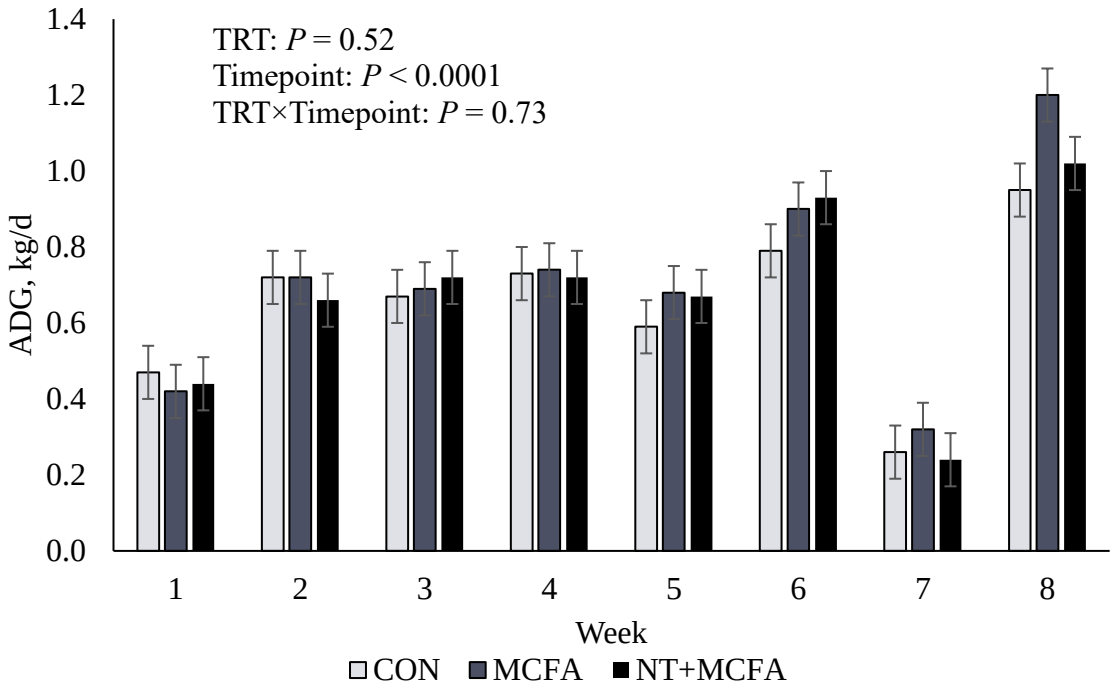

Figure 4.2. Weekly calf ADG by treatment from week 1 to 8. Calves were assigned to one of three treatments; control (CON) where calves were fed milk replacer (MR) with no C8:0-C10:0 oil added, medium chain fatty acid (MCFA) where calves were fed MR with 0.5% C8:0-C10:0 oil added, or NeoTec with medium chain fatty acid (NT+MCFA) where calves were fed MR with NeoTec5g™ (Provimi, Brookville, OH) and 0.5% C8:0-C10:0 oil added. * Indicates a significant difference ($P = 0.01$) between treatments.

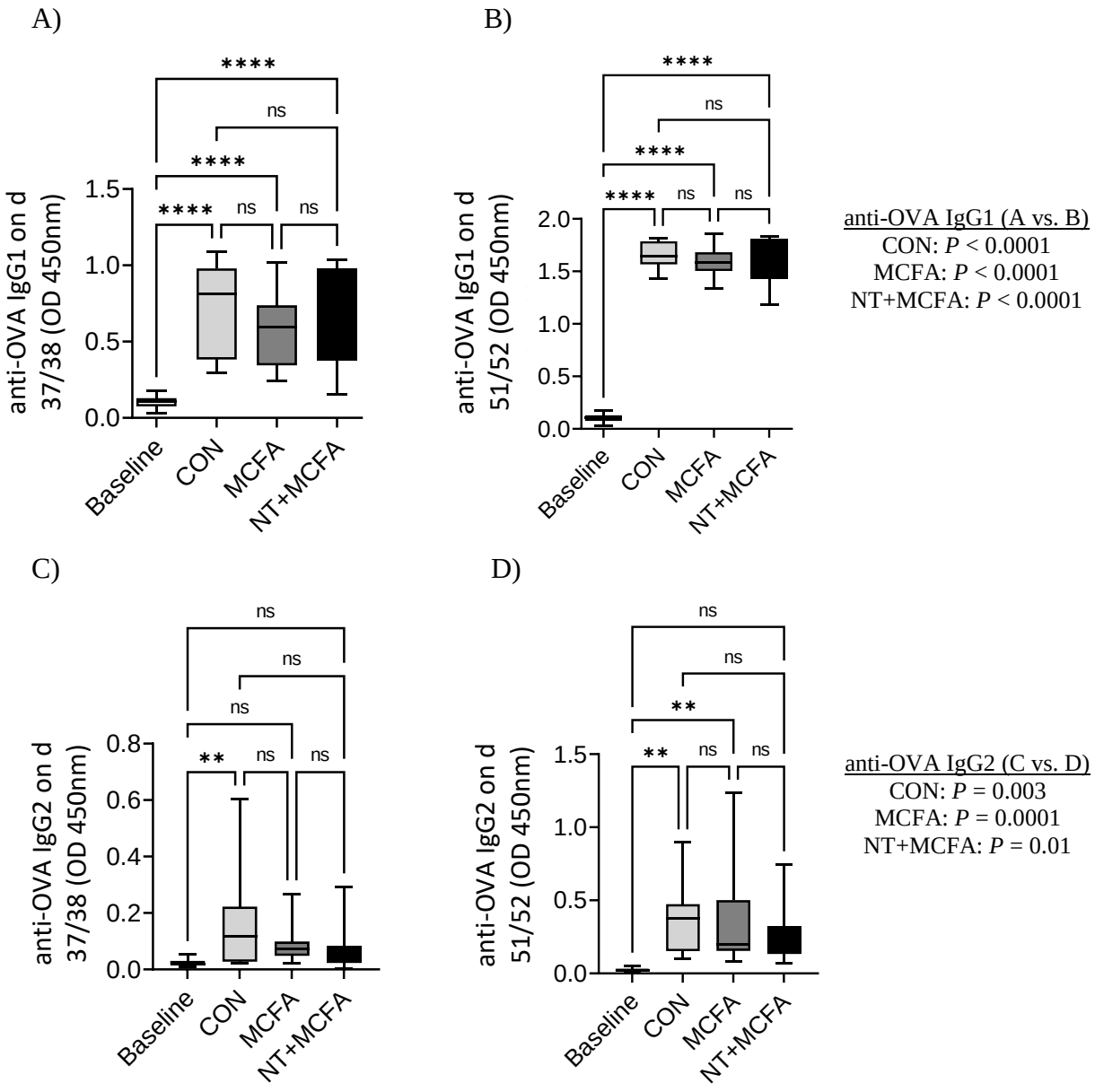

Figure 4.3. Antibody concentrations on d 37/38 (21 d post initial vaccination) and d 51/52 (14 d post booster vaccination) based on study treatments (CON = no C8:0-C10:0 oil added to milk replacer (MR); MCFA = 0.5% C8:0-C10:0 oil added to MR; NT+MCFA = NeoTec5g (Provimi, Brookville, OH) and 0.5% C8:0-C10:0 oil added to MR). **A)** anti-OVA IgG1 concentration on d 37/38, **B)** anti-OVA IgG1 concentration on d 51/52, **C)** anti-OVA IgG2 concentration on d 37/38, **D)** anti-OVA IgG2 concentration on d 51/52. ns: $P > 0.05$, **: $P \leq 0.01$, ****: $P \leq 0.0001$

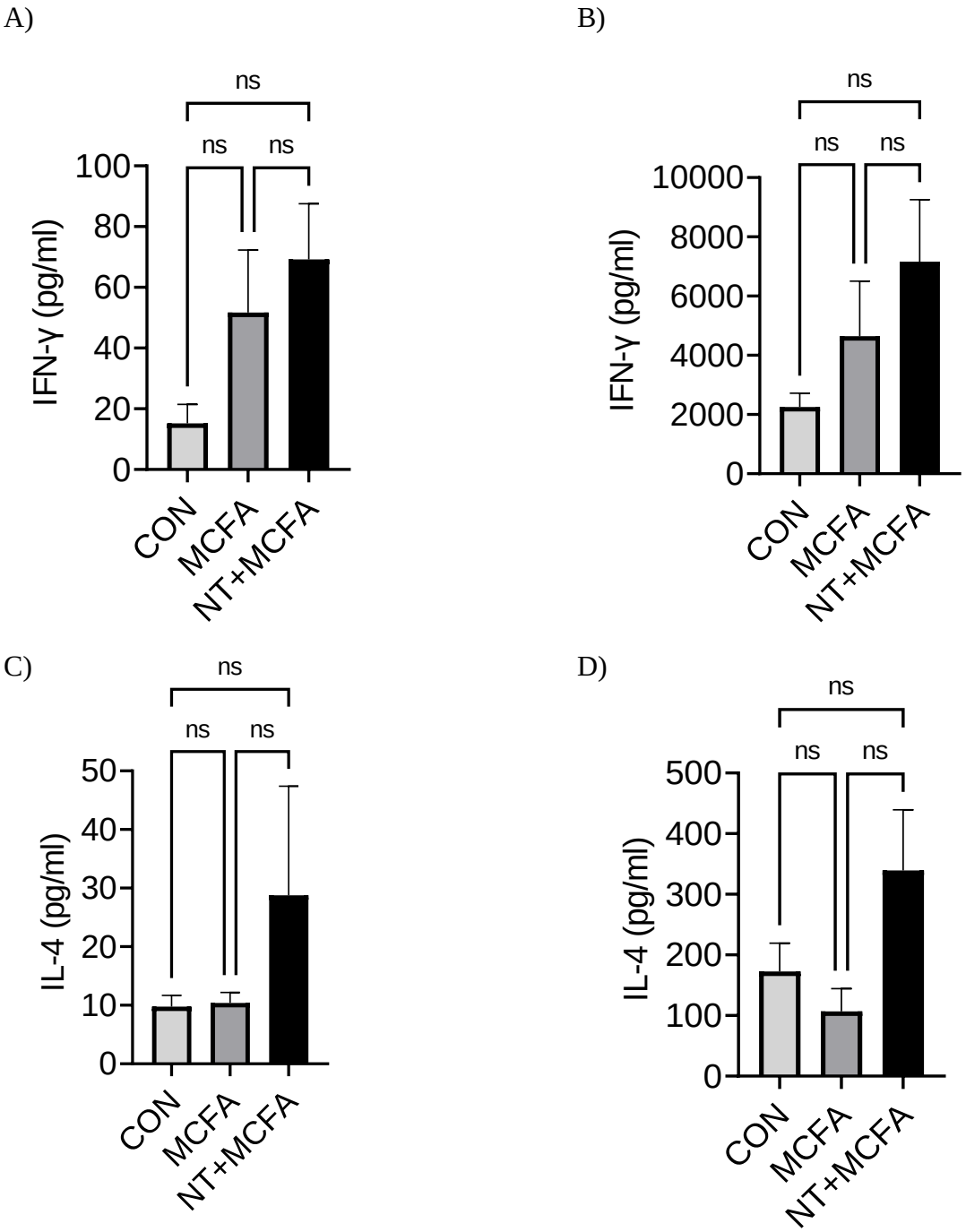

Figure 4.4. Cytokine concentrations on d 51/52 (14 d post booster vaccination) based on study treatments (CON = no C8:0-C10:0 oil added to milk replacer (MR); MCFA = 0.5% C8:0-C10:0 oil added to MR; NT+MCFA = NeoTec5g (Provimi, Brookville, OH) and 0.5% C8:0-C10:0 oil added to MR). A) Interferon-γ (IFN-γ) secretion by peripheral blood mononuclear cells (PBMCs) upon stimulation with ovalbumin (OVA), B) IFN-γ secretion by PBMCs upon stimulation with phytohemagglutinin (PHA), C) Interleukin 4 (IL-4) secretion by PBMCs upon stimulation with OVA, D) IL-4 secretion by PBMCs upon stimulation with PHA. ns: $P > 0.05$

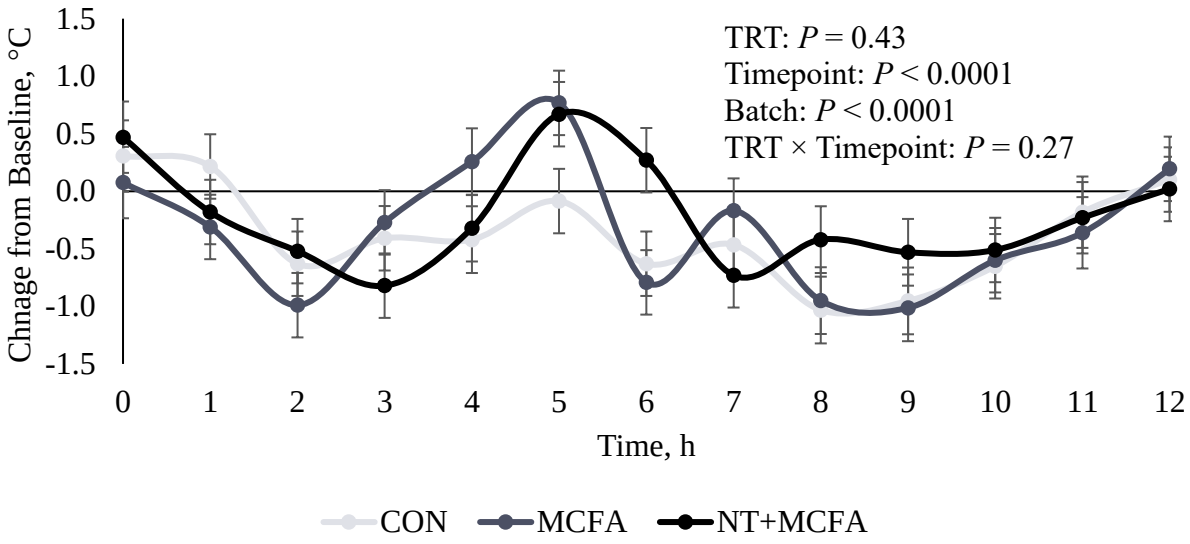

Figure 4.5. Change of temperature from baseline (average temperature over the 24 h prior to dosing) during the first 12 h in relation to dosing with 200 µg ovalbumin (OVA) and 1 mg aluminum hydroxide (AH) adjuvant on d 16/17. Calves were assigned to one of three treatments; control (CON) where calves were fed milk replacer (MR) with no C8:0-C10:0 oil added, medium chain fatty acid (MCFA) where calves were fed MR with 0.5% C8:0-C10:0 oil added, or NeoTec with medium chain fatty acid (NT+MCFA) where calves were fed MR with NeoTec5g™ (Provimi, Brookville, OH) and 0.5% C8:0-C10:0 oil added.

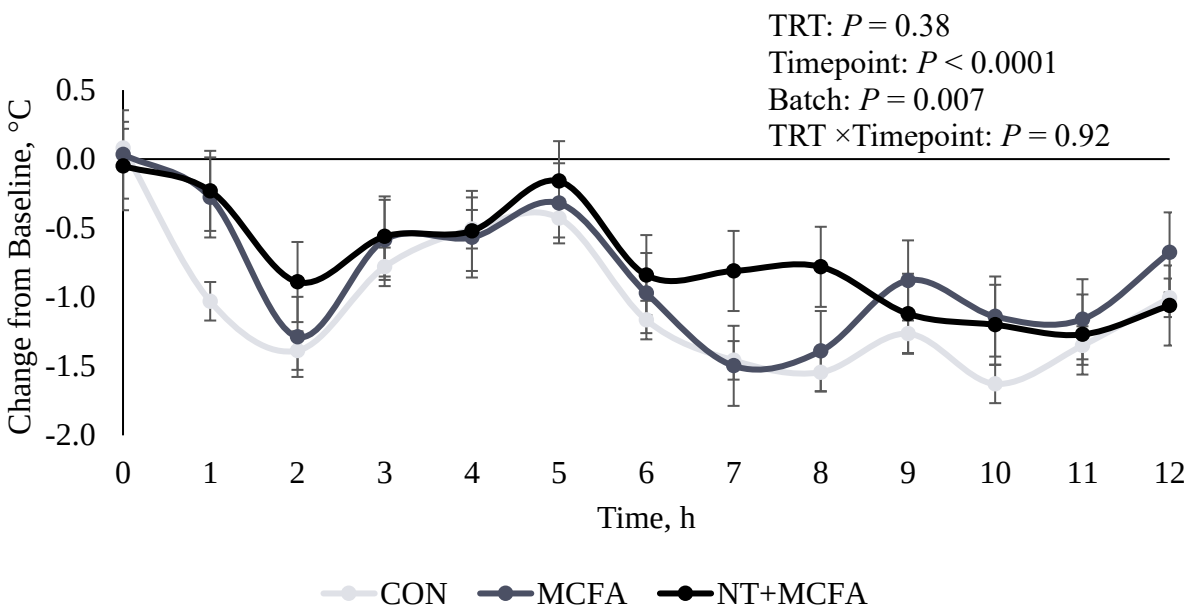

Figure 4.6. Change of temperature from baseline (average temperature over the 24 h prior to dosing) during the first 12 h in relation to dosing with 200 µg ovalbumin (OVA) and 1 mg aluminum hydroxide (AH) adjuvant on d 37/38. Calves were assigned to one of three treatments; control (CON) where calves were fed milk replacer (MR) with no C8:0-C10:0 oil added, medium chain fatty acid (MCFA) where calves were fed MR with 0.5% C8:0-C10:0 oil added, or NeoTec with medium chain fatty acid (NT+MCFA) where calves were fed MR with NeoTec5g™ (Provimi, Brookville, OH) and 0.5% C8:0-C10:0 oil added.

CHAPTER 5. RELATIONSHIP OF COW AND CALF CIRCULATING LIPIDOMES WITH COLOSTRUM LIPID COMPOSITION AND METABOLIC STATUS OF THE COW

Klopp, R. N., C. R. Ferreira, T. M. Casey, and J. P. Boerman. Relationship of cow and calf circulating lipidomes with colostrum lipid composition and metabolic status of the cow. *J. Dairy. Sci.* Accepted.

5.1 Abstract

Newborn calves rely on lipids in colostrum for energy and immune function. The lipid concentration in colostrum, however, is highly variable and little is known about its composition and maternal factors that influence its composition. The first objective was to measure plasma lipid composition of multiparous cows at 35 d before calving (**BC**; 35 ± 3 d; mean ± SD) and 7 d BC (7 ± 2 d), their colostrum, and serum lipid composition of calves (24 h postnatal) using multiple reaction monitoring (**MRM**) profiling, which is an exploratory and highly sensitive lipidomic analysis method that screens lipids based on chemical functionality. Secondly, data were analyzed to determine if there were relationships between circulating lipids in the cow, colostrum lipids, and calf serum lipids. Thirdly, relationships between markers of metabolic status of the cows and circulating and colostrum lipids were analyzed with correlation analysis. Blood was sampled and plasma prepared from multiparous cows (n=16) at 35 d and 7 d BC. Within 3 h of parturition, colostrum was collected from cows and fed to her calf. Calves received another feeding of colostrum within 12 h after birth and a serum sample was collected from each calf 24 h after the first feeding of colostrum. The metabolic status of cows was evaluated using insulin, glucose, and non-esterified fatty acid (**NEFA**) area under the curve (**AUC**) in response to an intravenous glucose tolerance test **(IVGTT)** performed at three wks BC. Lipids were extracted from plasma, colostrum and calf serum and analyzed using MRM profiling. Concentration of lipids were calculated using spiked in standards and expressed as percent of lipids identified. Data were uploaded into MetaboAnalyst 5.0 for multivariate and univariate analysis. Principal component analysis indicated that circulating lipids in the cow and calf were distinct from lipids in colostrum. Phosphatidylglycerol (**PG**) concentration was greater in colostrum and calf serum ($P < 0.05$) than in cow plasma, with 23 of the 24 PGs found in colostrum also found in calf serum. In response to IVGTT in late gestation, NEFA AUC was positively related to total triacylglycerols (**TG**) lipids

in 7 BC plasma (r = 0.63; P = 0.01) but negatively related to total membrane lipids in colostrum (r = -0.55; P = 0.03). Thus, the metabolic status of the dam influences circulating lipids and colostrum lipid content. Moreover, the circulating lipidome of the cow and calf are similar to one another and distinct from the colostrum lipidome, except for PG, where it appears that colostrum serves as the source for PG in the calf's circulation.

5.2 Introduction

Colostrum is the first secretion from the mammary glands and is an essential source of nutrients, energy, and antibodies for neonates immediately after birth. In the dairy industry, research has primarily focused on the immunoglobulin content of colostrum and the evaluation of successful passive transfer of immunity to calves. However, newborn calves rely partially on the lipids in colostrum as a source of energy to produce heat by both diet-induced thermogenesis and non-shivering thermogenesis in brown adipose tissue (Davis and Drackley, 1998). Calves are born with relatively low energy reserves, with adipose tissue making up only 3% of their birth body weight (Morrill et al., 2012). Lipids also play an important role in calf immune system function. Supplementing fatty acids (**FA**) to calves, specifically butyrate, medium-chained fatty acids (**MCFA**), and linolenic acid, via milk replacer reduced inflammation, scouring, and medical treatments, while increasing antibody production (Hill et al., 2011b). Through lipolytic activity in the gastrointestinal tract, milk triacylglycerols (**TG**) are converted to monoglycerol and FA, which both possess antimicrobial properties (Isaacs, 2001), that aid in protecting the host against gastrointestinal pathogens and infection (Sprong et al., 2002; Yoon et al., 2018).

The fat content of colostrum is greater on average than mature bovine milk (6.7% vs 4.0%, respectively; Godden, 2008). However, fat content in colostrum is highly variable (Kehoe et al., 2007; Morrill et al., 2012). Studies of lipid content of colostrum primarily evaluated the concentration in colostrum compared to total fat in transition milk and mature milk, with few studies evaluating lipid classes that make up colostrum (Bitman and Wood, 1990; Leiber et al., 2011; Contarini et al., 2014). Due to the vital role that colostrum plays in calf nutrition and health, the composition of lipids in colostrum needs to be further explored to enable the development of biologically relevant colostrum replacers and supplements. Moreover, there is a need for increased understanding of dairy cow physiological factors that affect colostrum composition and yield.

It is thought that colostrum formation begins three to four wk prior to parturition (Brandon et al., 1971), if not even earlier then 4 wks (Chandra et al., 2013). Colostrum is formed prior to tight junction closure between lactocytes in the mammary gland, and therefore occurs prior to secretory activation. Secretory activation is the onset of copious milk production (Hartmann et al., 1973), and is stimulated by the drop in circulating progesterone that occurs around parturition (Deis and Delouis, 1983; Nguyen et al., 2001). During colostrum formation, immunoglobulins and milk constituents are synthesized in lactocytes and transferred from maternal plasma into mammary secretions (Barrington et al., 2001). Fatty acids in milk are either taken up from circulating lipids or synthesized *de novo* in the mammary epithelial cells (Clegg et al., 2001). The current dogma is that the ability of mammary epithelial cells to synthesize FA *de novo* is not fully activated until secretory activation begins (Anderson et al., 2007), which occurs postnatal over a period of 24-48 h in cattle (Hartmann, 1973). Thus, the FA in colostrum are taken from circulating apolipoproteins and assembled into TG or plasma membrane lipids in the lactocytes for secretion into milk.

As dairy cattle transition from late gestation to early lactation, they experience an energy deficit, which leads to the mobilization of adipose tissue to meet energy demands. The relative amount of adipose tissue mobilization is reflected by the concentration of non-esterified fatty acids (**NEFA**) in circulation (McNamara, 1991). Highly elevated NEFA concentrations, which is often associated with over-conditioned cows, can lead to the development of disease in periparturient cattle (van Knegsel et al., 2005; Adewuji et al., 2005). In the first days of lactation, NEFA are used by the mammary gland to produce about 40% of milk fat (Adewuji et al., 2005), with elevated plasma NEFA concentrations related to alterations in FA composition in milk (Jorjong et al., 2014; Jorjong et al., 2015). Therefore, the metabolic status of the cow, as indicated by NEFA concentrations, is related to the lipid composition in milk (Newman and Verdin, 2017). It was surmised that since FA in colostrum are primarily derived from circulation, characterizing the lipids circulating during late gestation and the relationship of circulating and colostrum lipids to metabolic status of the cow, can increase the understanding of factors that regulate fat content of colostrum. Secondly, it was hypothesized that the lipidome of calf serum will be influenced by colostrum lipid composition, and therefore the circulating lipidome of the calf will be distinct from cow plasma lipidome. The first objective of this study was to measure lipidomes circulating in multiparous cows at 35 d before calving (**BC**) and 7 d BC, their colostrum sample, and 24 h

postnatal serum samples of calves using the multiple reaction monitoring (**MRM**) profiling method. The second objective was to determine if there was a relationship between circulating lipids in the cow, colostrum, and the calf serum. The third objective was to determine if there were relationships between colostrum lipid profiles and markers of metabolic status of the cows. Wherein metabolic status was determined by response in glucose, insulin and NEFA concentrations relative to an intravenous glucose tolerance test **(IVGTT**) at two wks prepartum.

5.3 Materials and Methods

5.3.1 Animals and Treatments

All procedures involving animals were reviewed and approved by the Purdue University Institutional Animal Care and Use Committee (Protocol # 1701001523). The study was performed at the Dairy Unit of the Purdue University Animal Sciences Research and Education Center in West Lafayette, Indiana. It utilized sixteen multiparous Holstein cows that were part of a larger study aimed at understanding the effect of exposure to chronic light-dark phase shift, which disrupts circadian clocks on metabolic status and mammary development (McCabe et al., 2021).

At 60 d before expected calving, milking of cows was ceased, and they were transitioned to a non-lactating diet. Cows were moved into a tie stall barn 35 d before expected calving, which was the start of the research trial. Cows were randomly assigned to one of two treatments, control (**CON**; n =8) or phase-shift (**PS**; n = 8) after being blocked by lactation number (2.88 ± 0.64 vs. 2.88 ± 0.64; mean ± SD) and previous lactation 305 d milk yield (12,087 ± 2,486 vs. 12,467 ± 2,497 kg; mean ± SD) for CON and PS cows, respectively. The CON cows were exposed to 16 h light and 8 h darkness. Whereas, PS cows were exposed to the same amount of light and dark, however, every 3 d there was a 6-h shift in the light-dark cycle until the animal calved [for further details on the study design, light-dark treatments, and diets fed please refer to McCabe et al., (2021)].

At approximately 5 d BC, cows were moved to box stalls in the maternity barn where they were exposed to control light-dark cycles until calving. After parturition, calves were removed from the box stall, and bottle fed 3.79 L of colostrum from their dam within 3 h of birth. If calves did not drink all 3.79 L, an esophageal tube was used to feed them the remaining colostrum. Calves

received another 3.79 L of colostrum within the first 12 h after birth. After receiving two feedings of colostrum, calves were switched to 1.89 L of a 20% fat: 20% protein milk replacer twice daily.

5.3.2 Sample Collection

Blood samples were collected using a 10 mL EDTA tube (Becton Dickinson, Franklin Lakes, NJ) from all cows (n = 16) via the coccygeal vessels at 35 ± 3 d BC and 7 ± 2 d BC. These time points were selected to profile circulating lipids in the cow prior to the initiation of colostrum formation and after, in order to identify differences potentially caused by the formation of colostrum. Samples were centrifuged within 1 h of collection at 4,000 x *g* for 15 min at 4°C, plasma was transferred to a microcentrifuge tube, and frozen at -20°C until lipid extraction. Colostrum was collected within 3 h of calving (n = 16) by milking out all four glands. Colostrum was either collected in the Purdue University milking parlor or by a portable milking unit that allowed for colostrum collection in maternity pens. Mode of colostrum collection was selected relative to the time of day of parturition. Specifically, if cow calved within two h prior to milking time at the farm, it was collected in the parlor, otherwise it was collected using the portable milking unit. Colostrum yield was recorded by weight and 15 ml of colostrum was frozen at -20°C until lipid extraction. Colostrum was assessed for quality using a digital Brix refractometer (MISCO, Cleveland, OH). A Brix refractometer uses the refraction of light that passes through the colostrum sample to estimate immunoglobulin G concentration (Quigley et al., 2013). Colostrum fat percent was measured using the creamatocrit approach (Lucas et al., 1978), using duplicate technical replicates. Blood samples were collected from calves 24 h after their first colostrum feeding (n = 15). Blood was not collected from one calf because it received colostrum replacer instead of colostrum due to its dam did not produce enough colostrum. Blood was allowed to coagulate, then centrifuged at 4,000 x *g* for 20 min at 4°C, the serum was harvested and transferred into microcentrifuge tubes, and frozen -20°C until lipid extraction.

As part of the larger project (McCabe et al., 2021), the relative metabolic state of cattle was evaluated. Biomarkers of cattle metabolic status included plasma concentrations of insulin, glucose, and NEFA area under the curve (**AUC**) in response to IVGTT. Blood samples were collected fourteen times over 180 min after dosing with 250 mg/kg BW of glucose using a 50% glucose solution, at two wks before expected calving, which indicated relative insulin sensitivity. Blood samples were also collected from each cow at 7 d BC, plasma was harvested as described above

and used to measure NEFA, insulin, glucose, and BHB concentrations to estimate metabolic status of the cow at the same time the lipidome of the cow was being evaluated.

5.3.3 Lipid Extraction and MRM-Profiling

Lipids were extracted from cow plasma taken at 35 and 7 d BC, colostrum samples, and calf serum taken 24 hours after colostrum feeding using the Bligh & Dyer protocol (Bligh and Dyer, 1959). The vacuum dried lipid extracts were re-suspended in 300 µl of methanol-chloroform (3:1) with butylated hydroxytoluene (BHT; 50 µg/ml; stock solution). Lipid extracts from colostrum samples were further diluted 1:1000, and plasma and serum samples were diluted 1:200 in acetonitrile: methanol:300 mM ammonium acetate (3:6.65:0.35). All samples were spiked with an isotopically labeled internal standard mix (**IS**; EquiSPLASH™ LIPIDOMIX®, Avanti Polar Lipids) at the concentration of 0.1 ng/µL per IS (total of 0.8 ng of each IS injected since 8 µL of diluted lipid extract was used). An Agilent G1367A 1100 series micro-autosampler was used to deliver 8 µL of each sample, at a flow rate of 7 µL/min, by flow injection to the ionization source of an Agilent QQQ 6410 quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) for each profile analysis.

Multiple reaction monitoring profiling is a highly sensitive and exploratory lipidomic analysis method based on chemical functionality screening (Xie et al., 2021). These features of the MRM profiling method are due to the use of a triple-quadrupole mass spectrometer which can run neutral loss (**NL**) and precursor ion (**Prec**) scans. Therefore, MRM profiling is highly efficient since the sample is directly injected into the mass spectrometer without previous chromatographic separation and a list of MRMs is considered as the chemical profile of the sample. Although highly rapid and sensitive due to the use of MRM scans, lipid attribution is performed only at species level, which means that the lipid class, total carbon length and unsaturation level (number of double bonds) are related to each MRM. Specifically, for the TG, only one fatty acyl group attached to the glycerol backbone is identified, the other two are not known as the strategy is based on neutral losses expected for fatty acyl chains (Li et al., 2014). Phospholipids are categorized by their functional group, for example phosphatidylcholine is identified as the total number of carbons and unsaturated bonds across the two fatty acyl chains. Thus, the identity of all the fatty acyl groups in TG and plasma membrane lipids is not known and information regarding species can only be inferred from the data.

The MRM profiling was divided into two different phases, a discovery and screening phase. For discovery phase, a pooled sample of all the plasma, serum, and colostrum samples was profiled. Discovery phase MRM profiling screened in pooled samples for the presence of 15 lipid classes and subclasses (phosphatidylcholine (**PC**), Lyso PC, sphingomyelin (**SM**), phosphatidylethanolamine (**PE**), Lyso PE, phosphatidylinositol (**PI**), Lyso PI, phosphatidylglycerol (**PG**), Lyso PG, phosphatidylserine, Lyso phosphatidylserine, TG, cholesteryl esters, acyl-carnitines and NEFA). An 'o' after PC or PG indicates the presence of an alkyl ether substituent, whereas a 'p' after PC or PG indicates the presence of a 1Z-alkenyl ether (plasmalogen) substituent (i.e. PCo, PCp, PGo). The discovery phase was therefore targeted based on the Lipid MAPS online database (http://www.lipidmaps.org/), as reported previously (de Lima et al., 2018; Dipali et al., 2019; Suarez-Trujillo et al., 2020). The discovery phase also included NL scans to screen for functional groups associated with particular lipid classes, as previously reported (de Lima et al., 2018; Dipali et al., 2019; Suarez-Trujillo et al., 2020; O'Neil et al., 2020). Lipids with intensities ≥ 1.3-fold in relation to the blank sample (injection solvent) were selected for the screening phase. The second phase of MRM-profiling, or the screening phase, was performed on individual plasma, serum, and colostrum samples for MRM selected as 1.3-fold higher than the blank sample during the discovery phase. For this second phase, MRMs generated during the discovery phase were divided into three profiling sessions, referred to as methods. They included 25 NL scans for fatty acyl residues and the MRMs based on the Lipid Maps database. Dividing analysis into multiple methods enables the acquisition of robust ion signals during each 1 min MRM profiling period per sample, which is followed by a wash injection with solvent to avoid cross-contamination. Each MRM profiling method examined the samples for lipids that were related by class or functional group. Screening method 1 profiled 171 MRMs including phospholipids, acyl-carnitines, sphingomyelins, and cholesteryl esters (Supplemental Table 5.1; https://purr.purdue.edu/publications/3902/1); screening method 2 profiled TG (192 MRMs; Supplemental Table 5.2; https://purr.purdue.edu/publications/3902/1) using MRM information from the Lipid Maps database and NL expected for FA 16:0, FA 16:1, FA 18:0, FA 18:1, FA 18:2 and FA 20:4 (Li et al., 2014); and screening method 3 profiled 200 MRMs detected by NL scan during the discovery phase (Supplemental Table 5.3; https://purr.purdue.edu/publications/3902/1). Therefore, a total of 563 MRMs were profiled in each sample during the screening phase.

Attributions of MRM are tentative and based on expected precursor ions and class-diagnostic product ions.

All MRM with an intensity1.3-fold greater than the blank were considered for profiling data analysis. Usually, only background/baseline subtraction using manual processing or specific algorithms is performed for profiling analysis of mass spectrometry data (Zhang and Yang, 2008; Smith et al., 2014; Hao et al., 2018). The 1.3-fold threshold cut-off was based on profiling experiments that employed calibration curves and observed MRM with ion signals 30% or lower relative to the blank sample did not exhibit a linear response (Xie et al., 2021). To estimate the relative ion quantification of membrane and TG lipids, intensities of MRM were divided by the intensity of the isotopically labeled internal standard amount of the lipid of the same class, corrected for the amount of internal standard injected, and corrected for dilution rate. Estimated relative ion quantification of cholesteryl esters, campesteryl esters, and stigmasteryl esters cannot be calculated using internal standards due to source fragmentation of the lipid class. Instead, the relative ion abundances of cholesterol esters were used for the analysis.

5.3.4 Statistics

The current research trial was highly exploratory and conducted as part of a larger research study. Therefore, a power analysis was not conducted based on the objectives of the current trial, but rather sample size was determined based on having sufficient power based on the main objective of the larger research study. The calculated relative ion quantities or relative ion abundances, described above, were uploaded into MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/). Data were normalized by auto-scaling (mean-centered and divided by the standard deviation of each variable) and Student's *t*-test was used for statistical analysis to identify MRMs differentially abundant between sample types. An adjusted *P*-value $\leq$ 0.05, was used to identify differentially abundant lipids. MetaboAnalyst 5.0 was used to create principal component analysis (**PCA**) scores plots and heatmaps to visualize the effect of treatment, day, and sample-type on MRM distribution. Supplemental data and files can be found at https://purr.purdue.edu/publications/3902/1.

The Mixed procedure in SAS v.9.4 (SAS Institute Inc., Cary, NC) was used to analyze the effect of treatment in the following continuous dependent variables; colostrum fat concentration, colostrum weight, colostrum Brix value, and plasma NEFA, BHB, insulin, and glucose 7 d BC.

Correlation analysis was also run to determine if there were linear relationships between variables of interest. The Correlation Procedure of SAS v.9.4 was used to determine correlations between lipid species (membrane and TG lipids) found in different sample types (35 BC plasma, 7 BC plasma, colostrum, and calf serum), metabolic status variables in the pre-partum dairy cow (insulin AUC, glucose AUC, NEFA AUC), and colostrum fat concentration. Coefficient of correlation (*r*-value) was set at $\geq |0.5|$ and *P*-values ≤ 0.05 was considered significant. The current trial was highly exploratory, which is why many variables were analyzed to explore all possible relationships that may exist.

5.4 Results

5.4.1 Colostrum and blood parameters and lack of an effect of treatment on lipidome

Colostrum fat concentration, as assessed by the creamatocrit approach, and IgG quantity, as assessed by Brix value, were highly variable across cows and ranged from 0.91 to 8.36% and 18.3 to 39.1, respectively (Table 5.1). The animals used in this study were part of a larger project that analyzed the effect of prepartum circadian disruption through exposure to chronic light-dark phase shift (PS) versus regular 24 h light-dark intervals (CON) on metabolic status and lactation performance (McCabe et al., 2021). Analysis of colostrum weight, fat and IgG quantity found no difference between PS and CON treatments. Plasma concentrations of NEFA, BHB, insulin, and glucose at 7 d BC were also not different between treatments (all *P*-values > 0.05).

PCA scores plots indicated no separation between plasma or colostrum lipid profiles based on treatment (CON vs. PS; Supplemental Figures 5.1-5.2; https://purr.purdue.edu/publications/3902/1). Student's *t*-test analysis found no effect of treatment ($P > 0.05$) on the quantity of individual lipids profiled in 7 d BC plasma nor in colostrum samples. Noting that 35 BC samples were collected prior to the start of treatment. Treatment was therefore removed from the model for all further analyses, which focused on describing the lipid profiles of plasma, serum, and colostrum and exploring potential relationships between the sample types.

5.4.2 Distribution and concentration of membrane lipids in plasma, serum, and colostrum

Principal component analysis score plots of membrane lipid classes (Figure 5.1A) demonstrated that calf serum and cow plasma samples clustered together and were not similar to

colostrum samples. A heat map and dendrogram of hierarchical cluster analysis of membrane lipids (Figure 5.1B), showed the greatest distinction by sample type, with pre-partum cow plasma samples clustering together and distinct from colostrum. The larger area spanned by 35 compared to 7 BC cow plasma samples in PCA plots indicated a greater degree of variation in 35 BC plasma lipid content between cows (Supplemental Figure 5.3; https://purr.purdue.edu/publications/3902/1). Student t-test-analysis indicated that more than half of the membrane lipid species (93 of 171) significantly decreased in abundance ($P < 0.05$) between 35 BC and 7 BC (Supplemental Table 5.4; https://purr.purdue.edu/publications/3902/1).

Data were analyzed to determine the percent of membrane lipid by class (PC, PG, PI, and SM) in cow plasma, colostrum, and calf serum samples (Figure 5.2). The most abundant membrane lipids found in plasma at 35 and 7 BC were PC, making up 84.7% and 84.3% of the total membrane lipids identified, respectively. The remaining membrane lipids detected in 35 BC and 7 BC plasma samples were PI, which made up 6.9% and 7.5%, and SM, which made up 8.2% and 7.8% of the membrane lipids, respectively. The PG lipids were the most abundant in colostrum samples accounting for approximately 75.9% of the membrane lipids measured. Whereas PC represented 22.6% and SM 1.5% of the membrane lipids detected in colostrum, with no PI detected. The most abundant membrane lipid in calf serum was PC at 71.9%, with 14.4% PG, 7.4% PI and 6.3% SM.

One hundred forty-eight individual membrane lipids were analyzed for their distribution across cow plasma, colostrum and calf serum samples (Figure 5.3). There were 78 MRMs related to membrane lipids shared between 35 BC plasma, 7 BC plasma, colostrum, and calf serum, 58 of which were PC, and the remaining were SM. There were 44 lipids found only in plasma of cows and serum of calves, but not in colostrum, and included 32 PC, five SM and seven PI. Colostrum contained 24 MRM with PG attribution, of which 23 were found in calf serum, while 35 BC and 7 BC plasma only contained one and two, respectively. No PI were present in colostrum, but seven species were found in cow plasma and calf serum. Cow plasma and calf serum contained 25 MRMs of SM while colostrum only contained 20 SM. Cholesteryl, campesteryl, and stigmasteryl esters varied between plasma, serum, and colostrum. Stigmasteryl esters were not present in colostrum, but one species was found in plasma and serum samples. There were sixteen cholesteryl esters and eight campesteryl esters species in plasma and serum samples compared to the thirteen and four in colostrum, respectively.

Membrane lipid distribution in cow plasma, colostrum, and calf serum was also analyzed for distribution of fatty acyl carbon length and level of unsaturated bonds (Figure 5.4A and 5.4B), which represents the sum between the two FA moieties. Plasma samples contained membrane lipids with the majority having 34 or 36 carbon and 1 or 2 unsaturated bonds. The most abundant membrane lipids in colostrum by total carbon number were 32 or 34 and number of unsaturated bonds were 0 or 1. Colostrum had a greater ($P < 0.05$) percentage of membrane lipids with 20, 30, and 32 carbons in the FA chains compared to those in the cow plasma and calf serum. Whereas cow plasma and calf serum had a greater ($P < 0.05$) percentage of 34, 36, 38, 40, and 42 carbons relative to colostrum. For number of unsaturated bonds, colostrum had a greater ($P < 0.05$) percentage of lipid species that were saturated, while plasma and serum samples had a greater ($P < 0.05$) percentage of membrane lipids with 1, 2, 3, and ≥ 5 unsaturated bonds.

The colostrum membrane lipids with less than 30 carbons in the FA chains, were all associated with PG, therefore the distribution of membrane lipids in cow plasma, colostrum, and calf serum was also analyzed without PG (Figure 5.4C and 5.4D). After removal of PG, colostrum had a greater ($P < 0.05$) percentage of membrane lipids with 30 and 32 carbons in the two FA chains compared to blood components, which had a greater ($P < 0.05$) percentage of 34, 36, 38, 40, and 42 carbons. Colostrum had a greater ($P < 0.05$) percentage of 0 and 4 unsaturated bonds across the two FA, whereas cow plasma and calf serum samples had a greater ($P < 0.05$) percentage of mono and polyunsaturated bonds up to 3 unsaturated bonds.

The ten most abundant membrane lipids in plasma samples made up 60-62% of the total membrane lipids. Nine of the ten most abundant membrane lipids in plasma were shared between 35 and 7 BC samples, all of which were PC (Table 5.2). Nine of the ten most abundant membrane lipid species in colostrum were PG. The vinyl ether plasmalogen phosphatidylcholine with 32 carbons and four unsaturations [PCp(32:4)] was the only lipid among the top ten most abundant in colostrum shared with 35 and 7 BC. Nine of the ten most abundant MRMs for membrane lipids found in calf serum were also among the most abundant found in cow plasma. Membrane lipid distribution in colostrum was also analyzed after removing PG from consideration. The ten most abundant membrane lipids, after PG removal, were all PC. Seven of the top ten were found in cow plasma (Table 5.3), with PCp(32:4) making up nearly 40% of all the non-PG membrane lipids in colostrum.

5.4.3 Distribution and concentration of TG lipids in plasma, serum, and colostrum

Principal component analysis of TG lipids in plasma, colostrum, and calf serum (Figure 5.5A) illustrated that serum and plasma samples clustered together and away from colostrum. To get a more detailed look at lipids circulating in cows compared to calves, colostrum was removed for hierarchical cluster analysis. Heat maps and dendrograms of hierarchical cluster analysis of TG lipids showed plasma samples clustered together and were distinct from calf serum (Figure 5.5B). There was no significant change in TG lipid composition ($P > 0.05$) between 35 and 7 BC. PCA showed that TG lipids in 35 BC and 7 BC plasma were similar and closely clustered (Supplemental Figure 5.4; https://purr.purdue.edu/publications/3902/1). Calf serum contained the greatest number of MRMs for TG (127) followed by colostrum (117), 7 BC (107) plasma, and then 35 BC (94) plasma.

The MRM profiling information regarding TG includes the identification of one fatty acyl group, followed by total carbon number and number of unsaturated bonds across all three FA (Xie et al., 2021). Analysis of the distribution of TG lipids by total carbon number in the fatty acyl chains found colostrum had more TG with 48, 50, and 56 carbons ($P < 0.05$) across the three FA chains compared to cow plasma at 35 BC and 7 BC (Figure 5.6A). The number of unsaturated bonds in TG also varied by sample type. Colostrum and calf serum had more 2 and 3 unsaturated bonds across fatty acyl groups, than plasma, but less TG with greater than or equal to 7 unsaturated bonds than cow plasma ($P < 0.05$; Figure 5.6B). The most common TG fatty acyl group for all four sample types was FA 18:1, followed by FA 18:0 and FA 16:0. Colostrum had greater 14:0, 16:0, and 18:1 fatty acyl groups identified as part of TG compared to plasma samples ($P < 0.05$; Figure 5.6C). Calf serum had significantly greater FA 18:1 in TG profiled compared to plasma and colostrum ($P < 0.05$).

Plasma at 35 BC and 7 BC shared eight of the top ten TG MRMs while colostrum shared three of these (Table 5.4). Colostrum shared four of its top ten TG MRMs with 35 BC plasma, five with 7 BC Plasma, and six with colostrum.

5.4.4 Correlation analysis of percent fat of colostrum and markers of metabolic status with plasma and colostrum lipids

Correlation analysis was used to investigate whether there were plasma or colostrum lipids related to the variation in percent fat of colostrum. There were seven membrane lipids (PI 36:2, PI

38:3, PI 38:4, PCo 42:0, SM d18:1/20:0, SM d18:1/18:1, and SM d16:1/22:1) in 35 BC plasma that were negatively correlated ($P < 0.05$) with percent fat in colostrum (Supplemental Table 5.5; https://purr.purdue.edu/publications/3902/1). In colostrum, two membrane lipids (PC 32:0 and SM (d18:1/18:0)) were positively correlated with colostrum fat concentration. Nine TG in 35 BC plasma (TG 18:2_48:2, TG 16:1_52:8, TG 18:2_50:5, TG 18:2_54:5, TG 18:2_54:9, TG 18:3_50:4, TG 18:3_54:3, TG 18:2_54:10, TG 20:4_54:4) and one in 7 BC plasma (TG 16:1_52:8) were negatively correlated with percent fat in colostrum, whereas six TG species in colostrum (TG 16:0_52:4, TG 18:2_50:2, TG 16:1_50:5, TG 16:1_50:3, TG 16:1_50:4, TG 18:2_52:4) were positively correlated with fat concentration of colostrum (Supplemental Table 5.6; https://purr.purdue.edu/publications/3902/1).

To investigate whether cow metabolic status may be responsible for colostrum variation, the relationship between markers of metabolic status and the concentration of lipids by class (i.e. membrane or TG) were analyzed. The NEFA AUC, in response to IVGTT at two weeks pre-partum, was negatively correlated with the total membrane lipid concentration of colostrum ($r = -0.55$; Table 5.5). Whereas NEFA AUC was positively correlated with total TG content of plasma samples at 7 BC ($r = 0.63$).

The relationships between NEFA AUC and carbon length or unsaturation level of membrane and TG lipids were analyzed (Table 5.5). The AUC for NEFA was positively correlated with membrane lipids in 7 BC plasma, with 34 carbons in the FA chains and monounsaturated FA, and negatively correlated to membrane lipids in 7 BC plasma samples with 36, 40, and 44 carbons as well as 3 unsaturated bonds. The AUC for NEFA was positively correlated with membrane lipids in colostrum that had 34, 36, 38, 40, and 42 carbons in the FA chains plus 1, 2, 3, 4, and ≥5 unsaturated bonds, but negatively correlated with 24, 26, 28, and 30 carbons in the FA chains as well as saturated lipid species. Circulating TG species and NEFA AUC was positively correlated with 50 carbons at 35 BC, two unsaturated bonds at 35 BC, 50 carbons at 7 BC, and two and three unsaturated bonds at 7 BC. The NEFA AUC was negatively correlated with TG with 3 saturated FA at 35 BC, 54 and 56 carbons at 7 BC, and saturated TG or those with, five or greater unsaturated bonds at 7 BC. NEFA AUC was also negatively correlated with TG with 48 carbons and one unsaturated bond in colostrum but positively correlated with 52 carbon and three unsaturated bonds in colostrum. Correlation analysis between the carbon lengths and unsaturation levels of membrane lipids and TG in 35 BC plasma, 7 BC plasma, and colostrum, with glucose AUC, insulin AUC,

total PG in colostrum, and colostrum fat concentration were also evaluated (Supplemental Table 5.7; https://purr.purdue.edu/publications/3902/1).

5.5 Discussion

In general, the circulating lipid profiles of cows were distinct from colostrum lipid profiles. There was a higher concentration of PG in colostrum than in circulating lipids. Plasma lipid profiles of cows were highly similar between five wks and one wk before calving and marked by similar concentrations and distributions of TG lipids between the time points. The distribution of membrane lipids in cow plasma was also similar between time points, however, approximately half of the individual membrane lipids decreased in concentration from 35 BC to 7 BC. The lipid profiles of cow plasma and calf serum were also similar to one another, however, calf serum contained a high concentration of PG. The high abundance of PG lipids in colostrum was related to the twenty distinct PG lipids in calf serum. Whereas plasma from multiparous cattle only had one or two PG lipids. This finding indicates that colostrum was potentially the source of the PG in calf serum, but cow plasma was not the source of PG in colostrum.

Lack of similarity between lipids in colostrum and those circulating in plasma likely reflects the uptake and processing of lipids by the mammary gland. In the mammary gland, lipids coming from circulation are broken down into monoacylglycerides and free fatty acids by lipoprotein lipase in endothelial cells and then taken up by mammary epithelial cells (Bayly, 2014). Fatty acids are repackaged into TG molecules in the rough endoplasmic reticulum of mammary epithelial cells and released as fat droplets surrounded by a phospholipid monolayer. Fat droplets move to the apical membrane of the mammary epithelial cells, are encapsulated by the plasma membrane phospholipid bilayer through an apocrine process and are secreted as a milk fat globule (**MFG**) with a trilayer phospholipid membrane (Bauman et al., 2006). Thus, the different distribution of lipids in circulation and colostrum reflects mammary specific assembly of TG and lipid composition of the apical membrane of epithelial cells.

Despite similar distribution of classes of membrane lipids in 35 and 7 BC plasma samples, approximately 50% of lipid membranes decreased from 35 BC to 7 BC, without a significant change in TG lipids. In order for TG and cholesterol esters to be transported through circulation, they need to be assembled into plasma lipoproteins, which consists of a hydrophobic core of TG and cholesterol esters, surrounded by a hydrophilic membrane of phospholipids apolipoproteins,

and free cholesterol (Feingold and Grunfeld, 2000). The reduction in membrane lipids with no change in TG content suggests that the apolipoprotein molecules were larger, i.e. had greater volume of TG to lipid membrane envelope. When a cow experiences negative energy balance, which may occur during late gestation, and based on energy calculation derived from NRC (2001) six out of 16 cows were in negative energy balance at 7 BC (data not shown), lipids are released from peripheral tissues as NEFA and taken up by the liver. Hepatocytes export lipids back into circulation by synthesizing and secreting very low-density lipoproteins (VLDL; Katoh, 2002). The phospholipid composition of VLDL is low compared to low-density lipoproteins and high-density lipoproteins (Stead and Welch, 1975). This would suggest that increased VLDL could explain why membrane lipids decrease from 35 BC to 7 BC as cows experience negative energy balance and are mobilizing more adipose tissue.

Cow plasma and calf serum were similar in membrane and TG lipid concentrations and distributions; however, PG was present in calf serum but not cow plasma. Colostrum had a high abundance of PG lipids. The PG lipids found in colostrum and circulating in the calf had the same number of carbons and unsaturated bonds, suggesting that when a calf consumes colostrum, PG remain intact when absorbed by enterocytes and transported into circulation. The transport of intact PG molecules may be high in neonates, as prior to 24 h postnatal (Broughton and Lecce, 1970) the tight junctions between enterocytes are not formed (Weaver et al., 2000). The high abundance of PG in colostrum may indicate its importance in mammary function or neonate requirements. Phosphatidylglycerol is a precursor for cardiolipin (CL), which is an inner mitochondrial membrane lipid (Stillwell, 2016). Therefore, the presence of PG in the colostrum may be reflective of lactocyte mitochondria being secreted into milk, which has been suggested in sow milk (Suarez-Trujillo et al., 2021). Since colostrum composition likely reflects the calf's requirements and mitochondria generate 90% of a cell's energy, high levels of PG substrates may be needed to support mitochondrial biogenesis in cells of growing tissues. Another potential reason for selection for PG in colostrum is its potential use by the immune system of the calf. Research in mice has shown that PG are capable of blocking toll-like receptor mediated inflammation (Choudhary et al., 2019), leading to an improved immune status and reduced energy expenditure. Additionally, as a phospholipid, the basic structure of PG is a glycerol 3-phosphate backbone with FA bound to both sn-1 and 2. The PG in colostrum were the only membrane lipids with 20-28 carbons and therefore served as a source of MCFA for the calf. Calves supplemented with MCFA had improved immune

status as evident by increased serum titers after vaccinating with bovine viral diarrhea and parainfluenza-3, as well as increased cytokine production, and immune cell phagocytosis capability (Hill et al., 2011a; Garcia et al., 2015) and reduced scours and treatment events (Hill et al., 2011b). Improved immune responses with MCFA supplementation may be due to their antimicrobial and antiviral effects (Zentek et al., 2011). Therefore, a source of MCFA may be important to increase immune function in newborn calves. Alternatively, PG is very abundant in bacterial membranes (Stillwell, 2016), therefore if bacteria is introduced into colostrum during colostrum collection and processing, these bacteria could be a source of the PG identified in colostrum samples and in calf serum.

Similar to findings of others, colostrum fat content varied greatly across cows (Quigley et al., 1994; Morrill et al., 2012). Several highly unsaturated TG species in 35 BC plasma samples were negatively related to the percent fat of colostrum. The ingestion of high concentrations of polyunsaturated fatty acids (**PUFA**) coupled with an altered rumen environment causes milk fat depression in lactating cows (Bauman and Griinari, 2001). As some unsaturated FA are toxic to rumen microbes (Maia et al., 2007), they are converted to saturated FA in the rumen, in a process referred to as biohydrogenation. Biohydrogenation intermediates (i.e. C18:2 t-10, c-12) inhibit enzymes associated with milk fatty acid synthesis in the mammary gland resulting in reduced fat content of milk (Harvatine and Bauman, 2011). Diets that cause milk fat depression generally have increased PUFA concentrations and are associated with reduced *de novo* synthesis of FA in the mammary gland (Peterson et al., 2003). There is the potential that high levels of PUFA in circulation were indicative of the production of specific biohydrogenation intermediates in the rumen associated with milk fat depression. Although all of the cows were on the same diet, there is significant variation in the production of biohydrogenation intermediates for cattle fed the same diet (Calus et al., 2005).

The historical and current dogma is that FA in colostrum are obtained from circulation, as formation of colostrum is prior to secretory activation when *de novo* fatty acid synthesis is fully initiated (Anderson et al., 2007). Fatty acids with 14 carbons or less are considered *de novo* synthesized in the gland and derived from butyrate and acetate. Milk FA with 18 or more carbons are taken up directly from circulation. Whereas 16 carbon FA are derived either from circulation or synthesized *de novo* in the gland. The two membrane lipids in colostrum that correlated with total fat content were PC 32:0, which on average would be constituted by two Fsaturated 16 carbon

fatty acyl groups, and SM (d18:1/18:0), which has two 18 carbon fatty acyl groups. The six TG in colostrum that were positively correlated with the total fat content in colostrum ranged in length from 50-52 carbons. Due to the carbon length of the six TG and the identified acyl group being 16 and 18 carbons in length, it is likely that these FA that comprise the TG are derived from circulation. These six TG also had a high level of unsaturated bonds across the three fatty acyl groups and the identified group included alpha-linolenic (C18:3) and linoleic (C18:2) acids. Essential fatty acids, and likely their derivatives (e.g. arachidonic acid C20:4) are preformed fats in colostrum. Thus, the mechanisms controlling uptake of preformed fatty acids from circulation by the mammary gland during late gestation are affecting the total fat content of colostrum. That said, it is important to note that the PG in colostrum had 20-28 carbons across the fatty acyl groups, indicating on average these FA ranged from 10-14 carbons in length, and accordingly *de novo* synthesized. Additionally, colostrum had more membrane lipids with 30 and 32 carbons compared to blood components, which had more 34, 36, 38, 40, and 42 carbons. This also supports the idea that FA in colostrum are derived from *de novo* fatty acid synthesis, despite secretory activation not being fully activated yet.

In colostrum, nearly 40% of the non-PG membrane lipids were PCp(32:4). PCp is a plasmalogen, which is a naturally occurring phospholipid that has a vinyl ether fatty alcohol attached to the glycerol backbone at the *sn*-1 position (Naudí et al., 2015). Plasmalogens are commonly found in the cell membranes of electrically active tissues such as brain, heart, and myelin (Thompson et al., 2004). Also, immune cells, such as leukocytes, neutrophils, and macrophages, contain significant levels of plasmalogens (Nagan and Zoeller, 2001). The presence of PCp(32:4) in colostrum would suggest that colostrum is providing the calf with cell components needed for the development of the nervous, cardiovascular, and immune system.

It is important to note, that in this study the lipid content of colostrum was evaluated by separating out the fat globules from the colostrum. The remaining colostrum fraction, however, also contains phospholipids as a part of extracellular vesicles (**EV**), which are small membrane-enclosed vesicles secreted from most cells and are therefore present in body fluids like blood, urine, saliva, and milk (Ascanius et al., 2021). The specific phospholipid content of the EV present in bovine colostrum is unclear, but in bovine milk it is estimated that EV make up 35-40% of the phospholipids present in membrane structures (Huang and Kuksis, 1967). Therefore, it is likely

that calves will end up consuming slightly more phospholipids via colostrum than just those present in the fat globules that were analyzed for this study.

The NEFA AUC was positively correlated with membrane lipids in colostrum that had 34-42 carbons, and 1 to ≥5 unsaturated bonds, but negatively correlated with saturated membrane lipids with 24-30 carbons in the FA chains, which were all PG. Bovine colostrum milk fat globule membranes (**MFGM**) contain a greater proportion of unsaturated fatty acids compared to the MFGM in mature bovine milk (Zou et al., 2015). This could indicate that the unsaturated FA in circulation when NEFA concentrations are high, are contributing to the unsaturated FA in the MFGM of colostrum. Moreover, high NEFA may suppress the synthesis of PG and the associated *de novo* synthesized MCFA in the mammary gland. Further work is needed to understand if less available PG is detrimental to the neonate. Also needed, is a greater understanding if high circulating NEFA is detrimental to lipid composition of colostrum, or whether altered plasma membrane lipid profiles of colostrum related to high NEFA are simply reflective of lipids available for uptake and secretion from the gland when rates of lipolysis are high in the cow.

The negative relationship of high PUFA in 35 BC plasma with colostrum fat content may also be indicative of lipids involved in inflammatory cascades. During the transition period, when dairy cows undergo increased adipose tissue mobilization, they have an increased risk for inflammation-based diseases such as metritis, mastitis, and laminitis (Sordillo et al., 2009). *In vitro* studies found that treating bovine endothelial cells with NEFA increased mRNA expression of the cyclooxygenase 2 enzyme involved in eicosanoid biosynthesis, and the production of eicosanoids 9- and 13-hydroxyoctadecadienoic acids in culture (Contreras et al., 2012). Eicosanoids are components of inflammatory processes. The increased production of eicosanoids and expression of enzymes involved in eicosanoid biosynthesis when exposed to NEFA indicates that elevated NEFA concentrations are associated with the vascular inflammatory response in cattle. Eicosanoids are synthesized from PUFA and include prostaglandins and leukotrienes, which function in the induction and inhibition of inflammation (Calder, 2001). Therefore, PUFA in circulation can be used by the body to synthesize components of the immune system that lead to inflammation. There appears to be a relationship between negative energy balance, inflammation, and immunosuppression associated with disease and lower milk production (Esposito et al., 2014). Further studies are needed to understand the negative relationship of TG with highly unsaturated FA and colostrum fat content.

Additionally, NEFA AUC had relationships with total membrane and TG lipids and the carbon length and unsaturation level of membrane and TG lipids in both plasma and colostrum samples. Cows with high NEFA concentrations are at a greater risk for developing metabolic disease. Studies of humans have found high levels of NEFA are associated with dyslipidemia and development of diseases associated with metabolic syndrome (Gutierrez et al., 2009). A negative relationship was found between NEFA AUC and the total membrane lipid concentration and total PG in colostrum. The NEFA AUC was positively correlated with total TG content of plasma samples at 7 BC. These findings would indicate that increased NEFA led to increased concentrations of TG lipids in circulation at 7 BC but also led to decreased membrane lipids and PG in colostrum. Prior research evaluating high concentrations of circulating NEFA in hyperketonemic cows reported increased milk fat (Adewuyi et al., 2005; Palmquist, 2006). When circulating NEFA is high, it leads to increased TG but potentially lower membrane lipid concentrations in plasma. This increased TG to membrane lipid ratio in circulating might be impacting MFG synthesis, leading to larger MFG (increased TG to membrane lipid ratio) in colostrum, which is supported by prior research that found that NEFA has an important role in the ratio of TG to membrane lipids and therefore MFG diameter (Argov-Argaman, 2019).

A limitation of this study was sampling protocols only captured a snapshot of what is circulating at each time point. It is unclear if the lipid species identified are coming directly from the diet, mobilized from adipose tissue, or processed by the liver. Another limitation of this study was plasma samples were prepared from the cow blood, whereas serum samples were prepared from the calves. Ideally both samples would have been plasma due to the increased reliability in lipid measurements compared to serum (Ishikawa et al., 2014). It is also important to note that the high variability in colostrum fat content from cow to cow (n = 16) could impact the lipidome profile analysis of the colostrum, colostrum with lower fat might have significantly less lipid species present. Lastly, this study used MRM profiling and although it is highly efficient and cost effective, it is an exploratory method and does not determine the FA composition of esterified lipid species, and so these can only be inferred from the total carbon number and level of unsaturation.

5.6 Conclusions

Lipidome analysis of colostrum found profiles distinct from the circulating lipids of the cow and calf, which were similar to each other. However, a significantly greater amount of phosphatidylglycerol was identified in the circulation of the calf compared to the cow. High levels of phosphatidylglycerol in calf serum were related to the high levels in colostrum, and were of the same species, and suggest direct transfer from the dam to the neonate through the milk. Circulating non-esterified fatty acid concentrations were related to lipids in circulation at 35 d and 7 d before calving, and the lipids found in colostrum, indicating that the metabolic status of the cow can influence the lipids present in circulation and the lipids that are available to the calf in colostrum. Next steps should include determining the specific role phosphatidylglycerols play in calf health, nutritional work evaluating if altering the diet of the cow can beneficially alter the colostrum lipidome profile and comparing calf blood lipidome before and after colostrum feeding.

5.7 Acknowledgments

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Table 5.1. Colostrum parameters (fat concentration, brix value, and weight) and plasma non-esterified fatty acid (NEFA), beta-hydroxybutyrate (BHB), insulin, and glucose at 7 d before calving (BC) based on study treatments (control (CON) vs. phase-shift (PS)).

	Treatment			
Item	CON	PS	SEM	*P*-value
Plasma at 7 BC (n = 16)				
NEFA, mmol/L	0.29	0.33	0.11	0.80
BHB, mmol/L	0.71	0.83	0.06	0.18
Insulin, mIU/mL	0.49	0.45	0.13	0.86
Glucose, mg/dL	78.5	78.0	2.69	0.90
Colostrum (n = 16)				
Fat[1], %	5.5	5.5	0.75	0.99
Fat range, %	0.9-8.4	2.8-8.2	-	-
Brix value[2]	23.9	28.4	2.05	0.14
Brix value range	18.3-39.1	20.6-33.4	-	-
Weight, kg	8.3	6.7	1.49	0.46
Weight range, kg	1.8-15.2	2.6-16.9	-	-

[1]Fat percent was determined using the creamatocrit approach.
[2]Brix value was determined using a Brix refractometer.

Table 5.2. Top 10 membrane lipid species (phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylinositol (PI), and sphingomyelin (SM)) and their percentage of the total membrane lipids in 35 d before calving (BC) plasma (n = 16), 7 d BC plasma (n = 16), colostrum (n = 16), and calf serum (n = 15).

35 BC Plasma		7 BC Plasma		Colostrum		Calf Serum	
Lipid species	%	Lipid species	%	Lipid species	%	Lipid species	%
PC (36:2)	14.48	PC (36:2)	15.31	PG (20:0)	10.57	PC (36:2)	11.95
PC (34:2)	13.51	PC (34:2)	14.25	PG (32:0)	9.84	PC (34:1)	10.36
PC (34:1)	6.96	PC (34:1)	6.87	PCp[2] (32:4)	8.90	PC (34:2)	10.14
PC (36:1)	6.70	PC (36:1)	6.54	PG (30:0)	8.79	PC (36:1)	6.62
PC (36:3)	4.44	PC (36:3)	4.49	PG (34:1)	7.72	PC (36:3)	5.42
PC (30:1)	3.16	PCp[2] (32:4)	3.37	PGo[1] (20:0)	5.77	PCp[2] (32:4)	4.03
PC (38:3)	2.95	PC (38:3)	2.87	PG (32:1)	4.36	PG (34:1)	2.20
PCp[2] (32:4)	2.94	PC (30:1)	2.75	PG (28:0)	4.09	PC (38:4)	2.12
PC (38:4)	2.81	PC (38:4)	2.65	PG (24:0)	3.08	PC (36:4)	2.02
PC (36:4)	2.08	PI (38:4)	2.29	PG (34:2)	2.70	PI (38:4)	1.98

[1]PGo indicates the presence of an alkyl ether substituent.
[2]PCp indicates the presence of a 1Z-alkenyl ether (plasmalogen) substituent.

Table 5.3. Top 10 membrane lipid species (phosphatidylcholine (PC), phosphatidylinositol (PI), and sphingomyelin (SM), excluding phosphatidylglycerol) and their percentage of the total membrane lipids in colostrum (n = 15).

Colostrum (no PG)	
Lipid species	%
PCp[1] (32:4)	39.93
PC (34:4)	12.10
PC (34:1)	3.68
PCo[2] (34:4)	3.59
PC (30:1)	3.16
PC (32:0)	2.38
PC (36:2)	2.32
PC (36:1)	2.22
PC (34:5)	2.19
PC (34:2)	2.19

[1] PCp indicates the presence of a 1Z-alkenyl ether (plasmalogen) substituent.
[2] PCo indicates the presence of an alkyl ether substituent.

Table 5.4. Top 10 triacylglycerol (TG[1]) species and their percentage of the total TG in 35 d before calving (BC) plasma (n = 16), 7 d BC plasma (n = 16), colostrum (n = 16), and calf serum (n = 15).

35 BC Plasma		7 BC Plasma		Colostrum		Calf Serum	
Lipid species	%	Lipid species	%	Lipid species	%	Lipid species	%
TG 18:1_52:1	5.52	TG 18:1_52:1	5.53	TG 16:0_50:2	9.51	TG 18:1_52:2	11.59
TG 18:0_52:1	5.34	TG 18:0_52:1	5.28	TG 16:0_48:1	7.02	TG 16:0_50:2	7.26
TG 18:1_52:2	3.92	TG 18:1_52:2	4.45	TG 18:1_50:2	6.65	TG 18:1_50:2	4.71
TG 18:0_52:0	3.54	TG 16:0_50:2	4.36	TG 18:1_52:2	6.06	TG 16:0_52:2	4.31
TG 16:0_52:1	3.51	TG 16:0_52:1	3.51	TG 18:1_48:1	5.76	TG 18:1_52:3	3.31
TG 16:0_50:2	3.49	TG 18:1_50:2	2.89	TG 18:1_50:3	3.41	TG 18:1_54:3	3.10
TG 18:0_54:1	2.80	TG 18:0_52:0	2.76	TG 14:0_48:1	2.93	TG 18:1_52:1	3.07
TG 18:1_50:2	2.36	TG 16:0_52:2	2.50	TG 16:0_52:2	2.85	TG 18:1_50:3	2.88
TG 16:0_52:0	2.33	TG 18:0_54:1	2.32	TG 16:0_50:3	2.74	TG 18:2_52:3	2.66
TG 18:1_54:2	2.30	TG 16:0_48:0	2.02	TG 16:1_48:1	2.35	TG 16:0_48:1	2.49

[1]TG nomenclature shows one fatty acyl group attached to the glycerol backbone, followed by total carbon length and unsaturation level of the TG.

Table 5.5. Correlations between triacylglycerol (TG) lipids in 7 d before calving (BC) plasma (n = 16), total membrane lipids in colostrum (n = 16), and the carbon lengths and unsaturation levels of membrane lipids and TG in 35 d BC plasma (n = 16), 7 d BC plasma, and colostrum with non-esterified fatty acid (NEFA) area under the curve (AUC).

	NEFA AUC	
Item	*r*-value	*P*-value
Total TG lipids in 7 BC plasma	0.63	0.01
Total membrane lipids in colostrum	-0.55	0.03
7 BC plasma membrane lipids		
34 C	0.78	0.0006
36 C	-0.62	0.01
40 C	-0.56	0.03
44 C	-0.55	0.03
1 unsaturation	0.67	0.006
3 unsaturations	-0.74	0.002
Colostrum membrane lipids		
24 C	-0.52	0.05
26 C	-0.66	0.008
28 C	-0.66	0.008
30 C	-0.65	0.008
34 C	0.60	0.02
36 C	0.67	0.007
38 C	0.67	0.007
40 C	0.79	0.0005
42 C	0.76	0.0009
0 unsaturations	-0.72	0.003
1 unsaturation	0.53	0.04
2 unsaturations	0.74	0.002
3 unsaturations	0.58	0.02
4 unsaturations	0.64	0.01
≥5 unsaturations	0.61	0.02
35 BC plasma TG lipids		
50 C	0.53	0.04
0 unsaturations	-0.53	0.04
2 unsaturations	0.69	0.004
7 BC plasma TG lipids		
50 C	0.78	0.0007
54 C	-0.73	0.002
56 C	-0.53	0.04
0 unsaturations	-0.61	0.02
2 unsaturations	0.83	0.0001
3 unsaturations	0.66	0.008
5 unsaturations	-0.57	0.03
6 unsaturations	-0.57	0.03
≥7 unsaturations	-0.74	0.002
Colostrum TG lipids		
48 C	-0.68	0.006
52 C	0.69	0.004
1 unsaturation	-0.74	0.002
3 unsaturations	0.63	0.01

A)

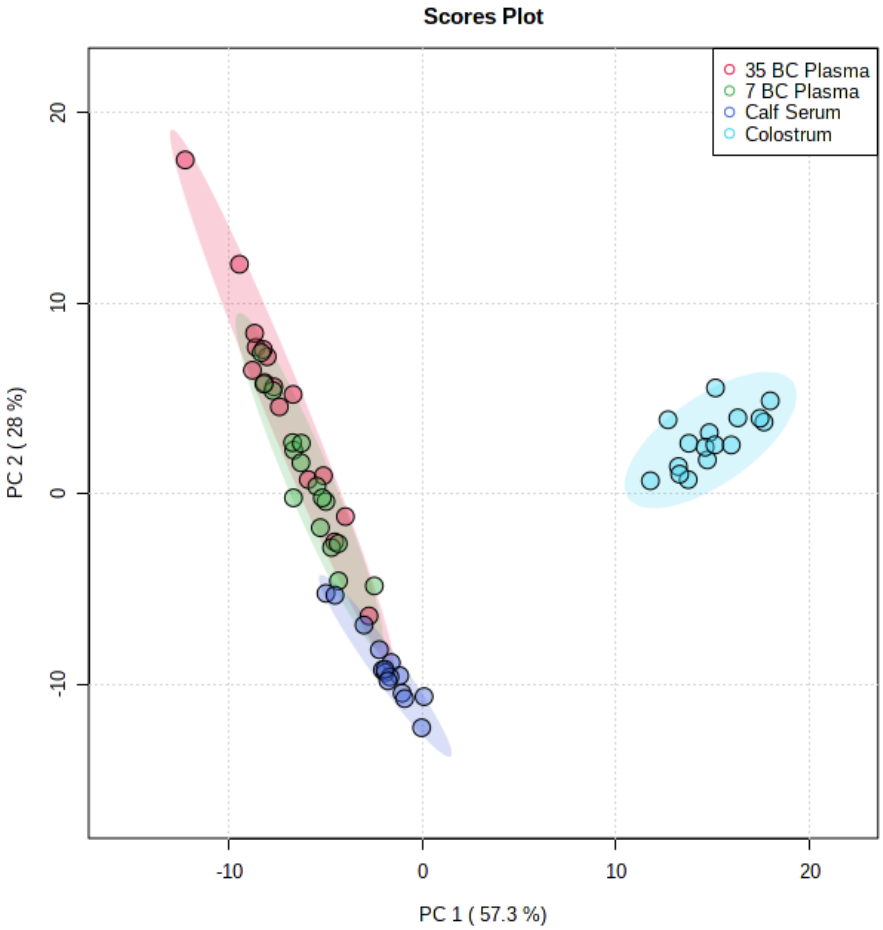
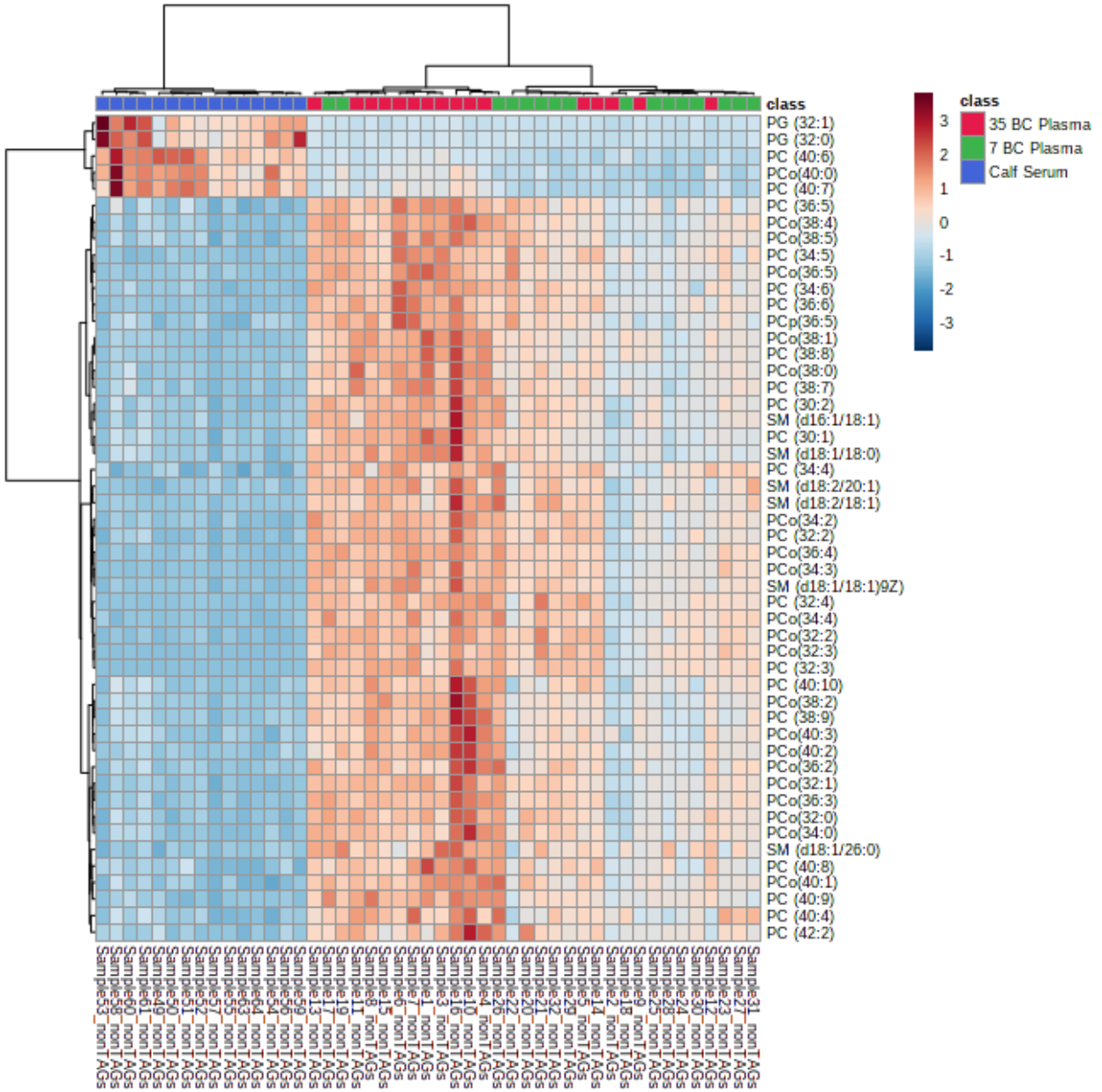


Figure 5.1. Principal component analysis scores plot (A) of membrane lipids in 35 d before calving (BC) plasma (n = 16), 7 d BC plasma (n = 16), colostrum (n = 16), and calf serum (n = 15) and heat map of hierarchical cluster analysis (B) for membrane lipids in 35 BC plasma (n = 16), 7 BC plasma (n = 16), and calf serum (n = 15).

Figure 5.1 continued

B)



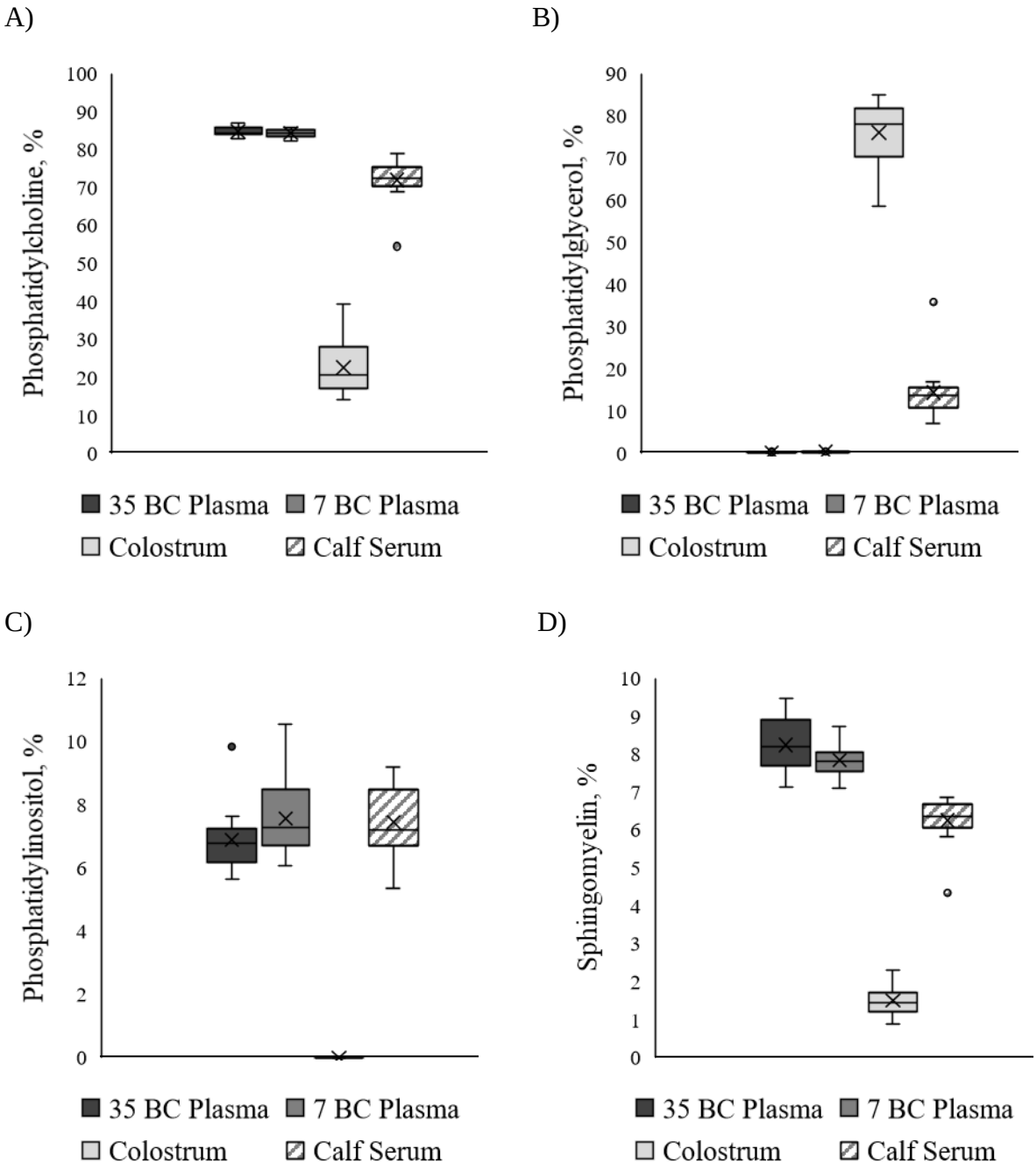


Figure 5.2. Membrane lipid concentration breakdown by lipid class (A: phosphatidylcholine, B: phosphatidylglycerol, C: phosphatidylinositol, and D: sphingomyelin) for cow plasma at 35 d before calving (BC; n = 16) and 7 d BC (n = 16), colostrum (n = 16), and calf serum (n = 15). Error bars represent minimum and maximum.

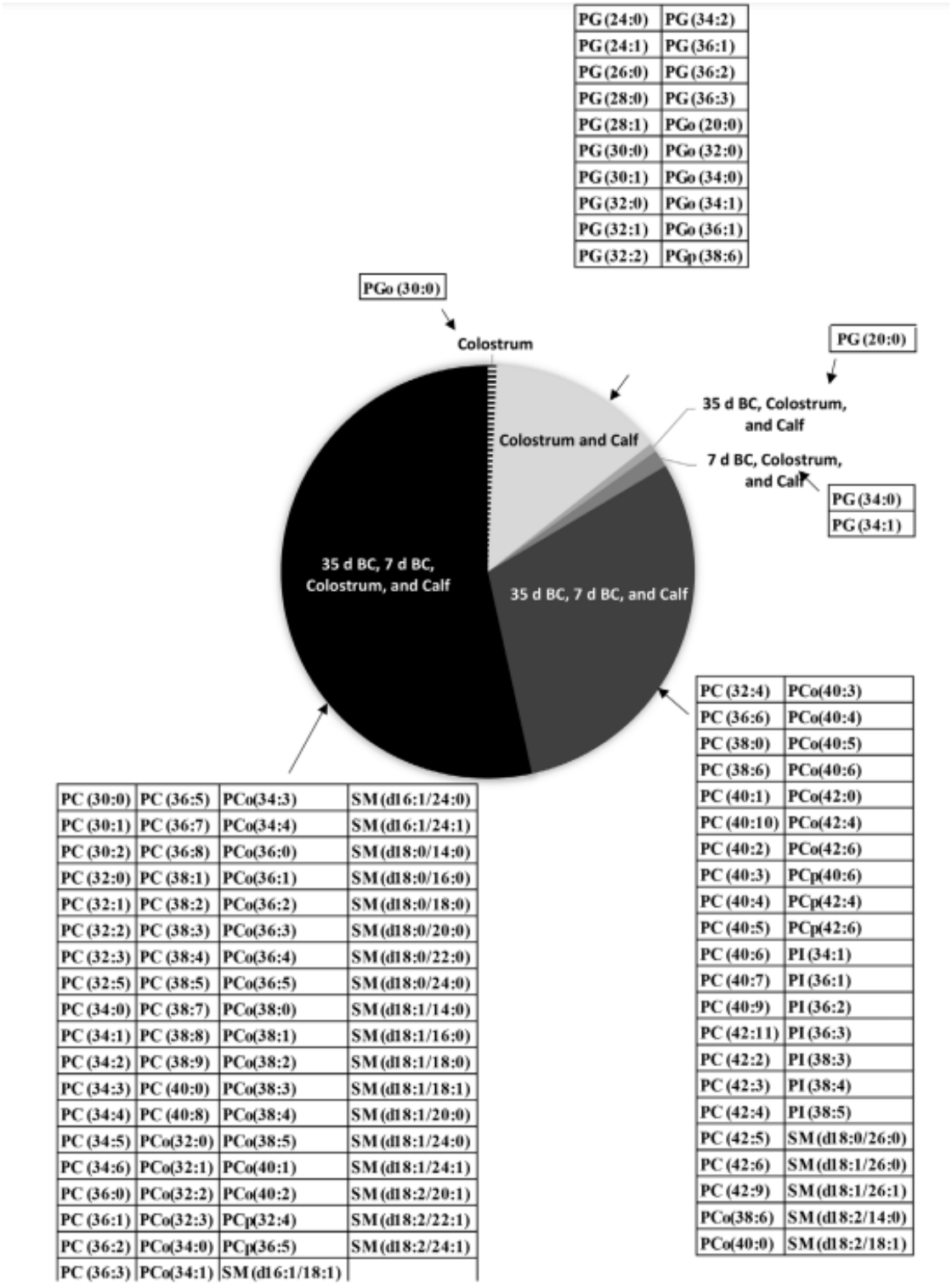


Figure 5.3. Pie chart summarizing membrane lipids (phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylinositol (PI), and sphingomyelin (SM)) shared and distinct between 35 d before calving (BC) plasma (n = 16), 7 d BC plasma (n = 16), colostrum (n = 16), and calf serum (n = 15).

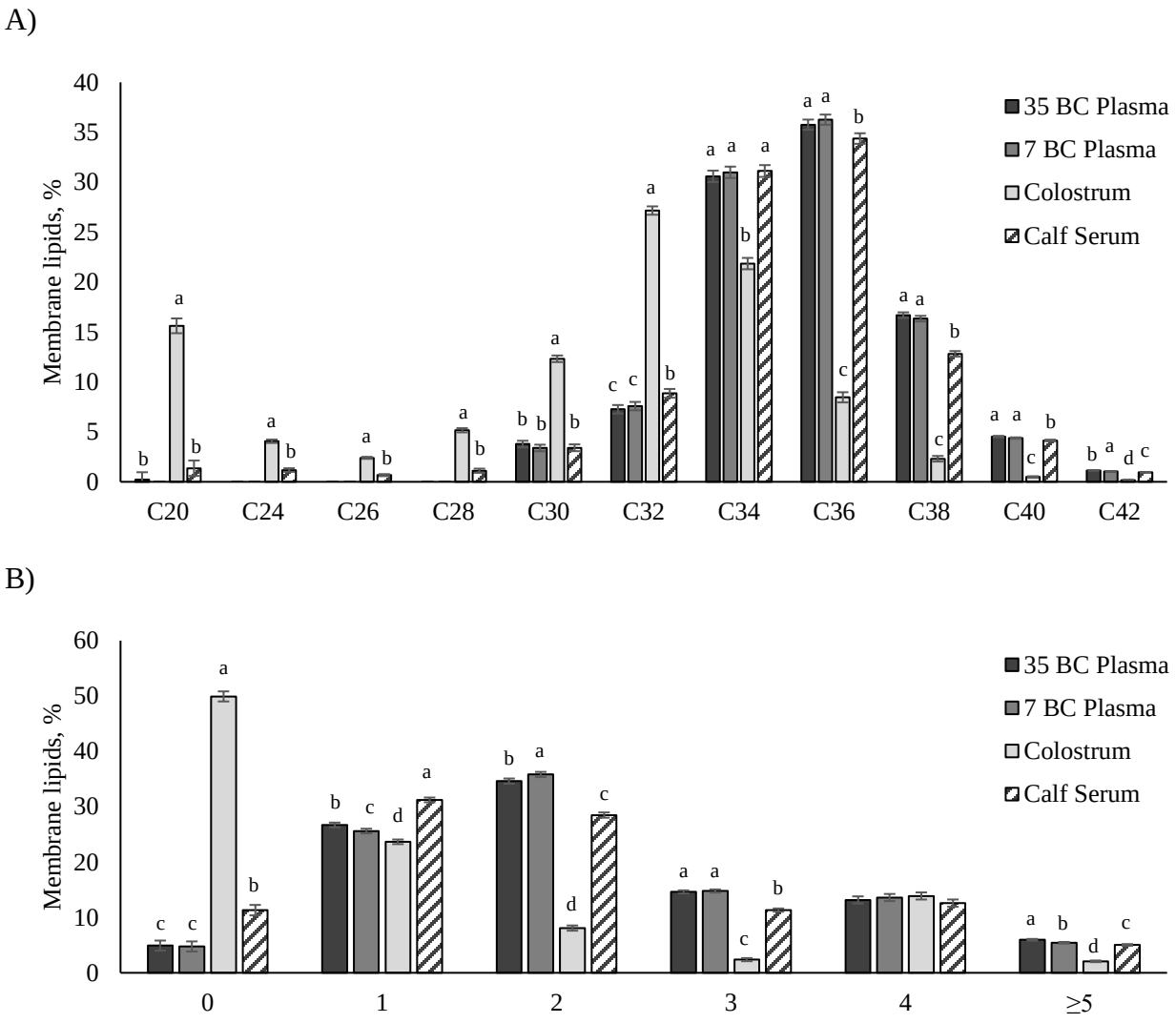
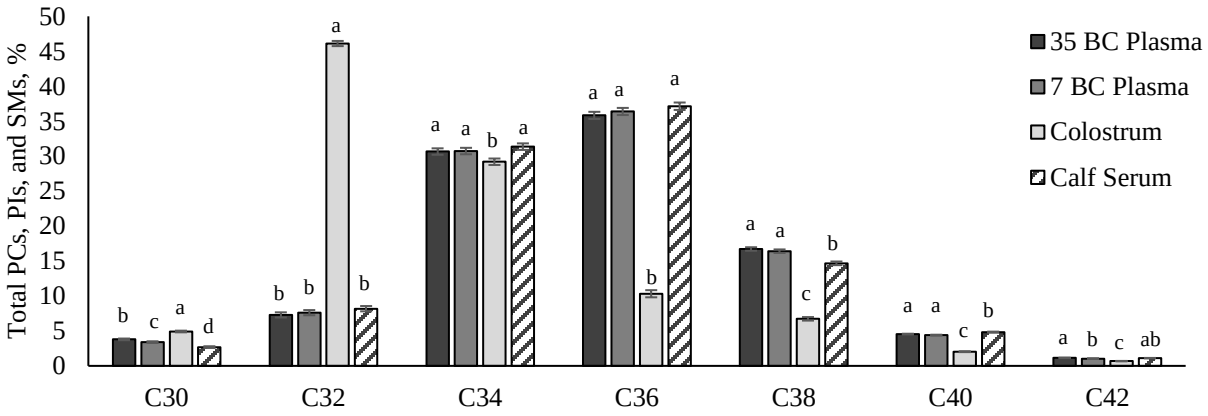


Figure 5.4. Concentration of all identified membrane lipids (phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylinositol (PI), and sphingomyelin (SM)) based on A) carbon length and B) saturation level present in 35 d before calving (BC) plasma (n = 16), 7 d BC plasma (n = 16), colostrum (n = 16), and calf serum (n = 15) and concentration of membrane lipids after removing PG based on C) carbon length and D) saturation level present in 35 BC plasma (n = 16), 7 BC plasma (n = 16), colostrum (n = 16), and calf serum (n = 15). Letters (a-d) signify significant differences ($P < 0.05$) between sample types. Error bars represent SEM.

Figure 5.4 continued

C)



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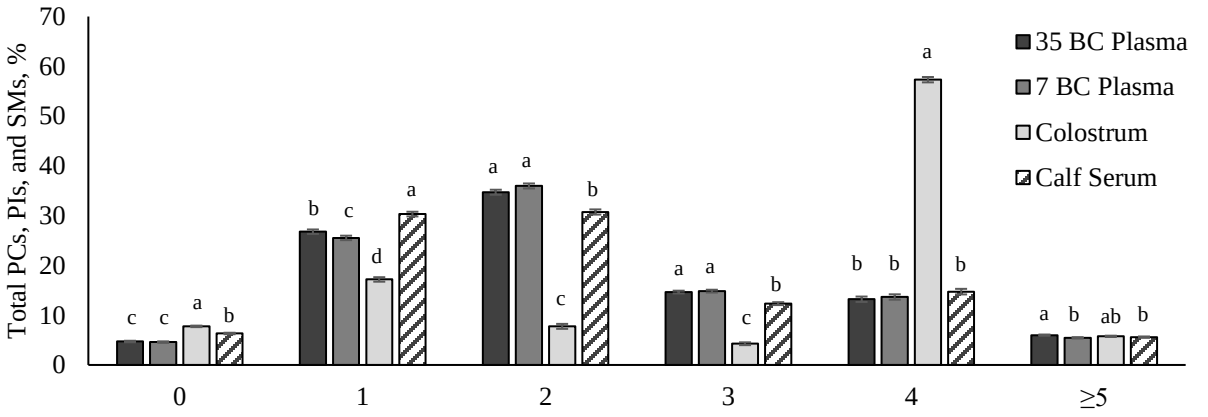


A)

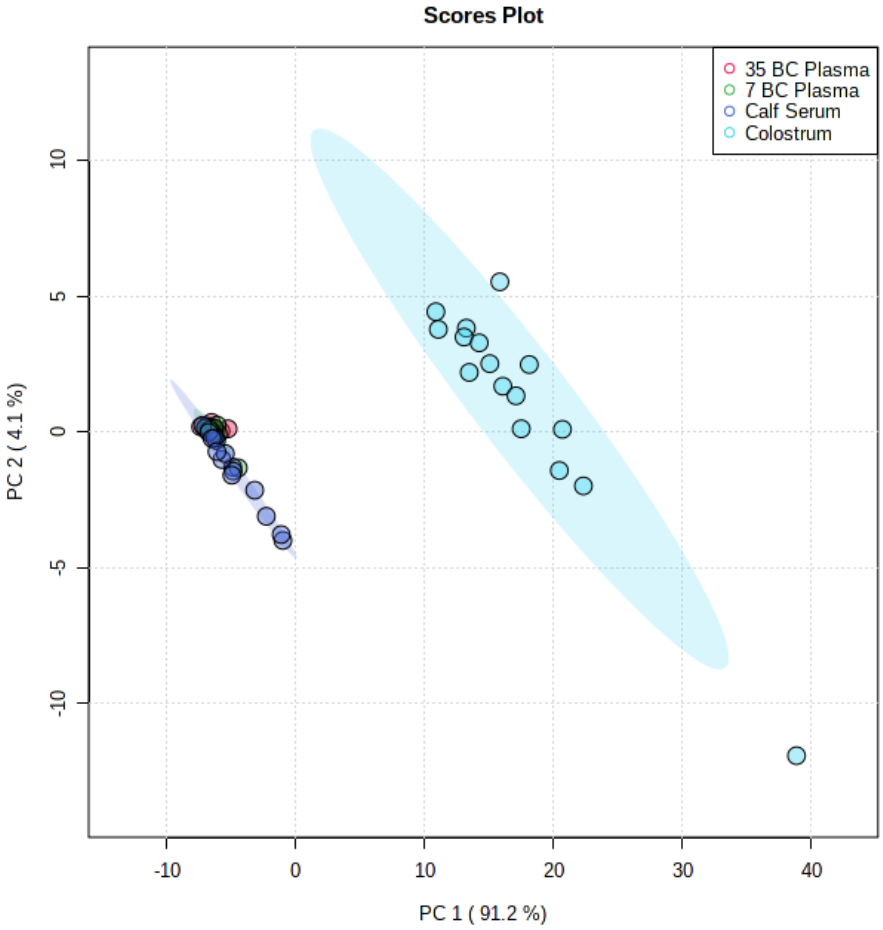
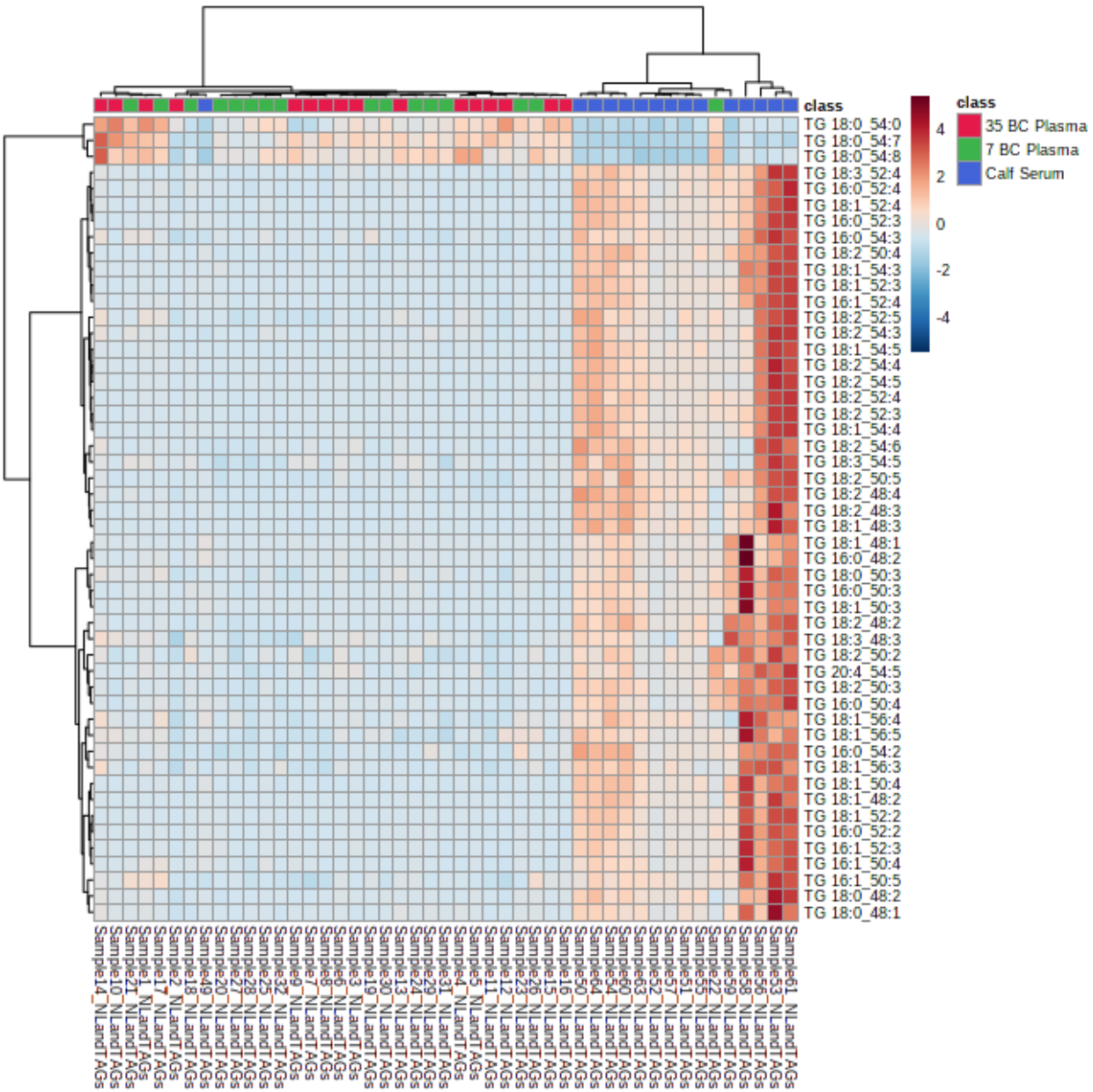


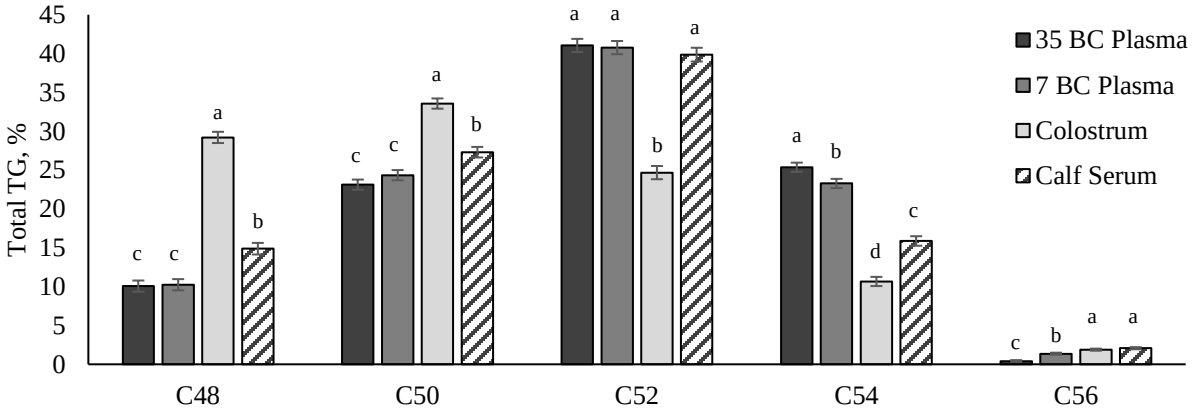
Figure 5.5. Principal component analysis (A) for triacylglycerol (TG) lipids in 35 d before calving (BC) plasma (n = 16), 7 d BC plasma (n = 16), colostrum (n = 16), and calf serum (n = 15) and heat map of hierarchical cluster analysis (B) for TG lipids in 35 BC plasma (n = 16), 7 BC plasma (n = 16), and calf serum (n = 15).

Figure 5.5 continued

B)



A)



B)

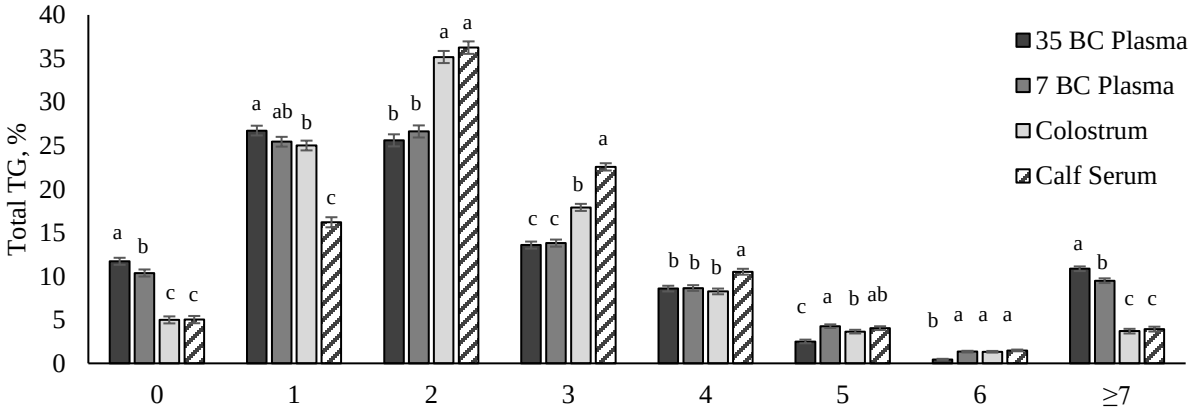
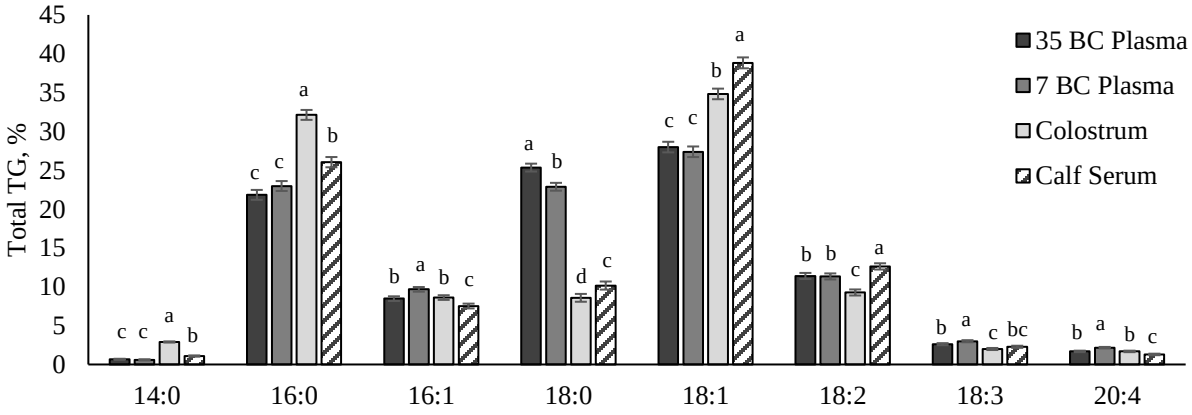


Figure 5.6. Concentration of all identified TG lipids based on A) carbon length, B) saturation level, and C) FA present in 35 d before calving (BC) plasma (n = 16), 7 BC plasma (n = 16), colostrum (n = 16), and calf serum (n = 15). Letters (a-c) signify significant differences ($P < 0.05$) between sample types. Error bars represent SEM.

Figure 5.6 continued

C)



CHAPTER 6. CONCLUSION

Early life calf health and development continue to be areas of focus for researchers and professionals in the dairy industry. Early life calf nutrition, management, and health status provide the foundation for future production and health. However, calf morbidity and mortality continue to be areas of opportunity for improvement in the dairy industry as current nationwide averages remain greater than the target rates for morbidity and mortality, which are <25 and <5%, respectively. Sick calves have reduced productivity and negatively impact farm economics. In addition, animal agriculture is committed to reducing the use of antibiotics due to the concern of antibiotic resistant bacteria. Researchers and producers are faced with the challenge of identifying strategies to reduce calf morbidity and mortality, while also decreasing reliance on antibiotics.

One strategy to improve the health status and performance of dairy calves is feeding nutraceuticals, which describes feed or nutrients that have natural health benefits, for example, *Saccharomyces cerevisiae* fermentation products. Feeding calves *Saccharomyces cerevisiae* fermentation products in milk replacer and solid feeds until 4 months of age improved post-weaning average daily gain and feed efficiency and reduced the treatment incidence for respiratory disease, thereby, reducing antibiotic use. The *Saccharomyces cerevisiae* fermentation products increased the acute innate immune response, as determined by increased TNF-α, glucose, and respiration rate during an intravenous lipopolysaccharide challenge. It also increased the evenness of the fecal microbiome and altered beta-diversity post-weaning. These findings indicate that *Saccharomyces cerevisiae* fermentation products may be minimizing the negative effects observed in calves after a stress event (weaning and lipopolysaccharide challenge).

Another potential nutraceutical used to improve health and growth in dairy calves is medium chain fatty acids (C8:0 and C10:0) supplemented in milk replacer. Feeding C8:0 and C10:0 to calves influenced metabolite concentrations related to energy balance around weaning as evident by decreased plasma NEFA concentrations prior to weaning when compared to control calves. No performance or health differences were observed in calves when feeding C8:0 and C10:0. However, vaccinating calves at three weeks of age with ovalbumin combined with an aluminum hydroxide adjuvant is an effective way to evaluate adaptive immune responses in calves, as seen by the production of antibodies post-vaccination and a further increase in production after booster vaccination.

Last, directly supplementing calves is not the only way to influence calf growth and health, maternal factors can also impact calf nutrition and health through the consumption of colostrum by the calf. The metabolic status of the cow affects circulating lipids in the cow and the lipid content of colostrum. The lipidome of colostrum is distinct from the circulating lipidome of the calf, which is similar to the circulating lipidome of the cow, except for phosphatidylglycerol. It appears that colostrum is the source for the phosphatidylglycerol present in the circulation of the calf and the phosphatidylglycerol in colostrum were the only membrane lipids with 20-28 carbons and therefore served as a source of MCFA for the calf. When discussing calf health and development, a more holistic approach should be taken that evaluates the dam's nutrition and environment during the transition period and the long-term effects they have on colostrum and the health and growth of the calf.

Nutritional strategies affect the growth and health of dairy calves. This includes direct nutrition, feeding nutraceuticals directly to the calf, and indirect nutrition, maternal factors that impact colostrum composition and therefore the first nutrients the calf consumes. Nutrition is the key to reducing calf morbidity and mortality while also decreasing reliance on antibiotics, therefore, research should continue to evaluate other nutraceuticals and the benefits they may have on calf health as well as how maternal factors influence colostrum.

APPENDIX A. CHAPTER 2 SUPPLEMENTARY DATA

Supplementary Table 6.1. List of accession numbers for each fecal sample.

Accession	Sample Name	SPUID	Organism	Tax ID	BioProject
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SAMN17773588	Calf71_d0	Calf71_d0	bovine gut metagenome	506599	PRJNA699317
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SAMN17773590	Calf73_d0	Calf73_d0	bovine gut metagenome	506599	PRJNA699317
SAMN17773591	Calf74_d0	Calf74_d0	bovine gut metagenome	506599	PRJNA699317
SAMN17773592	Calf75_d0	Calf75_d0	bovine gut metagenome	506599	PRJNA699317
SAMN17773593	Calf76_d0	Calf76_d0	bovine gut metagenome	506599	PRJNA699317
SAMN17773594	Calf77_d0	Calf77_d0	bovine gut metagenome	506599	PRJNA699317
SAMN17773595	Calf78_d0	Calf78_d0	bovine gut metagenome	506599	PRJNA699317
SAMN17773596	Calf79_d0	Calf79_d0	bovine gut metagenome	506599	PRJNA699317
SAMN17773597	Calf80_d0	Calf80_d0	bovine gut metagenome	506599	PRJNA699317
SAMN17773598	Calf81_d0	Calf81_d0	bovine gut metagenome	506599	PRJNA699317
SAMN17773599	Calf82_d0	Calf82_d0	bovine gut metagenome	506599	PRJNA699317
SAMN17773600	Calf83_d0	Calf83_d0	bovine gut metagenome	506599	PRJNA699317
SAMN17773601	Calf84_d0	Calf84_d0	bovine gut metagenome	506599	PRJNA699317
SAMN17773602	Calf85_d0	Calf85_d0	bovine gut metagenome	506599	PRJNA699317
SAMN17773603	Calf86_d0	Calf86_d0	bovine gut metagenome	506599	PRJNA699317
SAMN17773604	Calf87_d0	Calf87_d0	bovine gut metagenome	506599	PRJNA699317
SAMN17773605	Calf88_d0	Calf88_d0	bovine gut metagenome	506599	PRJNA699317
SAMN17773606	Calf89_d0	Calf89_d0	bovine gut metagenome	506599	PRJNA699317
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SAMN17773614	Calf70_d112	Calf70_d112	bovine gut metagenome	506599	PRJNA699317
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SAMN17773628	Calf85_d112	Calf85_d112	bovine gut metagenome	506599	PRJNA699317
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SAMN17773633	Calf61_d28	Calf61_d28	bovine gut metagenome	506599	PRJNA699317
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SAMN17773640	Calf69_d28	Calf69_d28	bovine gut metagenome	506599	PRJNA699317
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SAMN17773642	Calf71_d28	Calf71_d28	bovine gut metagenome	506599	PRJNA699317
SAMN17773643	Calf72_d28	Calf72_d28	bovine gut metagenome	506599	PRJNA699317
SAMN17773644	Calf73_d28	Calf73_d28	bovine gut metagenome	506599	PRJNA699317
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SAMN17773651	Calf80_d28	Calf80_d28	bovine gut metagenome	506599	PRJNA699317
SAMN17773652	Calf81_d28	Calf81_d28	bovine gut metagenome	506599	PRJNA699317
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SAMN17773655	Calf85_d28	Calf85_d28	bovine gut metagenome	506599	PRJNA699317
SAMN17773656	Calf86_d28	Calf86_d28	bovine gut metagenome	506599	PRJNA699317
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SAMN17773676	Calf79_d56	Calf79_d56	bovine gut metagenome	506599	PRJNA699317
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SAMN17773678	Calf81_d56	Calf81_d56	bovine gut metagenome	506599	PRJNA699317
SAMN17773679	Calf82_d56	Calf82_d56	bovine gut metagenome	506599	PRJNA699317
SAMN17773680	Calf83_d56	Calf83_d56	bovine gut metagenome	506599	PRJNA699317
SAMN17773681	Calf84_d56	Calf84_d56	bovine gut metagenome	506599	PRJNA699317
SAMN17773682	Calf85_d56	Calf85_d56	bovine gut metagenome	506599	PRJNA699317
SAMN17773683	Calf86_d56	Calf86_d56	bovine gut metagenome	506599	PRJNA699317
SAMN17773684	Calf88_d56	Calf88_d56	bovine gut metagenome	506599	PRJNA699317
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SAMN17773687	Calf61_d84	Calf61_d84	bovine gut metagenome	506599	PRJNA699317
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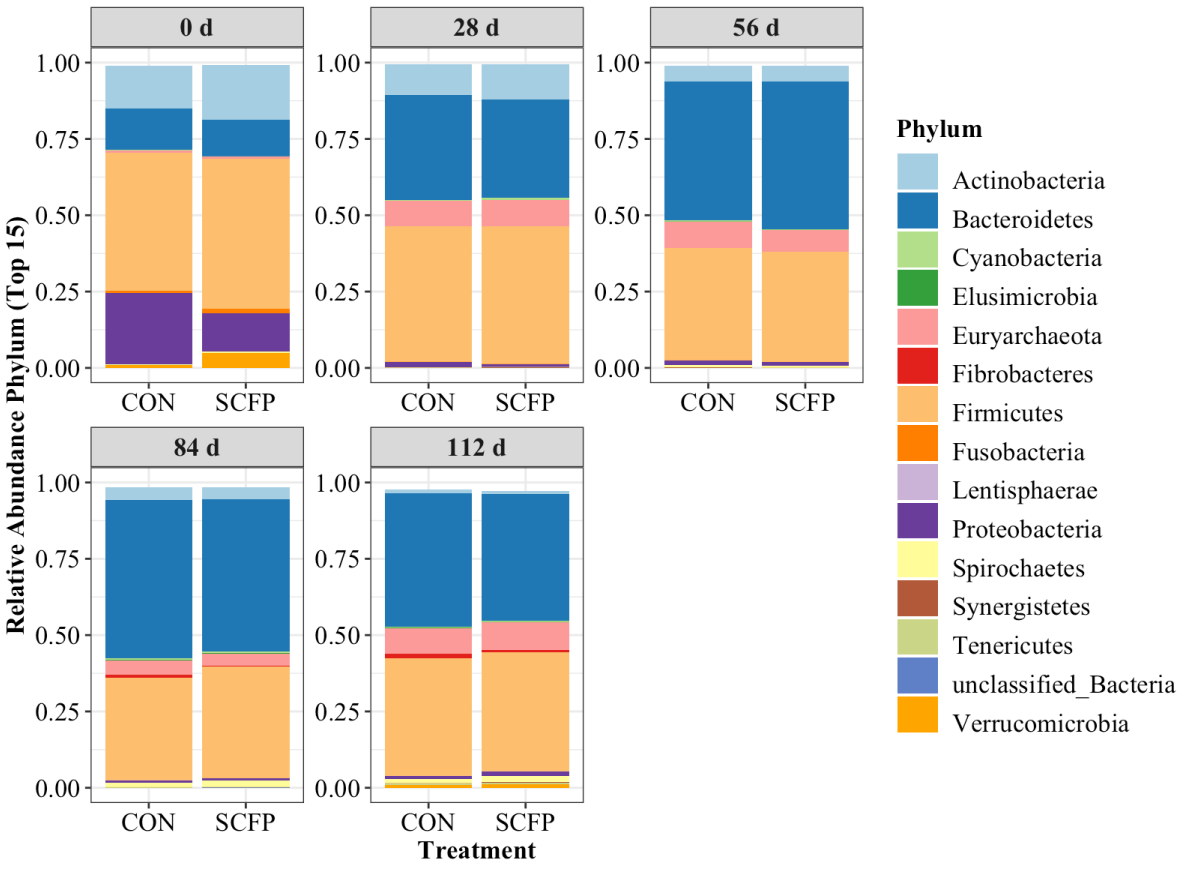
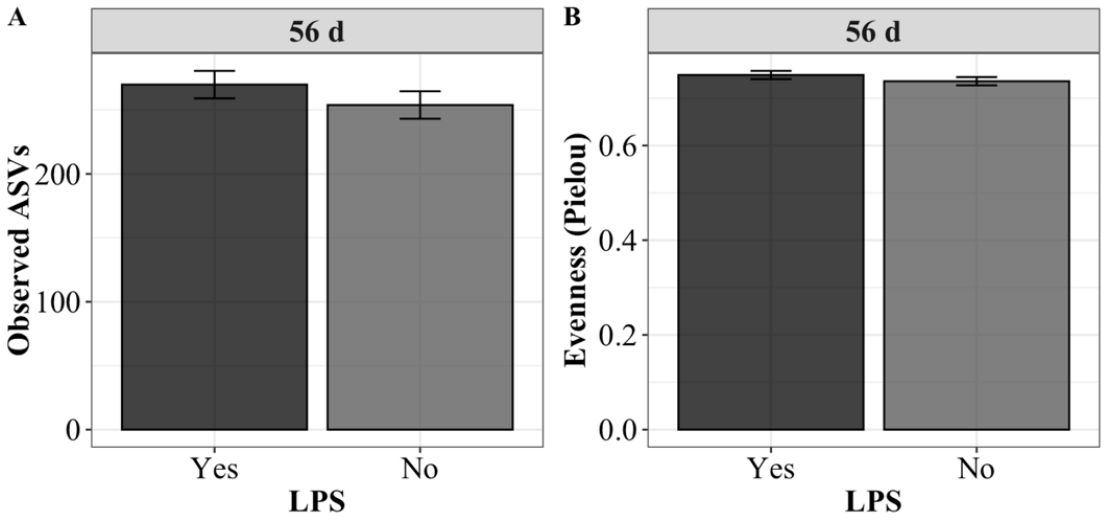
SAMN17773692	Calf68_d84	Calf68_d84	bovine gut metagenome	506599	PRJNA699317
SAMN17773693	Calf70_d84	Calf70_d84	bovine gut metagenome	506599	PRJNA699317
SAMN17773694	Calf71_d84	Calf71_d84	bovine gut metagenome	506599	PRJNA699317
SAMN17773695	Calf72_d84	Calf72_d84	bovine gut metagenome	506599	PRJNA699317
SAMN17773696	Calf73_d84	Calf73_d84	bovine gut metagenome	506599	PRJNA699317
SAMN17773697	Calf74_d84	Calf74_d84	bovine gut metagenome	506599	PRJNA699317
SAMN17773698	Calf75_d84	Calf75_d84	bovine gut metagenome	506599	PRJNA699317
SAMN17773699	Calf76_d84	Calf76_d84	bovine gut metagenome	506599	PRJNA699317
SAMN17773700	Calf77_d84	Calf77_d84	bovine gut metagenome	506599	PRJNA699317
SAMN17773701	Calf78_d84	Calf78_d84	bovine gut metagenome	506599	PRJNA699317
SAMN17773702	Calf79_d84	Calf79_d84	bovine gut metagenome	506599	PRJNA699317
SAMN17773703	Calf80_d84	Calf80_d84	bovine gut metagenome	506599	PRJNA699317

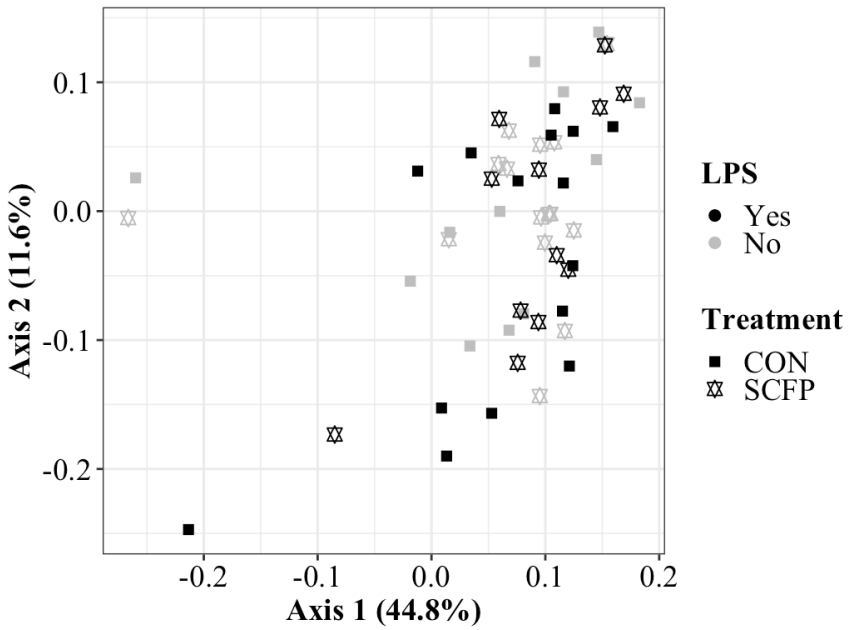
SAMN17773704	Calf81_d84	Calf81_d84	bovine gut metagenome	506599	PRJNA699317
SAMN17773705	Calf83_d84	Calf83_d84	bovine gut metagenome	506599	PRJNA699317
SAMN17773706	Calf84_d84	Calf84_d84	bovine gut metagenome	506599	PRJNA699317
SAMN17773707	Calf85_d84	Calf85_d84	bovine gut metagenome	506599	PRJNA699317
SAMN17773708	Calf86_d84	Calf86_d84	bovine gut metagenome	506599	PRJNA699317
SAMN17773709	Calf88_d84	Calf88_d84	bovine gut metagenome	506599	PRJNA699317
SAMN17773710	Calf89_d84	Calf89_d84	bovine gut metagenome	506599	PRJNA699317
SAMN17773711	Calf90_d84	Calf90_d84	bovine gut metagenome	506599	PRJNA699317
SAMN17773712	MOCK_1	MOCK_1	metagenome	256318	PRJNA699317
SAMN17773713	MOCK_2	MOCK_2	metagenome	256318	PRJNA699317
SAMN17773714	MOCK_3	MOCK_3	metagenome	256318	PRJNA699317
SAMN17773715	MOCK_4	MOCK_4	metagenome	256318	PRJNA699317
SAMN17773716	MOCK_5	MOCK_5	metagenome	256318	PRJNA699317
SAMN17773717	WATER_1	WATER_1	metagenome	256318	PRJNA699317
SAMN17773718	WATER_2	WATER_2	metagenome	256318	PRJNA699317
SAMN17773719	WATER_3	WATER_3	metagenome	256318	PRJNA699317
SAMN17773720	WATER_4	WATER_4	metagenome	256318	PRJNA699317
SAMN17773721	WATER_5	WATER_5	metagenome	256318	PRJNA699317
SAMN17773722	EmptySwab	EmptySwab	metagenome	256318	PRJNA699317



Supplementary Figure 6.1. Average relative abundance of Phylum. Stacked bar graph showing the relative abundance of the top 15 phylum found in the calf fecal microbiome in each timepoint: 0 d, 28 d, 56 d, 84 d, and 112 d based on dietary treatment (CON and SCFP).



Supplementary Figure 6.2. Calf fecal alpha-diversity microbiome measured by Observed ASV Index (A; $P = 0.30$) and Pielou Index which measures evenness (B; $P = 0.29$) at d 56 between calves that were enrolled in the LPS challenge and those that were not.



Supplementary Figure 6.3. Principal Coordinates Analysis plot illustrating the beta-diversity of the calf fecal microbiota between the animals that were challenged with LPS (Yes) and not challenge (No) (P = 0.002, R^2= 0.035) and under the effect of the dietary treatments estimated by the Weighted UniFrac distances (P = 0.98, R^2= 0.629) on 56 d.

APPENDIX B. CHAPTER 5 SUPPLEMENTARY DATA

Supplementary Table 5.1. Method 1 MRMs

Lipid name	Parent Ion	Neutral Loss
12:0 Cholesteryl ester	586.5219	369.1
14:0 Cholesteryl ester	614.5532	369.1
15:0 Campesteryl ester_simulated	642.6002	369.1
15:0 Cholesteryl ester	628.5689	369.1
15:1 Campesteryl ester_simulated	640.6002	369.1
16:0 Cholesteryl ester	642.5845	369.1
16:1 Cholesteryl ester	640.5689	369.1
16:2 Campesteryl ester	652.5689	369.1
16:2 Cholesteryl ester, zymosteryl palmitoleate	638.5532	369.1
16:3 Stigmasteryl ester	662.5532	369.1
17:0 Campesteryl ester_simulated	670.6002	369.1
17:1 Campesteryl ester_simulated	668.5845	369.1
18:0 Cholesteryl ester, 16:0 Sitosteryl ester	670.6158	369.1
18:1 Campesteryl ester	682.6158	369.1
18:1 Cholesteryl ester, 16:0 Stigmasteryl ester, 16:1 Sitosteryl ester	668.6002	369.1
18:2 Cholesteryl ester, zymosteryl oleate, 16:1 Stigmasteryl ester, 16:2 Sitosteryl ester	666.5845	369.1
18:3 Campesteryl ester	678.5845	369.1
18:3 Cholesteryl ester, 16:2 Stigmasteryl ester, 16:3 Sitosteryl ester	664.5689	369.1
20:2 Cholesteryl ester, 18:1 Stigmasteryl ester, 18:2 Sitosteryl ester	694.6158	369.1
20:3 Cholesteryl ester, 18:2 Stigmasteryl ester, 18:3 Sitosteryl ester	692.6002	369.1
20:4 Cholesteryl ester, 18:3 Stigmasteryl ester	690.5845	369.1
20:5 Cholesteryl ester	688.5689	369.1
22:5 Cholesteryl ester	716.6002	369.1
22:6 Cholesteryl ester	714.5845	369.1
lanosteryl palmitoleate, 18:2 Campesteryl ester	680.6002	369.1
PC (30:0)	706.5	184.1
PC (30:1)	704.5	184.1
PC (30:2)	702.5	184.1
PC (32:0)	734.6	184.1
PC (32:1)	732.6	184.1
PC (32:2)	730.5	184.1
PC (32:3)	728.5	184.1
PC (32:4)	726.5	184.1
PC (32:5)	724.5	184.1
PC (34:0)	762.6	184.1
PC (34:1)	760.6	184.1
PC (34:2)	758.6	184.1
PC (34:3)	756.6	184.1
PC (34:4)	754.5	184.1
PC (34:5)	752.5	184.1
PC (34:6)	750.5	184.1
PC (36:0); PCp(38:6)	790.6	184.1
PC (36:1)	788.6	184.1

PC (36:2)	786.6	184.1
PC (36:3)	784.6	184.1
PC (36:4)	782.6	184.1
PC (36:5)	780.6	184.1
PC (36:6)	778.5	184.1
PC (36:7)	776.5	184.1
PC (36:8)	774.5	184.1
PC (38:0)	818.7	184.1
PC (38:1)	816.6	184.1
PC (38:2)	814.6	184.1
PC (38:3)	812.6	184.1
PC (38:4)	810.6	184.1
PC (38:5)	808.6	184.1
PC (38:6)	806.6	184.1
PC (38:7)	804.6	184.1
PC (38:8)	802.5	184.1
PC (38:9)	800.5	184.1
PC (40:0)	846.7	184.1
PC (40:1)	844.7	184.1
PC (40:10)	826.5	184.1
PC (40:2)	842.7	184.1
PC (40:3)	840.6	184.1
PC (40:4)	838.6	184.1
PC (40:5)	836.6	184.1
PC (40:6)	834.6	184.1
PC (40:7)	832.6	184.1
PC (40:8)	830.6	184.1
PC (40:9)	828.6	184.1
PC (42:11)	852.6	184.1
PC (42:2)	870.7	184.1
PC (42:3)	868.7	184.1
PC (42:4)	866.7	184.1
PC (42:5)	864.6	184.1
PC (42:6)	862.6	184.1
PC (42:9)	856.6	184.1
PCo(32:0)	720.6	184.1
PCo(32:1)	718.6	184.1
PCo(32:2)	716.6	184.1
PCo(32:3)	714.5	184.1
PCo(34:0)	748.6	184.1
PCo(34:1)	746.6	184.1
PCo(34:2)	744.6	184.1
PCo(34:3)	742.6	184.1
PCo(34:4)	740.6	184.1
PCo(36:0)	776.6	184.1
PCo(36:1)	774.6	184.1
PCo(36:2)	772.6	184.1
PCo(36:3)	770.6	184.1
PCo(36:4)	768.6	184.1
PCo(36:5)	766.6	184.1
PCo(38:0)	804.7	184.1

PCo(38:1)	802.7	184.1
PCo(38:2)	800.6	184.1
PCo(38:3)	798.6	184.1
PCo(38:4)	796.6	184.1
PCo(38:5)	794.6	184.1
PCo(38:6)	792.6	184.1
PCo(40:0)	832.7	184.1
PCo(40:1)	830.7	184.1
PCo(40:2)	828.7	184.1
PCo(40:3)	826.6	184.1
PCo(40:4)	824.6	184.1
PCo(40:5)	822.6	184.1
PCo(40:6)	820.6	184.1
PCo(42:0)	860.7	184.1
PCo(42:4)	852.7	184.1
PCo(42:6)	848.6	184.1
PCp(32:4)	738.5	184.1
PCp(36:5)	764.6	184.1
PCp(40:6)	818.6	184.1
PCp(42:4)	850.7	184.1
PCp(42:6)	846.6	184.1
PG (20:0)	572.3294	383.3294
PG (24:0)	628.392	439.392
PG (24:1)	654.4076	465.4076
PG (26:0)	656.4233	467.4233
PG (28:0)	684.4546	495.4546
PG (28:1)	682.4389	493.4389
PG (30:0)	712.4859	523.4859
PG (30:1)	710.4702	521.4702
PG (32:0)	740.5172	551.5172
PG (32:1)	738.5015	549.5015
PG (32:2)	736.4859	547.4859
PG (34:0)	768.5485	579.5485
PG (34:1)	766.5328	577.5328
PG (34:2)	764.5172	575.5172
PG (36:1)	794.5641	605.5641
PG (36:2)	792.5485	603.5485
PG (36:3)	790.5328	601.5328
PGo (20:0)	544.3709	355.3709
PGo (30:0)	698.5066	509.5066
PGo (32:0)	726.5379	537.5379
PGo (34:0)	754.5692	565.5692
PGo (34:1)	752.5536	563.5536
PGo (36:1)	780.5849	591.5849
PGp (38:6)	796.5223	607.5223
PI (34:1)	854.5489	577.5489
PI (36:1)	882.5802	605.5802
PI (36:2)	880.5645	603.5645
PI (36:3)	878.5489	601.5489
PI (38:3)	906.5802	629.5802
PI (38:4)	904.5645	627.5645

PI (38:5)	902.5489	625.5489
SM (d16:1/18:1)	701.6	184.1
SM (d16:1/22:1)	757.6	184.1
SM (d16:1/24:0)	787.7	184.1
SM (d16:1/24:1)	785.6	184.1
SM (d18:0/14:0)	677.6	184.1
SM (d18:0/16:0)	705.6	184.1
SM (d18:0/18:0)	733.6	184.1
SM (d18:0/20:0)	761.6	184.1
SM (d18:0/22:0)	789.7	184.1
SM (d18:0/24:0)	817.7	184.1
SM (d18:0/26:0)	845.7	184.1
SM (d18:1/14:0)	675.5	184.1
SM (d18:1/16:0)	703.6	184.1
SM (d18:1/18:0)	731.6	184.1
SM (d18:1/18:1)9Z))	729.6	184.1
SM (d18:1/20:0)	759.6	184.1
SM (d18:1/24:0)	815.7	184.1
SM (d18:1/24:1)15Z))	813.7	184.1
SM (d18:1/26:0)	843.7	184.1
SM (d18:1/26:1)17Z))	841.7	184.1
SM (d18:2/14:0)	673.5	184.1
SM (d18:2/18:1)	727.6	184.1
SM (d18:2/20:1)	755.6	184.1
SM (d18:2/22:1)	783.6	184.1
SM (d18:2/24:1)	811.7	184.1

Supplemental Table 5.2. Method 2 MRMs

Lipid name	Parent Ion	Neutral Loss
	1028.4	671.396
	1172.4	759.396
	531.5	271.296
	658.6	655.326
	768.4	495.396
	768.4	523.396
	774.6	389.596
	774.6	429.596
	774.6	503.596
	776.6	429.596
	776.6	503.596
	776.6	505.596
	778.6	431.596
	778.6	505.596
	786.4	467.396
	788.6	491.596
	788.6	493.596
	790.4	493.396
	790.4	495.396
	790.4	519.396
	790.4	567.396
	792.6	491.596
	792.6	493.596
	792.6	495.596
	792.6	519.596
	792.6	521.596
	792.6	569.596
	794.6	437.596
	794.6	495.596
	794.6	497.596
	794.6	521.596
	794.6	523.596
	794.6	549.596
	794.6	571.596
	794.6	577.596
	796.6	523.596
	796.6	525.596
	796.6	551.596
	796.6	573.596
TG 16:1_48:8	808.4	537.396
TG 18:2_48:4	816.6	519.596
TG 18:3_48:4	816.6	521.596
TG 18:3_48:3	818.6	523.596
	818.6	595.596
TG 18:1_48:3	818.8	519.8
TG 18:2_48:3	818.8	521.8
TG 16:0_48:3	818.8	545.8
TG 16:1_48:3	818.8	547.8

	820.6	525.596
	820.6	597.596
TG 18:0_48:2	820.8	519.8
TG 18:1_48:2	820.8	521.8
TG 18:2_48:2	820.8	523.8
TG 16:0_48:2	820.8	547.8
TG 16:1_48:2	820.8	549.8
TG 14:0_48:1	822.4	577.396
	822.4	599.396
TG 18:0_48:1	822.8	521.8
TG 18:1_48:1	822.8	523.8
TG 18:2_48:1	822.8	525.8
TG 16:0_48:1	822.8	549.8
TG 16:1_48:1	822.8	551.8
	824.5	601.496
TG 18:0_48:0	824.8	523.8
TG 18:1_48:0	824.8	525.8
TG 16:0_48:0	824.8	551.8
TG 16:1_48:0	824.8	553.8
	832.3	537.296
TG 18:2_50:10	834.7	537.696
TG 16:1_50:10	834.7	563.696
	834.7	611.696
TG 18:1_50:9	836.2	537.196
TG 16:1_50:9	836.2	565.196
	840.8	569.796
TG 18:3_50:6	842.6	547.596
	842.6	619.596
TG 18:2_50:5	844.6	547.596
TG 18:3_50:5	844.6	549.596
TG 16:1_50:5	844.6	573.596
	844.6	621.596
TG 18:3_50:4	846.6	551.596
	846.6	623.596
TG 18:0_50:4	846.8	545.8
TG 18:1_50:4	846.8	547.8
TG 18:2_50:4	846.8	549.8
TG 16:0_50:4	846.8	573.8
TG 16:1_50:4	846.8	575.8
	848.6	503.596
	848.6	625.596
TG 18:0_50:3	848.8	547.8
TG 18:1_50:3	848.8	549.8
TG 18:2_50:3	848.8	551.8
TG 16:0_50:3	848.8	575.8
TG 16:1_50:3	848.8	577.8
	850.7	437.696
	850.7	503.696
TG 20:0_50:2	850.7	521.696
	850.7	627.696
TG 18:0_50:2	850.8	549.8

TG 18:1_50:2	850.8	551.8
TG 18:2_50:2	850.8	553.8
TG 16:0_50:2	850.8	577.8
TG 16:1_50:2	850.8	579.8
TG 18:0_50:1	852.8	551.8
TG 18:1_50:1	852.8	553.8
TG 16:0_50:0	852.8	579.8
TG 18:2_52:10	860.6	563.596
TG 18:1_52:9	862.8	563.796
TG 18:2_52:9	862.8	565.796
TG 16:1_52:9	862.8	591.796
TG 18:1_52:8	864.8	565.796
TG 16:1_52:8	864.8	593.796
TG 20:4_52:5	870.8	549.796
TG 18:2_52:5	870.8	573.8
TG 18:3_52:5	870.8	575.796
TG 16:0_52:5	870.8	597.8
TG 16:1_52:5	870.8	599.8
TG 18:3_52:4	872.7	577.696
TG 20:4_52:4	872.8	551.8
TG 18:1_52:4	872.8	573.8
TG 18:2_52:4	872.8	575.8
TG 16:0_52:4	872.8	599.8
TG 16:1_52:4	872.8	601.8
TG 18:3_52:3	874.5	579.496
TG 18:0_52:3	874.8	573.8
TG 18:1_52:3	874.8	575.8
TG 18:2_52:3	874.8	577.8
TG 16:0_52:3	874.8	601.8
TG 16:1_52:3	874.8	603.8
TG 18:0_52:2	876.8	575.8
TG 18:1_52:2	876.8	577.8
TG 18:2_52:2	876.8	579.8
TG 16:0_52:2	876.8	603.8
TG 16:1_52:2	876.8	605.8
TG 18:0_52:1	878.8	577.8
TG 18:1_52:1	878.8	579.8
TG 18:2_52:1	878.8	581.8
TG 16:0_52:1	878.8	605.8
TG 16:1_52:1	878.8	607.8
TG 18:0_52:0	880.8	579.8
TG 18:1_52:0	880.8	581.8
TG 16:0_52:0	880.8	607.8
TG 18:2_54:11	886.7	589.696
	886.7	591.696
TG 18:2_54:10	888.6	591.596
TG 18:1_54:9	890.7	591.696
TG 18:2_54:9	890.7	593.696
TG 18:0_54:8	892.7	591.696
TG 18:1_54:8	892.7	593.7
TG 18:0_54:7	894.8	593.8

TG 18:3_54:6	896.6	601.596
TG 20:4_54:6	896.8	575.8
TG 18:2_54:6	896.8	599.8
TG 18:3_54:5	898.7	603.696
TG 20:4_54:5	898.8	577.8
TG 18:0_54:5	898.8	597.8
TG 18:1_54:5	898.8	599.8
TG 18:2_54:5	898.8	601.8
TG 16:0_54:5	898.8	625.8
TG 18:3_54:4	900.6	605.596
TG 20:4_54:4	900.8	579.8
TG 18:0_54:4	900.8	599.8
TG 18:1_54:4	900.8	601.8
TG 18:2_54:4	900.8	603.8
TG 16:0_54:4	900.8	627.8
	902.7	607.696
TG 18:0_54:3	902.8	601.8
TG 18:1_54:3	902.8	603.8
TG 18:2_54:3	902.8	605.8
TG 16:0_54:3	902.8	629.8
TG 18:0_54:2	904.8	603.8
TG 18:1_54:2	904.8	605.8
TG 18:2_54:2	904.8	607.8
TG 16:0_54:2	904.8	631.8
TG 18:0_54:1	906.8	605.8
TG 18:1_54:1	906.8	607.8
TG 16:0_54:1	906.8	633.8
	908.4	609.396
TG 18:0_54:0	908.9	607.896
TG 18:2_56:1	914.7	617.696
	922.3	577.296
TG 20:4_56:6	924.8	603.8
TG 18:1_56:6	924.8	625.8
	924.8	629.796
TG 16:0_56:6	924.8	651.8
TG 20:4_56:5	926.8	605.8
TG 18:1_56:5	926.8	627.796
TG 16:0_56:5	926.8	653.8
TG 18:1_56:4	928.8	629.8
TG 18:1_56:3	930.8	631.796
TG 18:2_56:3	930.8	633.8
TG 18:1_56:2	932.9	633.9

Supplemental Table 5.3. Method 3 MRMs

Lipid name	Parent Ion	Neutral Loss
	520.5	195.496
	524.5	111.496
	530.5	177.496
	530.5	185.496
	530.5	231.496
	530.5	89.496
	532.5	177.496
	532.5	209.496
	536.5	207.496
	536.5	265.496
	538.5	267.496
	544.5	103.496
	544.5	131.496
	554.5	281.496
	570.6	347.596
	572.6	355.596
	572.7	299.696
	576.6	191.596
	584.6	313.596
	586.4	313.396
	588.6	147.596
	590.6	271.596
	598.5	299.496
	598.5	327.496
	598.5	375.496
	600.6	327.596
	600.6	355.596
	600.6	383.596
	602.6	161.596
	604.4	279.396
	610.6	313.596
	610.6	339.596
	612.6	313.596
	612.6	339.596
	612.6	341.596
	612.6	389.596
	614.5	313.496
	624.4	327.396
	624.4	401.396
	626.4	281.396
	626.4	297.396
	626.4	327.396
	626.4	353.396
	626.4	355.396
	626.4	381.396
	626.4	403.396
	626.6	409.596
	628.6	281.596

	628.6	327.596
	628.6	355.596
	628.6	383.596
	628.6	405.596
	628.6	411.596
	630.6	217.596
	630.6	357.596
	640.6	339.596
	640.6	341.596
	640.6	417.596
15:1 Campesteryl ester_simulated	640.6002	369.1
	641.7	296.696
	642.6	341.596
15:0 Campesteryl ester_simulated	642.6002	369.1
	650.7	355.696
	650.7	427.696
	652.4	355.396
	652.4	381.396
	652.4	429.396
	654.5	355.496
	654.5	381.496
	654.5	383.496
	654.5	409.496
	654.5	431.496
	656.6	355.596
	656.6	357.596
	656.6	383.596
	656.6	385.596
	656.6	411.596
	656.6	433.596
	664.5	367.496
18:3 Cholesteryl ester, 16:2 Stigmasteryl ester, 16:3 Sitosteryl ester	664.5689	369.1
18:2 Cholesteryl ester, zymosteryl oleate, 16:1 Stigmasteryl ester, 16:2 Sitosteryl ester	666.5845	369.1
	666.6	371.596
	668.6	339.596
	668.6	371.596
	668.6	395.596
	668.6	397.596
	668.6	445.596
18:1 Cholesteryl ester, 16:0 Stigmasteryl ester, 16:1 Sitosteryl ester	668.6002	369.1
	670.7	397.696
	678.5	381.496
	678.5	383.496
	678.5	407.496
	679.9	408.896
	680.5	381.496
	680.5	383.496
	680.5	407.496
	680.5	409.496
	680.5	457.496

	682.5	381.496
	682.5	383.496
	682.5	385.496
	682.5	409.496
	682.5	411.496
	682.5	437.496
	682.5	459.496
	684.6	383.596
	684.6	385.596
	684.6	411.596
	684.6	413.596
	684.6	439.596
	684.6	467.596
	686.6	413.596
	688.5	343.496
	688.5	367.496
20:5 Cholesteryl ester	688.5689	369.1
	690.5	371.496
20:4 Cholesteryl ester, 18:3 Stigmasteryl ester	690.5845	369.1
20:3 Cholesteryl ester, 18:2 Stigmasteryl ester, 18:3 Sitosteryl ester	692.6002	369.1
	694.4	397.396
	696.5	339.496
	696.5	397.496
	696.5	425.496
	700.4	355.396
	700.4	429.396
	702.5	429.496
	702.5	431.496
	702.6	355.596
	702.6	357.596
	702.6	383.596
	703.6	382.596
	704.4	355.396
	704.4	357.396
	704.4	375.396
	704.4	383.396
	704.4	407.396
	704.4	409.396
	704.4	431.396
	704.4	433.396
	706.5	351.496
	706.5	407.496
	706.5	409.496
	706.5	411.496
	708.5	407.496
	708.5	409.496
	708.5	411.496
	708.5	435.496
	708.5	437.496
	708.5	485.496
	710.5	355.496

	710.5	409.496
	710.5	411.496
	710.5	413.496
	710.5	437.496
	710.5	439.496
	710.5	487.496
	712.2	411.196
	712.2	439.196
	713.2	440.196
22:5 Cholesteryl ester	716.6002	369.1
	720.4	371.396
	722.5	425.496
	724.6	339.596
	728.7	383.696
	730.5	409.496
	730.5	411.496
	732.6	435.596
	732.6	437.596
	734.6	435.596
	734.6	437.596
	734.6	439.596
	734.6	463.596
	736.5	437.496
	736.5	439.496
	736.5	465.496
	736.5	513.496
	738.4	381.396
	738.4	383.396
	738.4	439.396
	738.4	465.396
	738.4	467.396
	738.4	515.396
	740.9	467.896
	740.9	469.896
	750.6	453.596
	752.5	339.496
	760.7	463.696
	760.7	465.696
	762.6	463.596
	762.6	465.596
	762.6	467.596
	764.5	411.496
	764.5	465.496
	764.5	467.496
	764.5	493.496
	764.5	541.496
	766.5	467.496
	766.5	493.496
	766.5	495.496
	766.5	543.496
	767.8	522.796

Supplemental Table 5.4. Log2 fold change of significantly different membrane lipid species between 35 BC (n = 16) and 7 BC plasma (n = 16).

Lipid Species	Log2 FC	*P*-Value
15:0 Campesteryl ester	-0.56	0.0001
15:0 Cholesteryl ester	-0.39	<0.0001
15:1 Campesteryl ester	-0.61	<0.0001
16:0 Cholesteryl ester	-0.57	<0.0001
16:1 Cholesteryl ester	-0.61	<0.0001
16:2 Campesteryl ester	-0.38	0.0001
16:3 Stigmasteryl ester	-0.70	<0.0001
17:1 Campesteryl ester	-0.59	<0.0001
18:0 Cholesteryl ester	-0.26	0.001
18:1 Campesteryl ester	-0.53	0.0001
18:1 Cholesteryl ester	-0.58	<0.0001
18:2 Campesteryl ester	-0.54	<0.0001
18:2 Cholesteryl ester	-0.50	0.0006
18:3 Campesteryl ester	-0.24	0.002
18:3 Cholesteryl ester	-0.58	0.0002
20:2 Cholesteryl ester	-0.20	0.002
20:3 Cholesteryl ester	-0.58	<0.0001
20:4 Cholesteryl ester	-0.73	<0.0001
20:5 Cholesteryl ester	-1.01	<0.0001
22:5 Cholesteryl ester	-0.56	0.0005
22:6 Cholesteryl ester	-0.49	<0.0001
PC (30:1)	-0.33	0.002
PC (30:2)	-0.23	0.01
PC (32:2)	-0.23	0.006
PC (32:4)	-0.23	0.01
PC (32:5)	-0.13	0.03
PC (34:5)	-0.19	0.0009
PC (34:6)	-0.28	0.0002
PC (36:5)	-0.24	0.003
PC (36:6)	-0.32	0.0006
PC (36:7)	-0.28	0.002
PC (36:8)	-0.35	0.001
PC (38:0)	-0.20	0.01
PC (38:1)	-0.40	0.0005
PC (38:2)	-0.25	0.01
PC (38:4)	-0.23	0.004
PC (38:5)	-0.32	0.001
PC (38:6)	-0.21	0.03
PC (38:7)	-0.34	0.0009
PC (38:8)	-0.48	0.0002
PC (38:9)	-0.43	0.0001
PC (40:3)	-0.22	0.02
PC (40:5)	-0.36	0.0009
PC (40:6)	-0.24	0.006
PC (40:7)	-0.31	0.001
PC (40:8)	-0.29	0.0002
PC (40:9)	-0.23	0.007

PC (40:10)	-0.32	0.0007
PC (42:3)	-0.28	<0.0001
PC (42:4)	-0.23	0.008
PC (42:5)	-0.26	0.005
PC (42:6)	-0.25	0.0009
PC (42:9)	-0.15	0.003
PC (42:11)	-0.22	0.005
PCo (32:0)	-0.18	0.02
PCo (32:1)	-0.20	0.006
PCo (34:0)	-0.22	0.006
PCo (34:1)	-0.30	0.0004
PCo (34:2)	-0.33	0.0001
PCo (36:0)	-0.26	0.007
PCo (36:1)	-0.42	0.0002
PCo (36:2)	-0.36	0.0006
PCo (36:3)	-0.23	0.004
PCo (36:4)	-0.17	0.02
PCo (36:5)	-0.25	0.007
PCo (38:0)	-0.38	0.0005
PCo (38:1)	-0.49	0.0002
PCo (38:2)	-0.44	0.0001
PCo (38:3)	-0.37	0.003
PCo (38:4)	-0.24	0.001
PCo (38:5)	-0.20	0.004
PCo (38:6)	-0.29	0.002
PCo (40:0)	-0.36	0.003
PCo (40:1)	-0.22	0.0008
PCo (40:2)	-0.32	0.002
PCo (40:3)	-0.42	<0.0001
PCo (40:4)	-0.30	0.0003
PCo (40:5)	-0.32	<0.0001
PCo (40:6)	-0.31	0.0002
PCo (42:0)	-0.28	0.0004
PCo (42:4)	-0.22	0.001
PCo (42:6)	-0.16	0.01
PCp (36:5)	-0.21	0.009
PCp (40:6)	-0.26	0.001
PCp (42:4)	-0.41	<0.0001
PCp (42:6)	-0.13	0.02
SM (d18:0/24:0)	-0.34	0.007
SM (d18:1/16:0)	-0.37	0.003
SM (d18:1/18:0)	-0.32	0.002
SM (d18:1/24:0)	-0.39	0.002
SM (d18:1/24:1)	-0.27	0.02
SM (d18:2/22:1)	-0.21	0.02
SM (d18:2/24:1)	-0.27	0.003

Supplemental Table 5.5. Membrane lipid species correlated to colostrum fat concentration in 35 BC plasma (n = 16) and colostrum (n = 16).

Sample Type	Lipid Species	R-Value	*P*-Value
35 BC Plasma	PI (36:2)	-0.64	0.01
	PI (38:3)	-0.59	0.02
	PI (38:4)	-0.57	0.02
	PCo(42:0)	-0.57	0.02
	SM (d18:1/20:0)	-0.53	0.04
	SM (d18:1/18:1)9Z)	-0.51	0.04
	SM (d16:1/22:1)	-0.51	0.05
Colostrum	PC (32:0)	0.51	0.04
	SM (d18:1/18:0)	0.56	0.02

Supplemental Table 5.6. TG species correlated to colostrum fat concentration in 35 BC plasma (n = 16), 7 BC plasma (n = 16), and colostrum (n = 16).

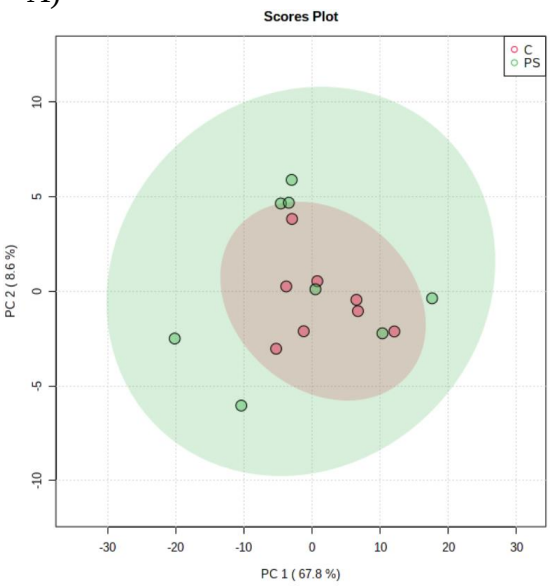
Sample Type	Lipid Species	R-Value	*P*-Value
35 BC Plasma	TG 18:2_48:2	-0.66	0.01
	TG 16:1_52:8	-0.59	0.02
	TG 18:2_50:5	-0.59	0.02
	TG 18:2_54:5	-0.58	0.02
	TG 18:2_54:9	-0.58	0.02
	TG 18:3_50:4	-0.57	0.02
	TG 18:3_54:3	-0.54	0.03
	TG 18:2_54:10	-0.53	0.03
	TG 20:4_54:4	-0.52	0.04
7 BC Plasma	TG 16:1_52:8	-0.53	0.04
Colostrum	TG 16:0_52:4	0.50	0.05
	TG 18:2_50:2	0.50	0.05
	TG 16:1_50:5	0.51	0.04
	TG 16:1_50:3	0.51	0.04
	TG 16:1_50:4	0.52	0.04
	TG 18:2_52:4	0.53	0.03

Supplemental Table 5.7. Correlations between the carbon lengths and unsaturation levels of membrane lipids and triacylglycerides (TG) in 35 BC plasma (n = 16), 7 BC plasma (n = 16), and colostrum (n = 16), with metabolic status parameters (NEFA AUC, glucose AUC, and insulin AUC), total PG in colostrum, and colostrum fat concentration.

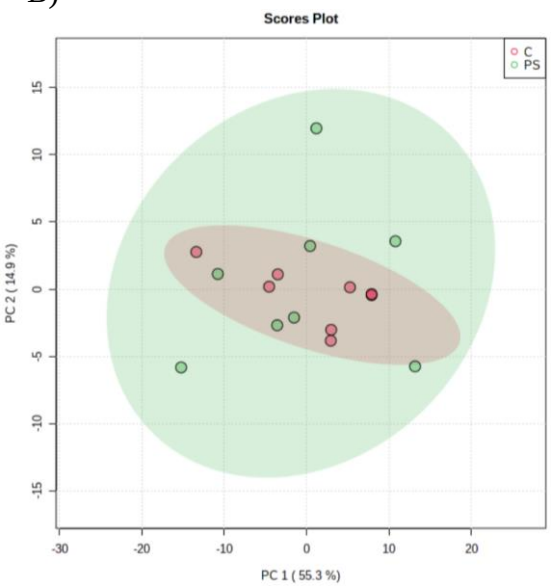
Item	*R*-Value	*P*-Value
NEFA AUC		
Total PG in colostrum	-0.57	0.03
Glucose AUC		
Colostrum membrane lipids		
30 C	-0.62	0.01
4 unsaturations	0.53	0.04
≥5 unsaturations	0.52	0.05
7 BC plasma TG lipids		
48 C	-0.59	0.02
52 C	0.56	0.03
0 unsaturations	-0.58	0.02
2 unsaturations	0.52	0.05
Colostrum TG lipids		
54 C	0.56	0.03
56 C	0.61	0.02
1 unsaturation	-0.59	0.02
5 unsaturations	0.55	0.03
6 unsaturations	0.58	0.02
≥7 unsaturations	0.69	0.05
Insulin AUC		
Colostrum TG lipids		
50 C	0.56	0.03
54 C	-0.56	0.03
56 C	-0.52	0.05
4 unsaturations	-0.52	0.04
5 unsaturations	-0.56	0.03
Total PG in Colostrum		
7 BC plasma membrane lipids		
34 C	-0.57	0.02
36 C	0.75	0.0009
Colostrum membrane lipids		
20 C	0.51	0.05
24 C	0.58	0.02
26 C	0.68	0.004
28 C	0.69	0.003
30 C	0.64	0.007
32 C	-0.56	0.02
34 C	-0.83	<0.0001
36 C	-0.75	0.0008
38 C	-0.91	<0.0001
40 C	-0.90	<0.0001
42 C	-0.82	0.0001
0 unsaturations	0.92	<0.0001
2 unsaturations	-0.69	0.003

3 unsaturations	-0.84	<0.0001
4 unsaturations	-0.94	<0.0001
≥5 unsaturations	-0.92	<0.0001
7 BC plasma TG lipids		
50 C	-0.56	0.02
3 unsaturations	-0.53	0.03
≥7 unsaturations	0.52	0.04
Colostrum TG lipids		
48 C	0.67	0.005
52 C	-0.65	0.007
54 C	-0.50	0.05
1 unsaturation	0.71	0.002
Colostrum Fat Concentration		
Colostrum TG lipids		
50 C	0.64	0.007
54 C	-0.54	0.03

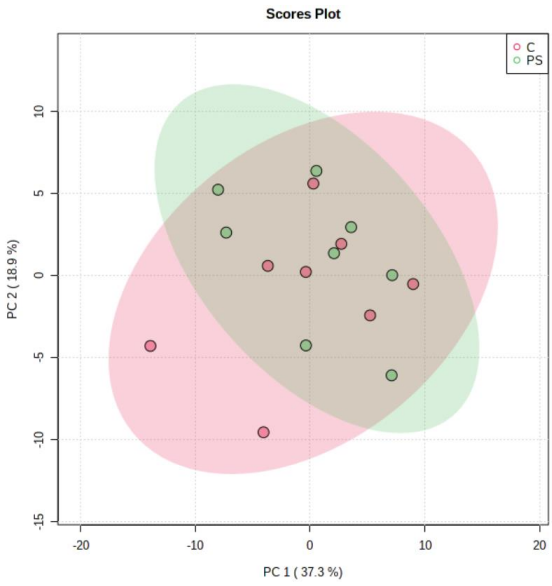
A)



B)

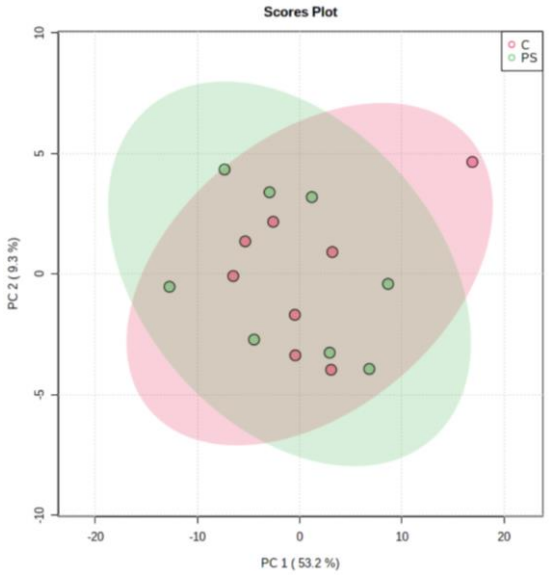


C)



Supplemental Figure 5.1. Principle component analysis for screening method 1 lipids in A: 35 BEC plasma (n = 16), B: 7 BC plasma (n = 16), and C: colostrum (n = 16) by study treatment (control vs. phase-shift).

A)



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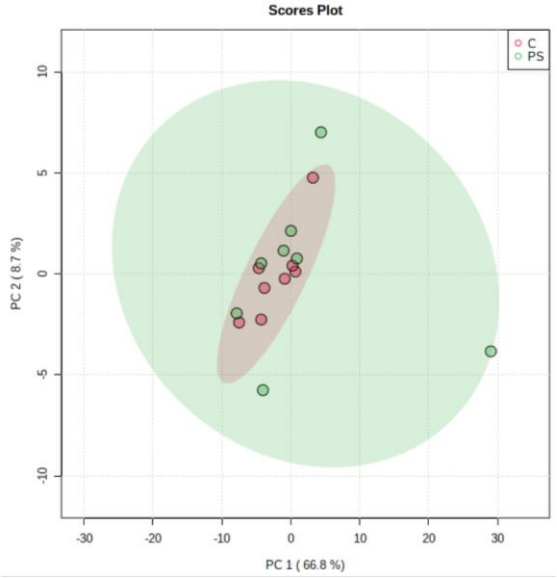
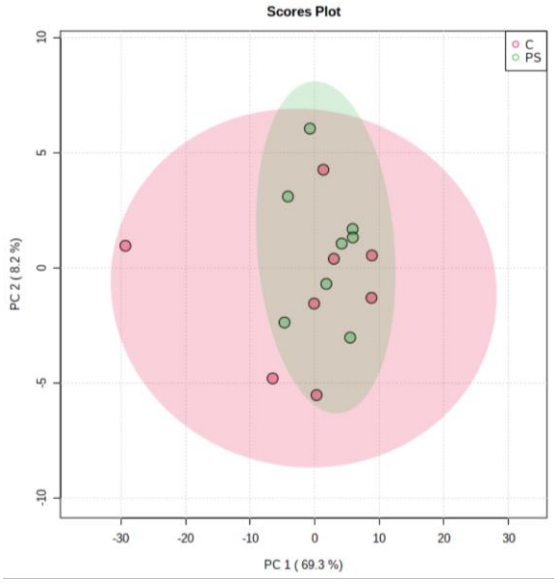
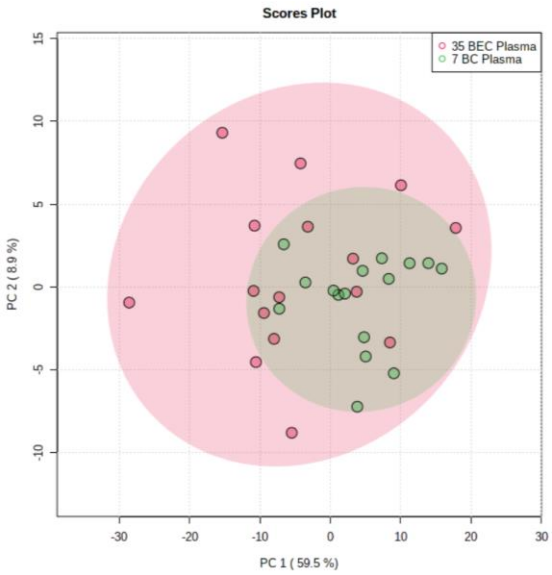


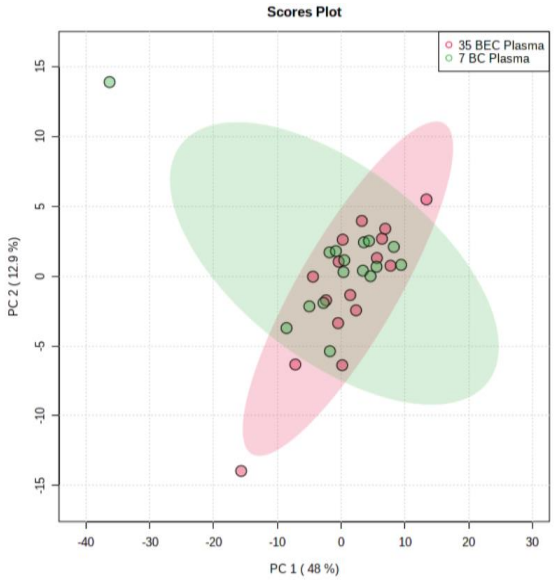
C)



Supplemental Figure 5.2. Principle component analysis for screening method 2 in A: 35 BEC plasma (n = 16), B: 7 BC plasma (n = 16), and C: colostrum (n = 16) by study treatment (control vs. phase-shift).



Supplemental Figure 5.3. Principle component analysis for screening method 1 lipids in bovine plasma based on sampling day (35 BEC (n = 16) vs. 7 BC (n = 16)).



Supplemental Figure 5.4. Principle component analysis for TG lipids in bovine plasma based on sampling day (35 BEC (n = 16) vs. 7 BC (n = 16)).