LYSOPHOSPHATIDYLCHOLINE AS AN IMMUNOMODULATOR IN NEONATAL DAIRY CALVES

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LYSOPHOSPHATIDYLCHOLINE AS A POTENTIAL IMMUNOMODULATOR IN NEONATAL DAIRY CALVES

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The neonatal bovine immune system is characterized as being immunonaïve at birth. Lysophosphatidylcholine (LPC) is an immunomodulator in non-ruminants that functions by enhancing bactericidal mechanisms of immune cells. Because the role of LPC in ruminants had not received prior attention, our objectives were to (1) determine whether LPC modulates neonatal bovine neutrophil oxidative burst, cytokine release, and *Escherichia* coli (*E. coli*) killing and (2) investigate the effects of subcutaneous (s.c.) and dietary LPC administration on immunity and growth in calves. For study 1, neutrophils were isolated from peripheral blood of pre-weaned Holstein heifer calves. The effects of LPC were evaluated on hydrogen peroxide (H_2O_2) production, tumor necrosis factor-α (TNFα) and interleukin (IL)-6 secretion, and *Escherichia coli* killing. We observed that LPC-18:0 enhanced phorbol myristate acetate-stimulated H_2O_2 production, potentiated the ability of lipopolysaccharide to stimulate TNFα and IL-6 secretion, and enhanced neutrophil-mediated *E. coli* killing. For study 2, forty-six Holstein heifer calves were randomly assigned to 1 of 4 treatments: a milk replacer diet unsupplemented with lecithin in the absence (CON; n=11) or presence of s.c. administered mixed (mLPC; 69% LPC-16:0, 25% LPC-18:0, 6% other; n = 11) or pure (pLPC; 99% LPC-18:0; n = 11) LPC, or a milk replacer diet supplemented with 3% lecithin enriched in lysophospholipids containing LPC in the absence of s.c. administered LPC (LYSO; n = 10). Starter grain and water were provided. Body measurements and clinical observations were recorded, and blood samples were collected. Rectal temperatures were higher in calves treated with mLPC or pLPC during administration, relative to CON. Calves

provided mLPC experienced lower body weight and average daily gain post-weaning, relative to CON. Calves receiving mLPC displayed increased serum aspartate aminotransferase, γ-glutamyltransferase, and glutamate dehydrogenase concentrations and a tendency for increased white blood cell counts during wk 3 of life, relative to CON. Plasma concentrations of SAA were significantly higher in mLPC and pLPC calves, relative to CON calves immediately prior to and following the final s.c. injection. We conclude that LPC enhanced bactericidal mechanisms of bovine neutrophils, and the s.c. injection of saturated LPC to healthy calves induced an acute febrile response, modified measures of liver and immune function, and impaired growth.

BIOGRAPHICAL SKETCH

Brianna Nicole Tate was born in Greensboro, North Carolina on May 14, 1994. Ever since she was a young child, Brianna nursed a passion for animals and science, having initial career aspirations to become a veterinarian. After graduating Grimsley High School with her International Baccalaureate diploma in 2012, Brianna was awarded a track and field scholarship to attend Appalachian State University in Boone, NC where she majored in Biology and minored in Chemistry. Balancing both her role as both a collegiate athlete as well as roles of leadership within her spheres of influence, Brianna was able to maintain a GPA of 3.47 throughout her undergraduate studies. During this time, she met Dr. Chishimba Nathan Mowa, who mentored her and supported her success within and outside of academia. After matriculating with her bachelor's degree in 2016, Brianna went on to pursue her master's degree at the same institution, joining Dr. Mowa's lab where she investigated the medicinal plant *Morigna oleifera* and its potential nutraceutical applications in a cell model of rheumatoid arthritis. During this time, Brianna was integral in establishing and developing mammalian cell culture techniques in the Mowa lab. In July 2018, Brianna completed her master's degree and forwent veterinary school, instead deciding to pursue a doctoral degree. In August 2018, Brianna joined the lab of Dr. Joseph McFadden in the Department of Animal Science at Cornell University. Throughout Brianna's graduate training, she explored the immunomodulatory roles of the lysophospholipid lysophosphatidylcholine within both bovine immune cell models and neonatal dairy calves. During her time at Cornell, Brianna received awards and honors such as the Maynard Award, the Cornelia Ye Outstanding Teaching Assistant Award, and was inducted as a member of the Edward A. Bouchet Honor Society. After defending her dissertation, Brianna will begin an NIH-funded postdoctoral position at University of Maryland's Medical Center under the tutelage of Dr. Marcela Pasetti.

This dissertation is dedicated to my parents (Caroline T. Tomlinson-Pemberton and William B. Tate, III), my brothers (Brian D. M. Pemberton and Brandon N. Pemberton), as well as all of my supportive extended family, both blood and chosen.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AGP	Alpha-1 acid glycoprotein
AP-1	Activating protein-1
AST	Aspartate aminotransferase
ADG	Average daily gain
BW	Body weight
CD	Cluster of differentiation
cAMP	Cyclic adenosine monophosphate
d	Day
DAG	Diacylglycerol
DMI	Dry matter intake
EC	Endothelial cells
EL	Endothelial lipase
ERK	Extracellular signal-regulated kinase
FPT	Failure of Passive Transfer
cytb558	Flavocytochrome b558
Fab	Fragment antigen binding
Fc	Fragment crystallizable
FcR	Fragment crystallizable receptors
$G_{\beta\gamma}$	G beta-gamma complex
GPCR	G protein-coupled receptors
G_{α}	G-alpha subunit
GGT	Gamma-glutamyl transferase
Gi	G-inhibitory protein
GLDH	Glutamate dehydrogenase
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
h	Hour
H_2O_2	Hydrogen peroxide

Ig	Immunoglobulin
IκB	Inhibitor of kappa B
IP3	Inositol-1,4,5-triphosphate
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low-density lipoprotein
LPC	Lysophopshatidylcholine
LPCAT	Lysophosphatidylcholine acyltransferase
MR	Milk replacer
MAPK	Mitogen-activated protein kinase
MYD88	Myeloid differentiation factor 88
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa beta
oxLDL	Oxidized low-density lipoprotein
PMA	Phorbol myristate acetate
PC	Phosphatidylcholine
PIP2	Phosphatidylinositol-4,5-biphosphate
PLA_2	Phospholipase A_2
PLC	Phospholipase C
Akt	Protein kinase B
PKC	Protein kinase C
ROS	Reactive oxygen species
s.c.	Subcutaneous
TH2	T helper type-2
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
wk	Week

Chapter 1: Introduction

The success and profitability of the dairy industry relies on the maintenance of a steady supply of milk production, which is contingent on the production and successful rearing of healthy replacement heifers. The prevention of disease-related mortality and morbidity is integral to ensuring adequate dairy calf health and welfare as adverse early-life health complications can negatively impact growth and milk production later in life. For example, in an 18-month longitudinal study of dairy heifer calves from birth to weaning, calves that experienced a disease throughout the pre-weaning period experienced a 4.7 times higher rate of mortality than calves with no disease (Urie et al., 2018). Additionally, heifers affected by diarrhea in calfhood required more inseminations to become pregnant and experienced a 325 kilogram (kg) reduction in 305-day (d) mature-equivalent milk production during their first lactation and a 50 g/d reduction in average-daily-gain (ADG) (Abuelo et al., 2021). Therefore, it is imperative that calves are immunologically protected from their pathogen-laden environment in order to prevent the contraction of parasitic, viral, and bacterial infections that may predispose them to poor performance later in life. Unfortunately, this presents a challenge, because the immune system of the neonatal dairy calf is deficient with respect to many of their protective mechanisms until months after birth (Chase et al., 2008). Indeed, the first few weeks of a dairy calf's life are termed its "window of susceptibility" due to the high incidence of mortality and morbidity that is experienced during this time (Chase et al., 2008). Of calves born alive, 7.8% die prior to weaning (NAHMS and USDA, 2007). Currently, the feeding of colostrum is the industry standard by which farmers attempt to confer immune protection in calves by means of passive transfer. Failure of passive transfer (FPT), either through the inadequate management or insufficient feeding of colostrum, can occur as often as 1 in every 5 calves in US dairy operations (Beam et al., 2009).

Failure of passive transfer renders the neonatal calf vulnerable to pathogenic infection. While antibiotics are often utilized to prevent calfhood diseases, their use is becoming increasingly discouraged by consumers (Wemette et al., 2021). Additionally, antibiotic mismanagement can potentially facilitate the emergence of resistant strains of bacteria to develop within the calf gastrointestinal tract (Berge et al., 2005). Other extra-label additives (e.g., Bovatec®) have been developed to improve neonatal calf immune function by nutritional methods; however, their efficacy is questionable. Therefore, the generation and evaluation of efficacious non-antibiotic therapies that can enhance immune function and mitigate disease in calves are imperative. This thesis is written to provide an overview of the development and deficiencies in the immune system of the calf as a means to identify potential targets for therapeutic immune interventions that can improve overall immune protection. I will also explore a novel potential immunomodulator for the calf called lysophosphatidylcholine (LPC).

Chapter 2: Literature Review

2.1. Development and Deficiencies of the Dairy Calf Immune System

The cotyledonary synepitheliochorial placenta of the bovine prevents the transport of immunoglobulins (Ig) and foreign antigens *in utero*, resulting in the neonate being born with an antigenically naïve immune system (Barrington and Parish, 2001; Lombard et al., 2020). As a result, the calf is hindered in its ability to mount a successful immune response due to the immaturity of its innnate immunoprotective mechanisms and inability of its adaptive immune cells to recognize foreign antigens. The entirety of this process results in a prolonged lag period of the immune response and low Ig production (Chase et al., 2008; Heinrichs, 2017). Additionally, endogenous corticosteroids and maternal hormone factors (e.g., estrogen) produced during parturition can further impact the functionality of both the calf's innate and adaptive immune systems (Chase et al., 2008). The interplay of these physiological and environmental stimuli modulates the development of the dairy calf immune system and ultimately influence both the onset and trajectories of both the adaptive and innate immune cells and their responses.

2.1.a. The Innate Immune System

The innate immune system serves as the body's first line of defense against pathogenic infection and disease. While physiological barriers such as skin and mucosal membranes are integral components of innate immunity, innate immune cells like neutrophils and monocytes are critical for the efficient clearance of pathogens that are able to breach these physical barriers (Vlasova and Saif, 2021). Therefore, it is important that the protective mechanisms of innate immune cells are responsive and functional to ensure that any invading pathogen is neutralized before they are able to illicit a pathophysiological response. Unfortunately, the cellular mechanistic

components of a dairy calf's innate immune system have been observed to be deficient at birth (Chase et al., 2008). For example, while the number of circulating neutrophils in newborn calves is approximately four times higher than in 3-week-old calves, neonates have an observably reduced phagocytic ability (Menge et al., 1998; Chase et al., 2008). The oxidative burst response and production of cytokines has also been found to be modulated by age and environmentally related factors (Chase et al., 2008).

2.1.a.1. Phagocytosis

In eukaryotes, phagocytosis is the process by which phagocytic immune cells, such as neutrophils and monocytes recognize and engulf pathogens in order to eliminate them. This process is initiated upon recognition of pathogen-associated molecular patterns, which are repetitive molecular structures expressed on the surface of pathogens, via pattern recognition receptors on the surface of phagocytes. Neutrophil pattern recognition receptors (PRRs) include nucleotide oligomerization domain-like receptors and Toll-like receptors (TLRs) (Bassel and Caswell, 2018; Rosales, 2020). Immunologic recognition can also be accomplished by opsonic receptors (i.e., Fc-receptors [FcR]) on the plasma membrane of neutrophils that recognize opsonins, or humoral proteins such as antibodies that are produced by activated B cells following antigen exposure (Heinrichs, 2017). Antibodies contain 2 key regions: a variable or antigen-specific region (fragment antigen binding [Fab]) and a constant or signaling region (fragment crystallizable [Fc]). The Fab region is specific to the antigen to which its parent B cell was activated while the Fc region translates an identical signal to phagocytic cells in order to initiate phagocytosis. If the Fc region is not quickly recognized by a phagocytic cell, circulating complement proteins may also recognize the bound IgG molecule and initiate a cascade of proteins leading to eventual lysis of the foreign cell (Heinrichs, 2017). These opsonic receptors recognize

opsonins bound to Fab regions on bacteria (Paape et al., 2003; Vlasova and Saif, 2021). Binding of opsonins allow the repellant forces between the negatively-charged cell walls of the pathogen and phagocyte to be overridden (Chew, 1996). Activation of these receptors initiates signaling cascades that modify the cell membrane of the neutrophil and results in the rearrangement of the actin cytoskeleton in order to allow the cell membrane to extend around the pathogen resulting in its subsequent engulfment and neutralization (Kobayashi et al., 2018). These actions are conserved within other innate immune cells such as macrophages and dendritic cells as well (Jönsson et al., 2012).

Complement is a protein that enhances the ability of antibodies and phagocytic cells to clear microbes and damaged cells from an organism. Circulating levels of complement in the dairy calf at birth are only 12 to 60% of those found in their adult counterparts and does not reach adult levels in calves until they are 6 months of age (Chase et al., 2008). Additionally, the expression of FcR on neutrophils is lower in neonatal and young calves than in cows (Zwahlen et al., 1992). This said, expression of the β2-integrin complement receptor 3 (iC3), which binds to the opsonin iC3b, was not found to be different between calves and cows (Zwahlen et al., 1992; Yamamori et al., 2000). Phagocytic capability can also be negatively impacted by glucocorticoids such as endogenous cortisol and estrogen produced by the dam which can suppress immune responses well after birth (Jacob et al., 2001; Chase et al., 2008). Indeed, calf neutrophils have been observed to have decreased phagocytic capabilities in response to these stimuli (Menge et al., 1998; Roach et al., 2020). As a result, the calf is rendered susceptible to infection as a result of its suppressed immune protection.

2.1.a.1. Oxidative burst

Bovine neutrophils employ another essential bactericidal mechanism critical in immune defense against bacterial infection: the generation of reactive oxygen species (ROS), and the release of these antimicrobial peptides into phagosomes (Yamamori et al., 2000). When a pathogen is recognized and bound by a neutrophil surface receptor, the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system becomes activated. In bovine neutrophils, as in other animal models, NADPH oxidase has been defined as a multicomponent enzyme consisting of a heterotrimeric dimer made up of two transmembrane proteins, gp91phox and gp22phox (also known as flavocytochrome b558 [cytb558]), and a regulatory complex consisting of three subunits that reside in the cytosol, p47phox, p67phox and Rac (Yamamori et al., 2000; Nguyen et al., 2017). Neutrophilic receptors that facilitate NADPH oxidase activation include the IgG Fc receptor (FcγR) and β2-integrin receptor CR3 (Yamamori et al., 2000). Upon activation of the neutrophil via receptor binding, the regulatory cytosolic subunits translocate to the plasma membrane and interact with cytb558, a step required for NADPH activation (Nguyen et al., 2017). A small GTPase protein, Rac2, also regulates the recruitment of the regulatory complex to the plasma membrane and the overall activation of NADPH oxidase. Once Rac2 becomes activated via the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP), it translocates to the membrane independently of the regulatory complex where it is essential for ROS production (Heyworth et al., 1994). Cross-linking of FcγR induces the activation of src family tyrosine kinases followed by the activation of Syk tyrosine kinase via coupling to immunoreceptor tyrosine-based activation motif (ITAM)-bearing molecules, thus facilitating tyrosine phosphorylation of FcγR by src family tyrosine kinases (Yamamori et al., 2000; Van Ziffle and Lowell, 2009). Neutrophils with defective Syk tyrosine kinase activity display significant decreases in phagocytosis and

superoxide production (Van Ziffle and Lowell, 2009). Additionally, FcγR cross-linking stimulates the tyrosine phosphorylation of phospholipase Cγ, resulting in an increase in intracellular [Ca^{2+}] and the production of diacylglycerol (DAG), an activator of protein kinase C (PKC) (Yamamori et al., 2000). Stimulation of FcγR and CR3 can also activate p38 mitogen-activated protein kinase (MAPK), a Ser/Thr kinase belonging to the family of MAPKs, in neutrophils via PKC and tyrosine kinases (McLeish et al., 1998). Both PKC and p38, as well as other kinases such as extracellular signal-regulated kinase (ERK) and Akt, play differing roles in phosphorylating p47phox, depending on which surface receptor is activated (Nguyen et al., 2017). Phosphorylation of p47phox facilitates its binding to p22phox and the subsequent activation of NADPH oxidase in a cell-free system (Fontayne et al., 2002). The interaction of the cytosolic and transmembrane components of the NADPH oxidase complex allows electrons to flow from cytoplasmic NADPH to cytb558-bound flavin adenine dinucleotide, to cytb558-bound heme groups, and finally to the heme-bound oxygen to form superoxide anion (Koshkin et al., 1997; Bokoch et al., 2009). The initiation of the NADPH oxidase cascade results in the sequential conversion of molecular oxygen to superoxide anion, hydrogen peroxide (H_2O_2), and finally, via myeloperoxidase (MPO) activity, to highly potent bactericidal component, hypochloride ions (OCl^-) (Chew, 1996). A second bactericidal pathway involving the reaction of superoxide with hydrogen peroxide generates hydroxy radicals (·OH) and singlet oxygen (·O,) which react with bacterial lipids to form bactericidal hydroperoxides (Chew, 1996). When all components of this complex, interrelated system work properly in tandem, the entire process culminates in the elimination of the invading pathogen.

Deficiencies in the oxidative burst response of neutrophils from young calves has been reported. Neonatal bovine neutrophils exhibited decreased reduction in cytochrome c (an enzyme

that transfers electrons from NADPH to several oxygenases) in response to phorbol myristate acetate (PMA), a calcium ionophore that serves as an agonist of NADPH oxidase and bypasses much of the signal transduction pathway and directly activates PKC (Lee and Roth, 1992). However, the PMA-stimulated intracellular [Ca^{2+}] rise of neutrophils in young calves has been observed to be similar to that in cows (Higuchi et al., 1997), indicating that the activity of PKC is similar among neonatal calves, young calves, and cows, which suggests that age-dependent changes in neutrophil bactericidal mechanisms may not be due to differences in PKC activity. Neutrophils from young calves also had significantly less myeloperoxidase activity than those from mature cattle (Lee and Roth, 1992). It has also been observed that neutrophils in dairy calves are significantly smaller diameter, volume, and surface area than in adult cows. It has been hypothesized that these size differences could play a role in the superoxide-generating deficiencies observed in neonatal bovine neutrophils (Dorè et al., 1990).

2.1.a.2. Cytokine production

The predominate acute phase cytokines during an innate immune response are interleukin (IL)-1 , IL-6 and tumor necrosis factor (TNF)-α. Interleukin-1 is necessary for release of IL-8, which is essential for neutrophil recruitment, by bovine pulmonary and gastrointestinal epithelial cells (Eicher et al., 2004). The activation of TLR4 initiates Toll/IL1 Receptor domain intracellular signaling through adaptor molecules, predominantly myeloid differentiation factor 88 (MYD88). This TLR4 and MYD88 signaling results in the activation of downstream kinases, ultimately resulting in the degradation of inhibitor of κB (IκB), which frees nuclear factor (NF)κB to translocate to the nucleus, where it binds κB sites in the promoter region of genes encoding proinflammatory cytokines, including IL-1β and IL-6 (Eicher et al., 2004; Cronin et al., 2012). While the functionality of dairy calf TLR signaling has not been evaluated in comparison to adult

cows, it has been demonstrated that TLR2 and TLR4 and acute phase cytokine expression can be altered by stress hormones, such as cortisol, and growth hormones, which, in turn, may decrease resistance to disease (Eicher et al., 2004). Since these hormonal cues are prevalent during parturition and early life phases (Hulbert and Moisá, 2016; Osorio, 2020), it is likely that these mechanisms decrease acute phase cytokine production in response to pathogenic stimuli and may play an indirect role in disease susceptibility. Oxidative stress has also been observed to negatively impact the expression of both protein and gene expression of cytokines (Cuervo et al., 2021). Conversely, blood immune transcriptome analyses has demonstrated that a surge in proinflammatory cytokine expression in dairy calves takes place circa parturition, though this is likely through non-TLR mediated mechanisms (Surlis et al., 2018). In all, the evidence points to a predominately suppressive effect of environmental factors on dairy calf cytokine production and signaling, though how these compare to the observed responses in adult cattle have yet to be explored. Further information regarding the inherent capabilities and deficiencies of bovine innate cells to elicit TLR signaling needs to be conducted.

2.1.b. The Adaptive Immune System

2.1.b.1. Cell-mediated immunity

The adaptive immune system of the neonatal dairy calf is suppressed by high concentrations of hormones (i.e., cortisol and prostaglandin E2) and cytokines (i.e., IL-4 and IL-10) produced during pregnancy and parturition, much like the innate immune system (Chase et al., 2008). This negatively impacts T cell function and biases the adaptive response towards a cluster of differentiation (CD)4+ T helper type-2 (TH2) response, which is anti-inflammatory and reduces the ability of the calf to invoke an inflammatory and pathogen-eradicating immune response.

Unfortunately, neonatal bovine B cells, key cells in the TH2 response, are present in very low numbers at birth and are not completely functional until 4 to 5 weeks of age (Chase et al., 2008). In addition to deficient B cell populations, CD4+ T cell numbers are also comparatively low in newborn calves making up only 16% of the peripheral T cell population (these cells account for 30% of peripheral T cells in adult cattle). Newborn calves have been documented as having unusually high numbers of circulating γδT cells (up to 60% of the lymphocyte pool) in place of these adaptive cells (Vlasova and Saif, 2021). This has been suggested to be a compensatory response that balances out the immaturity of neutrophils and macrophage functions in neonatal calves (Vlasova and Saif, 2021). It has also been hypothesized that, due to the limitations of the adaptive immune response, neonatal calves have evolved to rely more heavily on the innate functions of their immune system; a possible explanation for the very high proportions of peripheral γδ+ T cells, which are able to recognize antigen using receptors similar to those of innate immune cells. This allows γδ+ T cells to generate T cell type responses to foreign cells without the added step of antigen presentation. These γδ+ T cells are also able to perform a wider array of functions than either CD4+ or CD8+ T cells, including recruitment of innate cells, induction of cell death, and production of proinflammatory and anti-inflammatory cytokines (Heinrichs, 2017). High numbers of γδ+ T cells in circulation may grant the neonatal calf much-needed additional immunological plasticity and may compensate for the slower responses observed by other members of the adaptive system.

2.1.b.2. Humoral immunity

Due to the neonatal dairy calf's agammaglobulinemic state at birth, it is reliant on the intake of colostrum for Ig. The number of circulating B cells is greatly reduced in the neonate, representing only 4% of the total lymphocytes at 1 week of age compared with approximately 20%

to 30% in adults. The fraction of B cells in circulation increases gradually to 20% of total lymphocytes by 6 to 8 weeks of age (Chase et al., 2008). Reduced B cell populations coupled with the calf's production of corticosteroids and absorbed maternal hormones around the time of parturition, results in a prolonged lack of endogenous antibody response, despite] an apparent TH2 cytokine bias in neonates (Chase et al., 2008; Vlasova and Saif, 2021). In colostrum-deprived calves, production of IgM, which appears when an organism is exposed to an antigen for the first time, does not begin to appear in circulation until 4 days after birth(Chase et al., 2008). Moreover, levels of circulating IgA, which prevents mucosal infections, and IgG, the most predominant Ig in bovine milk and colostrum, do not reach appreciable levels until 16 to 32 days after birth (Chase et al., 2008; Hurley and Theil, 2011). As a result, the dairy calf is unable to mount an effective adaptive immune response nor confer antigenic "memory", contributing to increased and potentially recurrent, disease incidence.

2.2. Diet and Dairy Calf Immunity

2.2.a. Colostrum

During the process of colostrogenesis, which takes place approximately 3 to 4 weeks prior to parturition, the bovine mammary gland selectively transfers IgG from maternal blood circulation into the forming colostrum (Heinrichs, 2017). There are two subclasses of bovine IgG (IgG1 and IgG2) present in colostrum. The predominate Ig, however, is IgG1 which makes up 85 to 90% of the total IgG (Ulfman et al., 2018). Factors that can affect colostrum IgG concentration include time of colostrum collection relative to parturition, parity of the dam, dry period length, and energy intake during pregnancy (Fischer-Tlustos et al., 2021). The transfer of Ig from the cow to the calf via colostrum involves two key molecular transport processes: 1) the secretion of maternal Ig in

the colostrum, and 2) the transfer of colostral Ig from the gut intestines to the calf's tissues. Immunoglobulin is concentrated in the colostrum by an active receptor-mediated transfer of IgGl from the blood of the dam across the mammary gland secretory epithelium. This active transport is what allows for IgG1 to predominate the Ig colostral profile whereas IgM, IgA, and IgG2 are present in considerably lower concentrations. During transfer of IgGl to colostrum, IgGl diffuses across the vascular endothelium, is bound by specific IgG1- Fc receptors on the basal membrane of the mammary secretory epithelium, taken up by micropinocytotic vesicles that traverse the epithelial cell, and is secreted into the colostrum (Butler et al., 2015). This process results in colostral IgG1 concentrations 5 to 10 times higher than maternal serum concentrations, and the transfer of up to several hundred grams of IgG1 to the colostrum, a large enough transfer that it is associated with measurable decreases in the maternal serum IgG1 concentrations during this period. The transfer of colostral Ig from the colostrum to the body fluids of the calf is the result of a potentially neonatal Fc receptormediated macromolecular transport mechanism across the small intestinal absorptive epithelium (Barrington and Parish, 2001; Heinrichs, 2017; Fischer-Tlustos et al., 2021). The absorbed Ig molecules enter the bloodstream with via the thoracic duct. Finally, the protection from the passive immunity transferred to calves peaks 1-2 days and then starts declining (Vlasova & Saif, 2021) if sufficient quality colostrum is fed within 4 hours after birth (Fischer-Tlustos et al., 2021). The decline in maternally-derived passive immunity over time coupled with the lag in the development of immune responses in the dairy calf, results in what is known as the "window of susceptibility" during the first few weeks of life during which calves are disproportionally at risk for contracting diseases (Chase et al., 2008)

The benefits of colostrum on dairy calf immune function and maturation are well-documented. Colostrum deprived calves have been repeatedly shown to have high incidence of

mortality and morbidity (Raboisson et al., 2016). Key effector mechanisms of the innate and immune cells have also been documented to improve in functionality following absorption of colostrum. For example, ingestion of colostrum was found to increase the percentage of phagocytizing neutrophils and monocytes in dairy calves, an effect that was absent in colostrum-deprived animals. This pattern was the same for oxidative burst activity in monocytes (Menge et al., 1998). In regard to adaptive immunity, colostrum has been shown to significantly improve the serum complement content of calves (Yang et al., 2015). This allows for more efficacious recognition and clearance of invading pathogens by the neonatal bovine neutrophils.

The feeding of sufficient amounts of quality colostrum greatly bolsters the immunoprotective mechanisms of the dairy calf, however, FPT is an all too prevalent example of what can happen when colostrum management and protocols are not sound. Nearly 20 to 40% of newborn calves may experience this phenomenon and resulting mortality rates linked to FPT can range from 8 to 25% (Raboisson et al., 2016). Despite the advancements made in studying colostrum and optimizing its bioactive properties, dairy calf morbidity and mortality are still rampant in the dairy industry (NAHMS and USDA, 2007). Therefore, the identification, exploration, and refinement of other means of conferring immune protection in calves is needed.

2.2.b. Milk and milk replacer

Over decades, a variety of dairy calf feeding strategies have been developed and employed in order to adequately meet the nutritional needs of the calf and its developing gastric system. Milk replacer (MR), whole milk, and waste milk are the most prevalent means by which calves are fed during the pre-weaning period and each has its benefits and detriments relative to the plane of nutrition they provide and their cost efficiency. In 2006, about 70% of dairy operations in the United States reported feeding their calves milk replacer at any point prior to weaning. Compared

to this, about 33% and 30% of operations reported this statistic for waste milk and whole milk, respectively (USDA, 2007). During the weaning period, which typically lasts about a week or so, liquid feed intake is reduced in order to stimulate solid feed consumption and, thus, facilitate development of the rumen. This accelerated weaning strategy can provide a source of stress for the animal and can potentially impact immunity and health. Therefore, it is essential that the calf's dietary needs are met and maintained throughout this time in order to optimize the growth and performance of the animal during this time.

A majority of dairy calves rely on commercial MR in order to meet their nutritional demands through the pre-weaning period. Therefore, it is imperative that MRs are formulated to closely recapitulate maternal bovine milk not only so that the calf is not deficient in the essential nutrients needed for proficient growth and development but also to fuel the neonate's developing immune system. The nutritional content as well as the feeding regimen and plane of nutrition of MR fed to calves throughout the pre-weaning period has been found to have significant impacts on the development and responses of dairy calf immune cells. For example, one study found that calves fed higher planes of MR nutrition (higher crude protein content) had greater neutrophil oxidative burst intensities at d 77 when cocultured with *E. coli* (Ballou, 2012). Relative volumes of MR fed to calves has also been shown to affect calf immunity. Calves fed higher amounts of MR early in the pre-weaning period, then less in the latter half of the pre-weaning period had higher plasma IgG and lower TNFα levels, relative to calves whose milk replacer was increased over the course of the pre-weaning period (Wu et al., 2021). Interestingly, the number of feedings per day could also affect calf immunity, as evidenced by a study where calves fed once a day tended to have more circulating neutrophils at 27 d of age, greater expression of L-selectin on neutrophils at 31 and 45 d of age, and greater intensity of phagocytosis at 45 d of age relative to

calves fed twice daily (Hulbert et al., 2011a). Additionally, once-fed calves secreted less TNF-α during *ex vivo* lipopolysaccharide (LPS) challenges at 45 d of age compared with twice-fed calves. Clearly, modifications in the volumes, feeding rates, and plane of nutrition play a defined and important role in the immune health and performance of diet calves in the pre-weaning period.

The process of weaning calves by restricting the intake of liquid MR in order to facilitate the early transition to a solid feed diet presents a source of physiological stress that affect the calf's immunity. For example, weaning was found to suppress innate immune responses such as the expression of selectin on neutrophils and oxidative burst activity (Hulbert et al., 2011b). In this study, calves that ate more starter after early weaning had reduced circulating neutrophils and calves that did not increased feed intake during early weaning had reduced monocyte function (Hulbert et al., 2011b; Hulbert and Moisá, 2016). These results indicate that the consumption of solid feed during weaning allows the animal to cope with the rise in endogenous markers of stress such as increases in circulating glucocorticoids which can potentially suppress immune function (Hulbert and Moisá, 2016). This rise in glucocorticoids is likely due to the onset of physiological stress that is initiated by the immune process. For example, it was found that weaning disrupts redox balance, thus triggering oxidative stress and apoptosis in piglets (Song et al., 2016; Li et al., 2020). Studies have shown that antioxidant enzyme activity is the main indicator of the liver function in weaned piglets (Li et al., 2015; Meng et al., 2018), therefore, weaning-induced oxidative stress can potentially have deleterious effects on liver and overall health. It should be noted, however, that in instances where immune cell responses such as oxidative burst are augmented, oxidative stress can become exacerbated and cause tissue damage. Therefore, it is possible that the observed suppression of innate immune responses during the weaning period could be a protective mechanism utilized to prevent the overstimulation and activation of immune

cell, thus mitigating potential damage and stress that this may cause illicit in the host. A fine balance between immune cell functions and host health, that is, facilitating a response that is not too robust to cause harm to the host but also not too deficient as to render the host susceptible to infection, must be identified and maintained, especially in the development of immunomodulatory therapies to improve animal health.

2.3. Lysophosphatidylcholine Metabolism

Lysophosphatidylcholine (LPC), a component of lysolecithin, is a class of endogenous bioactive lysophospholipids. The LPC molecule consists of one long hydrophobic fatty acyl chain and one hydrophilic polar choline head group, attached to a glycerol backbone. This unique structure grants LPC its amphipathic nature, allowing it to form micelles and giving it surfactant and detergent-like properties in the presence of cell membranes (Schmitz and Ruebsaamen, 2010). Lysophosphatidylcholine is primarily synthesized during the iterative diacylation and reacylation of phosphatidylcholine, otherwise known as the Land's Cycle (**Figure 1**).

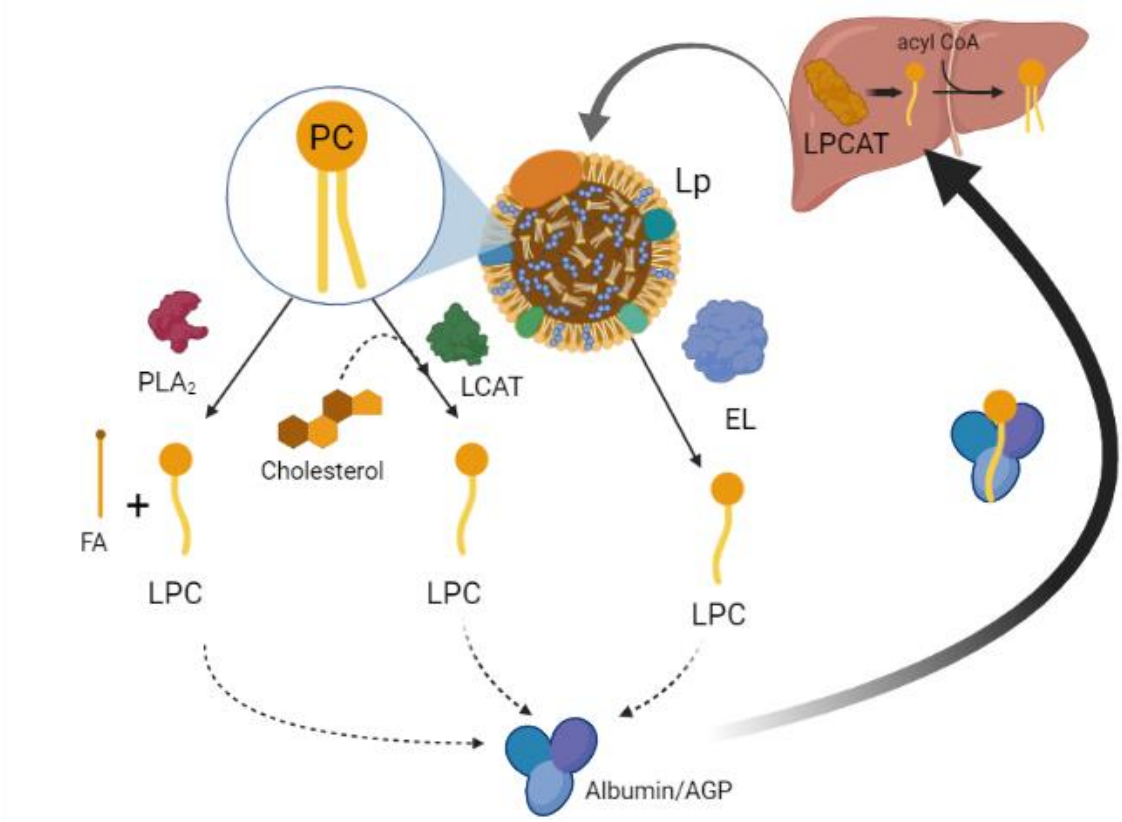

Figure 2.1. Metabolism of lysophosphatidylcholine. Phosphatidylcholine (PC) is synthesized in the liver and secreted as a component of lipoprotein (Lp). PC can then be degraded via hydrolysis by phospholipase A_2 (PLA_2) or converted by lecithin-cholesterol acyltransferase (LCAT) into lysophosphatidylcholine (LPC). The enzyme endothelial lipase (EL) can also catalyze the production of LPC by acting on high-density lipoprotein (HDL)-associated PC. The resulting LPCs can then be transported back to the liver by binding to either albumin or alpha-1 acid glycoprotein (AGP) and then are cleared by LPC acyltransferase (LPCAT) in the presence of acyl-CoA. This cycle of PC degradation and regeneration is termed the Lands cycle.

During this process, the *sn*-2 acyl bond of phosphatidylcholine is catalytically hydrolyzed by phospholipase A_2 (PLA_2), releasing a fatty acid and LPC. Alternatively, LPC can be produced via the action of lecithin-cholesterol acyltransferase (LCAT) which catalyzes the acyl esterification of cholesterol by transferring an acyl chain from phosphatidylcholine (Law et al., 2019). There is a third reaction by which LPC can be generated, which entails the cleavage action of endothelial lipase, a serum lipase anchored on the surface of vascular endothelial cells, on HDL-associated

phosphatidylcholine. In non-ruminants, this pathway yields predominately unsaturated forms of LPC including LPC-18:1, -18:2, and -20:4 in addition to saturated LPC-16:0 (Liu et al., 2020). In humans, physiological LPC concentrations can range from 100 to 170 µmol/mL, but have been observed to be greater in instances of disease such as cardiovascular diseases and diabetes (Law et al., 2019). The LPC profile has not been adequately evaluated in cattle but is important to consider because the profile is likely influenced by diet and physiological state, and type of LPC may influence immune outcomes (Rico et al., 2021).

While levels of LPC are comparatively high in mammals, relative to other lipid signaling molecules such as lysophosphatidic acid and sphingosine-1-phosphate (Tan et al., 2020), plasma LPC is rapidly cleared from circulation by transport proteins such as albumin and alpha-1 acid glycoprotein (AGP) for the synthesis of phosphatidylcholine in liver (Law et al., 2019). Albumin and AGP bind LPC in a 1:1 molar ratio and a 3:1 ratio, respectively (Ojala et al., 2006). While a majority of LPC is bound to albumin (approximately 80%), AGP binds LPC with a higher binding affinity, allowing AGP to complement albumin as an LPC-scavenging protein, especially during inflammatory conditions in which albumin-sequestering capacity is weakened (Ojala et al., 2006). Binding of these proteins has been observed to attenuate observed biological effects of LPC, suggesting that the bioactive properties of LPC are muted when bound to albumin (Vuong et al., 2001; Ojala et al., 2006; Kim et al., 2007). Lipoproteins, including low-density lipoprotein (LDL) and especially oxidized LDL (oxLDL), also contain LPC, though their relative levels are not adequately defined (Law et al., 2019; Liu et al., 2020). During instances of reduced albumin availability, incorporation of LPC into these lipoproteins increases significantly (Vuong et al., 1999). Once LPC is incorporated into lipoproteins, or conjugated to albumin or AGP, it is then transported to the liver or acted upon by catabolic enzymes in its vicinity to yield new metabolites.

For example, when autotaxin hydrolyzes phosphodiester bonds on LPC, lysophosphatidic acid can be formed (Law et al., 2019). The conversion of LPC back into phosphatidylcholine can also be facilitated via lysophosphatidylcholine acyltransferase (LPCAT) in the presence of acyl-CoA (Mapelli-Brahm et al., 2020). The cooperative action of anabolic and catabolic enzymes in the Land's Cycle keeps overall circulating and tissue levels of LPC relatively stable (Law et al., 2019).

2.4. The Role Lysophosphatidylcholine in Immunity

The biological effects of LPC are diverse and well-documented in non-ruminants. Numerous studies have highlighted LPC's modulatory effects on processes such as immune cell functionality, cardiometabolic disorders such as atherosclerosis, and insulin-stimulated glucose utilization (Bas et al., 2016; Law et al., 2019; Liu et al., 2020). The pervasiveness of LPC's roles in immune and metabolic cascades underpins its versatility as a biomarker and as a potential therapeutic for mitigating the deleterious cascades and downstream effects of many inflammatory processes and diseases. Therefore, in order to further comprehend and elucidate the mechanisms of LPC's role in these processes, it is imperative to understand the intra- and extracellular cascades that LPC initiates, potentiates, or attenuates within biological models. Unfortunately, the modulatory effects of LPC within the context of the bovine model is much less characterized. While associations have been made between disease/inflammatory status and the levels and relative ratios of LPC and its metabolites (Drobnik et al., 2003; Park et al., 2014), the lack of explicative bovine studies delineating the bioactive properties of LPC and the downstream cascades that it attenuates and elicits presents a gap in knowledge in the underlying biochemical pathways LPC modulates within the animal. A better understanding of these pathways could potentially provide better understanding of the etiology of common illnesses contracted by cattle and can aid in the development of interventions and therapeutics to better prevent and treat disease.

2.4.a. Lysophosphatidylcholine receptor-mediated signaling and its role in immunomodulation

Biological processes are primarily initiated and maintained through the ligand binding and resulting downstream cascades facilitated via membrane receptors. The effector mechanisms of LPC are largely directed and modulated through its role as a ligand to receptors found on the surface of tissue and immune cells. The reported receptors of LPC include G protein-coupled receptors (GPCRs) as well as TLRs (Liu et al., 2020). G protein-coupled receptors are composed of seven transmembrane domains consisting of an extracellular N-terminus and an intracellular C-terminal cytoplasmic tail (Vines and Prossnitz, 2004; Rehman et al., 2020). These cell-surface receptors have a large variety of ligands, such as sensory stimuli, lipids, and hormones. Moreover, they transduce extracellular signals through intracellular effector pathways via G protein (guanine nucleotide binding protein) activation (Vines and Prossnitz, 2004; Rehman et al., 2020). G proteins coupled with the receptor typically exist in the form of a heterotrimer consisting of a G_{α} subunit interacting with a $G_{\beta\gamma}$ complex. GPCR-mediated signal transduction is initiated by the binding of a ligand to the receptor, resulting in its conformational change brought about by the disruption of strong ionic interactions between the transmembrane helices (Rosenbaum et al., 2009). This conformational change allows for the otherwise hidden cytoplasmic surface of the receptor to be exposed, thus facilitating guanine nucleotide exchange (the release of guanosine diphosphate [GDP] and binding of guanosine triphosphate [GTP]) on the Gα subunit to which the receptor is coupled. The binding of GTP to the Gα subunit results in the activation of the heterotrimeric G protein complex and the subsequent dissociation of both the Gα subunit and Gβγ complex from the receptor (Rosenbaum et al., 2009). The dissociated Gα subunit and Gβγ complex can then go on to modulate a variety of signaling pathways through either the modulation of key downstream enzymes and/or ion channels (Rosenbaum et al., 2009).

A variety of G protein heterotrimers can be coupled to a GPCRs and can elicit divergent responses across cell types: there are at least 18 different Gα proteins to which GPCRs can be coupled, which, in turn, can form heterotrimeric complexes with Gβ and Gγ subunits, both of which have at least 5 and 11 different types respectively (Kroeze et al., 2003). Gα proteins are classified into four families: Gαi/o, Gαs, Gαq/11 and Gα12/13(Rehman et al., 2020). Gαs proteins primarily stimulate adenylyl cyclase activity while Gαi/o proteins have the opposite effect. Adenylyl cyclase is responsible for the formation of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate which, in turn, modulates cellular functions through the activation of cAMP-dependent protein kinases (Robin Lorenz, Daniela Bertinetti and Herberg, 2015). Gαq/11 proteins mainly act by activating phospholipase C (PLC), an enzyme responsible for breaking down phosphatidylinositol-4,5-biphosphate (PIP2), a component of membrane phospholipids, into DAG and inositol-1,4,5-triphosphate (IP3). Membrane-bound DAG can then activate PKC while IP3 can diffuse through the cytoplasm and bind to ligand-gated calcium channels, triggering the release of calcium ions into the cytosol. This release of calcium ions can go on to stimulate the activity of calcium-dependent protein kinases (Mizuno and Itoh, 2009). Gα12/13 proteins primarily stimulate the activity of the Rho family of GTPases (Suzuki et al., 2009). It is believed that the action of these subunits and the intracellular processes that they initiate serve as the primary underlying mechanism by which LPC elicits its effector mechanisms.

In addition to GPCRs, two major toll-like receptors, TLR2 and TLR4, also mediate LPC function, albeit the modulatory role of LPC on signaling cascades via TLR is less defined than GPCRs. The activation of these pathways can induce the production of proinflammatory mediators, thus propagating and regulating the systemic response to inflammatory and infectious diseases. It has been shown that LPC can elevate the expression of TLR4, MyD88 and NF-κB p65

(Chen et al., 2018). The activation of TLR stimulation elicits conserved inflammatory pathways, culminating in the activation of NF- κB and activating protein-1 (AP-1).(Kawai and Akira, 2006). The protein NF-κB is a dimeric transcription factor that is thought to be a heterodimer composed of the p65 and p50 subunits in most types of cells. In unstimulated cells, NF-κB is sequestered in the cytoplasm as an inactive form via its interaction with IκB proteins. Upon stimulation with various TLR ligands, canonical NF-κB activation is induced via the degradation of IκBα triggered through its site-specific phosphorylation by a multi-subunit IκB kinase (IKK) complex. This IKK complex is composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit named NF-κB essential modulator (NEMO) or IKKγ. The IKK complex can be activated by many different stimuli, including, but not limited to, cytokines, microbial components and stress agents (Liu et al., 2017). Upon activation, IKK phosphorylates IκBα at two N-terminal serines and triggers ubiquitin-dependent IκBα degradation in the proteasome. The phosphorylation targets IκBs for ubiquitination and degradation by the 26S proteasome, allowing for the transient nuclear translocation of NF-κB into the nucleus and its binding to the κB site. (Liu et al., 2017). This ultimately allows for the transcription of proinflammatory mediators such as cytokines.

Lysophosphatidylcholine can serve as a ligand of the receptor GPR4 as well as GPR132 (G2A), for which its affinity remarkably higher than relative to GPR4 (Liu et al., 2020). The GPCR G2A, otherwise known as GPR132, is an orphan GPCR expressed on major cells of both the innate (i.e., macrophages and neutrophils) and adaptive (i.e., T and B lymphocytes) immune systems (Kabarowski, 2009). Lysophosphatidylcholine can bind to G2A and activate ERK mitogen-activated protein kinase (MAPK) via G-protein α subunits and G-protein βγ subunit signaling to Ras, thus inducing downstream cascades such as lymphocyte migration and neutrophil extracellular trap formation (Cargnello and Roux, 2011). The activation of p38 MAPK has also

been implicated in the maturation and release of granulocytes in neutrophils and monocytes (Hong et al., 2010). Granule maturation and fusion is essential during the phagocytic process, during which phagocytes such as neutrophils engulf extracellular pathogens into vesicles known as phagosomes and neutralize them by fusing them with their granules containing antimicrobial compounds such as myeloperoxidase and superoxide anions to form phagolysosomes (Hurst., 2012). It has been shown that LPC amplifies the fusion of neutrophilic azurophil granules with phagosomes, thus enhancing neutrophilic bactericidal functionality (Hong et al., 2010). This action was confirmed to be mediated by p38 MAPK, which LPC has been shown to activate via the action of G_i/G_o proteins on PKC (Jing et al., 2000).

Studies in mice have demonstrated that G2A mediates potentiating effects of LPC on bacterial clearance and provides protection against sepsis and endotoxemia. For example, high concentrations of 18:0 LPC (up to 30 μM) potentiated the generation of H_2O_2 by neutrophils, thus enhancing their bactericidal activity. Treatment with anti-G2A polyclonal antibody inhibited this effect (Yan et al., 2004). Another recent study demonstrated that LPC (and structurally related lysolipids) produced by PLA_2 during inflammation activates G2A signaling in neutrophils leading to increases in calcium flux (Frasch et al., 2008). In this study, LPC administration to neutrophils stimulated calcium flux in a pertussis toxin-sensitive manner, implicating the involvement of $G_{\alpha i}$ signaling to PLC. This signaling response was ablated by addition of anti-G2A antibody. Recent data suggest that, rather than binding directly to G2A, LPC may alter G2A's distribution within the cell by increasing its translocation from the interior of the cell to the cell surface or by preventing its movement away from the cell surface to the cell interior (Wang et al., 2005). In cell types which have internal stores of G2A in membrane-bound secretory vesicles, such as neutrophils, G2A-containing vesicles continuously merge with and move back out of a cell's

surface membrane (Lan et al., 2014). By increasing the surface expression of G2A, LPC would potentially be able to amplify G2A-mediated effects, thus contributing to the mechanisms responsible for the initiation and/or resolution of inflammation in response to infection and, consequently, modifying host susceptibility to sepsis and other infectious diseases by facilitating the efficient clearance of bacterial pathogens (Kabarowski, 2009). Additionally, LPC itself has a bactericidal effect by increasing cell membrane permeability (Miyazaki et al., 2017), enhancing the antibiotic sensitivity of antibiotic-resistant bacteria (Parra Millán et al., 2016), and increasing bacterial clearance within the peritoneum in mouse models of systemic infection (Yan et al., 2004).

Engagement of TLRs also results in the activation of MAPK p38 and JUN kinases (JNK) (Kawai and Akira, 2006). These pathways are involved in various physiological and immunological processes. For example, the JNK pathway regulates cellular processes such as apoptosis T-cell activation and differentiation, and cytokine production (Hammouda et al., 2020). In mice and cell models, it has been observed that LPC can activate the NF-kB, p38 MAPK, and c-Jun N-terminal JUN signaling pathways by combining the TLR2 and TLR4 receptors (Liu et al., 2020). Due to LPC's documented roles in these processes (Huang et al., 1999; Yan et al., 2004; Perrin-Cocon et al., 2006; Liu et al., 2020), it is possible that these activities are activated or potentiated via the activation of the JNK pathway via LPC ligand stimulation of TLRs. The potential synergistic downstream cascades of both TLR and GPCR activation by LPC and how these processes modulate the host response to inflammation and physiological stress remains to be elucidated.

The documented immunomodulatory roles, though molecular in scale, have profound systemic implications on the host organism. Preemptive treatment with LPC was found to significantly improve survivorship in mice with experimentally induced sepsis via cecal ligation

puncture (Yan et al., 2004). Similarly, humans with sepsis have been found to have significantly lower circulating levels of LPC relative to their health counterparts (Drobnik et al., 2003) and certain species of LPC have been so closely correlated with outcomes of sepsis that serial measurements of LPC have been considered as a predictor for mortality (Park et al., 2014). These findings underpin the protective role of LPC in instances of systemic infection and disease and highlights LPC as a potential therapeutic target for the development of methods to mitigate sepsis-related mortality.

2.4.b. The role of lysophosphatidylcholine in the development of oxidative stress and inflammation

Lysophosphatidylcholine can play a key role in the development and progression of oxidative stress through the role of both the G2A and GPR4 receptors (Liu et al., 2020). Atherosclerotic studies have documented that the receptor GPR4 enhances the expression of adhesion molecules in endothelial cells involved in the migration of inflammatory cells, and impairs endothelial barrier functions (Gräler and Goetzl, 2002). It has been documented that when endothelial cells (ECs) are incubated with LDL, as much as 40% of oxLDL is hydrolyzed into LPC via the enzymatic action of PLA_2 (Steinbrecher et al., 1984). Lysophosphatidylcholine can potently activate ECs and induce monocyte adhesion by transcriptionally upregulating intercellular adhesion molecule-1 (ICAM-1) gene expression in human umbilical vein Ecs (Kume et al., 1992). Inhibitors of ROS and intracellular calcium are able to block LPC-induced ICAM-1 gene expression in these cells (Erdogan et al., 2007), implicating the necessity of Ca^{2+} mobilization and NADPH oxidase in these processes. Upon activation of the endothelium and upregulated expression of adhesion molecules such as ICAM-1 and vascular cell adhesion molecule-1,

monocytes and lymphocytes are recruited to the site (Kong et al., 2018). These migrated cells generate different proinflammatory cytokines, which will in return further activate local Ecs to produce adhesion molecules. Other adhesion molecules such as E-selectin and P-selectin, also can contribute to the recruitment of immune cells to the site. These actions indicate that LPC may have a stimulating role in recruiting and activating immune cells thus driving the progression of oxidative stress (Matsumoto et al., 2007; Zhang et al., 2021). Additionally, LPC activates proinflammatory transcription factors, such as NF-κB and activator protein-1 (AP-1) (Sugiyama et al., 1998). The promoter regions of LPC-inducible genes in endothelial cells have several binding sites for transcription factors such as NF-κB and AP-1, and the activities of these transcription factors are known to be in part modulated by PKC signal transduction system (Sugiyama et al., 1998). Thus, LPC could regulate the DNA-binding activities of these transcription factors through the mechanisms involved in a PKC-mediated pathway, leading to the induction of a number of endothelial genes implicated in atherosclerosis. Indeed, LPC has been shown to induce biphasic regulation of NF-κB activity in human vascular endothelial cells and this effect was confirmed to be partly mediated through a PKC-dependent pathway (Sugiyama et al., 1998). Together, AP-1 and NF-κB play a role in the regulation of cyclooxygenase-2 and inducible nitric oxide synthase expression, and are crucial for the induction of genes which are involved in the inflammation process (Tewari et al., 2018).

2.5. Lysophosphatidylcholine and insulin signaling

Lysophosphatidylcholine has also been shown to play a modulatory role in insulin signaling. Lysophosphatidylcholine induces insulin secretion through cAMP production in both pancreatic β-cell lines and intact pancreas. Incretin hormones, such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), are well known to bind to their

endogenous $G_{\alpha s}$-protein-coupled receptors expressed in pancreatic β-cells, and induce glucose-dependent insulin secretion by accumulating intracellular cAMP via adenylate cyclase activation (Drucker, 1998; Yip and Wolfe, 1999). Adenylate cyclase can be activated directly via the receptor-linked stimulatory $G_{\alpha s}$-protein however, though the presence of a $G_{\alpha s}$-protein-coupled receptor for LPC has been suggested (Yuan et al., 1996), previously identified LPC receptors, G2A and GPR4, are G_i-protein-coupled receptors and are not expressed in the pancreas (Xu, 2002). Therefore, in order to explain LPC's observed modulatory effects on cAMP and, in turn, insulin signaling, a $G_{\alpha s}$-protein-coupled receptor for LPC that is expressed in pancreatic β-cells needed to be identified. Interestingly, the GPCR GPR119, a $G_{\alpha s}$-protein-coupled receptor, was found to induce intracellular cAMP accumulation by LPC (Soga et al., 2005). Lysophosphatidylcholine has also been shown to play a role in fatty acid induced insulin resistance. Addition of exogenous LPC to myoblasts attenuated the phosphorylation of insulin receptor substrate (IRS)-1 Tyr612 and Akt Ser473 in response to insulin (Han et al., 2011). Phosphorylation of IRS-1 Ser307 and JNK was also induced by LPC, indicating that exogenous LPC inhibits insulin signaling through JNK phosphorylation. This finding supports the suggestion that LPC induces insulin resistance through JNK activation. This action was found to be pertussis toxin-sensitive, implicating the role of LPC signaling through GPCR/G_{ai}. Pertussis toxin reversed the decreased insulin-induced IRS-1 Tyr612 and Akt phosphorylation by LPC, suggesting this mechanism is indiced through GPCR/G_{ai} signaling (Han et al., 2011).

In all, the documented modulatory effects of LPC are extensive and diverse. Immune responses, metabolic processes, the onset of both pro- and anti-inflammatory cascades, and the development and progression of diseases have all been reported to be affected by or correlate with LPC and the downstream cascades that it elicits. Unfortunately, while these effects are explored

and well-documented in non-ruminant species, how LPC modulates immune and physiological responses, including growth, within a bovine model has remained unexplored. The immunodeficient neonatal dairy calf has much to gain from the potential protective immunomodulatory mechanisms observed in response to LPC therapy in murine models. The multitude of biological processes that are associated with LPC highlight its therapeutic potential as a target for attenuating the systemic effects of inflammatory and immunological challenges within calves in order to prevent or mitigate illness and disease. Therefore, in order to further define LPC and its canonical roles in calves in order to develop efficacious therapeutics, we must define the effects of LPC on bovine immunity and metabolic function.

2.6. Lecithin Feeding in Dairy Cattle

The term lecithin refers to amphiphilic phospholipids derived from animal or plant sources. It is composed in part of LPC but also other phospholipids such as PC and phosphatidylethanolamine. The incorporation of lecithin into livestock diets is a feeding strategy utilized widely in the dairy industry in order to enhance intestinal lipid digestion and improve production. During digestion, following the modification of dietary lipids via the catabolic action of pancreatic digestive enzymes, triglyceride, cholesterol, and PC are packaged with apoproteins (i.e., apo-B48) into lipoprotein particles called chylomicrons which are then secreted from enterocytes into the lymphatic system (Lo and Coschigano, 2020). Chylomicron-containing lymph then empties into venous blood circulation at the thoracic duct, whereafter their lipid components can be utilized by tissues such as the mammary gland and skeletal muscle as energy substrates. In order to efficiently digest and absorb dietary fats, emulsifying compounds such as lysolecithin (i.e., LPC) are required for the formation of micelles, a key feature of lipid digestion.

Micelles contain components such as bile acids and salts, lysolecithin, and fatty acids that ensure lipid absorption across the aqueous surface layer of intestinal microvilli. Owing to its amphiphilic nature, LPC possesses excellent emulsifying properties and is used widely as such for not only cosmetic and pharmaceutical applications, but also to improve lipid digestion and performance in livestock species (Xie and Dunford, 2015). In broilers, dietary LPC supplementation was found to improve total tract digestibility of palmitic, oleic, and linoleic fatty acids (Zhang et al., 2011). In cattle, lecithin feeding has been found to improve feed efficiency and modify milk composition (Fontoura et al., 2021). Additionally, dietary soy lecithin was found to enhance growth, antioxidative protection, and thermal tolerance in fish species (Ciji et al., 2021; Xu et al., 2022). While the effects of lecithin feeding on immune responses in bovine have not been largely explored, it has been shown to increase plasma levels of LPC (Wang et al., 2021), which could have potential effects on immune modulation.

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Chapter 3: Lysophosphatidylcholine enhances the oxidative burst response, inflammatory cytokine secretion, and *Escherichia coli* killing in polymorphonuclear neutrophils isolated from Holstein heifer calves

3.1. Introduction

Newborn ruminant calves are immunologically naïve at birth due to underdeveloped innate and adaptive immune cell mechanisms (as reviewed by Chase et al., 2008). This is due in part to the protective environment of the maternal womb, which prohibits antibody transfer and limits pathogen exposure (Chase et al., 2008; Windeyer and Gamsjäger, 2019). Although feeding of colostrum is considered the most effective way to immunologically protect young calves, FPT from colostrum increases an animal's vulnerability to infection and disease (NAHMS, 2007). In the calf, innate immune cells such as neutrophils and macrophages have reduced phagocytic capacity as well as reduced trafficking capabilities (Chase et al., 2008). The immature adaptive immune system is characterized by low lymphocyte responsiveness and a lack of circulating antibodies. This collective deficiency in immune function is cause for increased incidence of disease that takes place during the first 3 weeks of life, which has been defined as the "window of susceptibility" for calves (Chase et al., 2008). Indeed, immunodeficiency is the cause for high morbidity and mortality in neonatal calves, as compared to mature animals (NAHMS and USDA, 2007). Calves are especially vulnerable to enteric infections from the bacterium *Escherichia coli (E. coli)*, which is one of the most common cause for diarrhea and septicemia (Kolenda et al., 2015; Urie et al., 2018). Moreover, the development of an early-life illness predisposes the dairy calf to impaired growth, fertility, and milk production later in life (Waltner-Toews et al., 1986; Buczinski et al., 2021).

Neutrophils are responsible for the initial clearance of bacterial and fungal pathogens during the early stages of infection, and they are deemed essential for the successful resolution of

an infection in mammals (Chase et al., 2008; Panousis et al., 2018). Neutropenia will provoke recurrent bacterial and fungal infections (Aratani et al., 2000). In the dairy calf, although the size of the neutrophil population is not diminished, neutrophilic bactericidal mechanisms function at a reduced capacity compared to those in their adult counterparts (Roland et al., 2014; Roach et al., 2020). This includes low phagocytic capabilities, respiratory burst activity, and myeloperoxidase concentrations (Higuchi et al., 1997). Cytokines also play an important role in the development and function of neutrophils. For example, tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6) upregulate production of bactericidal effector molecules and enhance chemotaxis respectively (Fielding et al., 2008; Wright et al., 2010; Selders et al., 2017). These mechanisms assist in facilitating a robust immune response to ensure effective clearance of pathogens such as *E. coli* (Lee et al., 2003; Jones et al., 2006). Therefore, in the calf, it is imperative that these key neutrophilic mechanisms are functioning at full capacity to protect itself from pathogen exposure.

The lysophospholipid lysophosphatidylcholine (LPC) has received attention for its observed protective immunomodulatory effects in both murine and human models of sepsis (Hong and Song, 2008; Kabarowski, 2009). Plasma concentrations of LPC are markedly low in patients with sepsis (Drobnik et al., 2003a). The subcutaneous administration of stearoyl-LPC (LPC-18:0), prior to induction of experimental sepsis by cecal ligation procedure, increased survival over a 10-d period in mice (Yan et al., 2004). The protective effects of LPC are being defined in non-ruminants. Administration of saturated and unsaturated LPC to murine and human neutrophils enhanced their production of reactive oxygen species (ROS), a key function that is imperative to the successful clearance of pathogens by neutrophils (Yan et al., 2004; Ojala et al., 2007). Administration of LPC-18:0 was also found to enhance *E. coli* DH5-α killing by human neutrophils (Hong et al., 2010). These findings demonstrate that LPC have the ability to enhance

neutrophil bactericidal mechanisms and increase host survival during instances of bacterial infection, likely through signaling involving G-coupled protein receptor G2A (Kabarowski, 2009).

The effects of LPC on bovine neutrophil functionality have not been considered in dairy cattle. Therefore, our objective was to study the effects of LPC on neonatal dairy calf neutrophil activation and function. Our hypothesis was that LPC enhance the production of bactericidal products such as ROS and inflammatory cytokines as well as increase the clearance of pathogenic *E. coli* in bovine neutrophils. Such work is important to consider for the development of non-antibiotic therapies to manage failure of passive transfer and prevent infection and disease in dairy calves.

3.2. Materials and Methods

3.2.a. Bovine Neutrophil Isolation

Neonatal bovine neutrophils were isolated using a method outlined by Roach et al. (2020). In brief, blood samples from clinically healthy Holstein heifer calves aged 2 to 5 weeks (18.4 ± 3.6 d of age [mean ± SD]) were collected via jugular venipuncture into heparinized Vacutainer® (Becton, Dickinson and Company, Franklin Lakes, NJ) glass tubes, transported on ice, and analyzed within 1 h of collection. Neutrophils were isolated using a Ficoll® density-gradient (1.077 g/mL; Cytiva, Marlborough, MA) centrifugation followed by hypotonic lysis of residual erythrocytes with sterile ice-cold deionized water. Neutrophils were then washed with Hanks Buffered Saline Solution (HBSS) without Ca^{2+} and Mg^{2+} and enumerated using a hemacytometer. Neutrophil purity and viability were assessed by Diff-Quik staining (PolySciences, Warrington, PA) and trypan blue (Thermo Fisher Scientific, Waltham, MA) exclusion, respectively. For each experiment, 3 calves were used with biological and technical replicates performed in duplicate.

3.2.b. Lysophosphatidylcholine Preparation

Palmitoyl-, stearoyl, and oleoyl-LPC (LPC-16:0, -18:0, and -18:1, respectively) were obtained from Cayman Chemical Company (Ann Arbor, MI) and varying stock concentrations were created by dissolving LPC into HBSS without Ca^{2+} and Mg^{2+} either in the presence or absence of 5% fetal bovine serum (FBS) or 50 µM bovine serum albumin (BSA). The solutions were then sonicated in 5 sec intervals until solubilized, and aliquots stored at -20 ℃ until use.

3.2.c. Ex Vivo Hydrogen Peroxide Quantification

In order to determine whether LPC modulates the production of H_2O_2, a byproduct of the oxidative burst response, neutrophils (1×10^6 cells) were seeded in 96-well plates in 100 µL of Hank's Buffered Salt Solution (HBSS) without Ca^{2+} and Mg^{2+} with 65 µM luminol (Sigma-Aldrich, St. Louis, MO) and pre-treated with either vehicle HBSS without Ca^{2+} and Mg^{2+} or variable concentrations (5 to 30 µM) of unbound LPC-16:0, -18:0, or -18:1. The plate was then placed in a Veritas™ Luminometer (Veritas Technologies LLC, Santa Clara, California) and the emitted luminescence measured for 5 min at room temperature before cells were stimulated with 1 µM of phorbol myristate acetate (PMA), a known agonist of protein kinase C (i.e., the oxidative burst). The resulting luminescence was then read every 3 min for a duration of 90 min. Outputs are expressed in arbitrary units (A.U.).

3.2.d. Cytokine Quantification by ELISA

Neutrophils (5×10^6 cells) were seeded in 12-well plates in 0.5 mL RPMI 1640 media (Thermo Fisher Scientific, Waltham, MA) and pre-treated with either vehicle HBSS without Ca^{2+} and Mg^{2+} or LPC [LPC-18:0 or -18:1] at a concentration of 100 µM + 50 µM BSA for 30 min prior to stimulation with O55:B5 lipopolysaccharide (O55:B5 LPS, Sigma Aldrich, St. Louis, MO; 100 µg/mL). Cells were then cultured at 37℃ and 5% CO_2 for 6 h prior to collection of cell suspensions. This incubation duration was chosen as 6 hours is an observed time for cytokine detection in cell media (Yan et al., 2004). Cells were then spun down at 350 × g for 10 min and the supernatants collected and frozen at -80 ℃. Secreted TNFα and IL-6 were quantified in the supernatants using commercial ELISA kits from Thermo Fisher Scientific (Waltham, MA; catalog #: EBTNF and ESS0029). Inter- and intra-assay coefficients of variation were 11.3% and 9.1% respectively.

3.2.e. E. coli Killing Assay

For evaluation of the effects of LPC on neutrophil-mediated *E. coli* killing, neutrophils (5×10^6 cells) suspended in 1 mL of HBSS without Ca^{2+} and Mg^{2+} were pretreated with LPC-18:0 or LPC-18:1 for 30 min in 15-mL polypropylene conical tubes prior to co-culture in a 1:10 ratio with *E. coli* K99 in HBSS without Ca^{2+} and Mg^{2+} containing 10% Luria broth (Invitrogen, Waltham, MA). For both LPC, a 30 µM treatment prepared in the absence of albumin and a 100 µM treatment prepared in 50 µM BSA were tested for their effects on neutrophil-mediated *E. coli* killing. Additionally, a 0 µM LPC control (containing both *E. coli* and neutrophils) and an *E. coli* only (no neutrophils) control without LPC were also tested. These concentrations of LPC were chosen as these are previously established concentrations that yielded significant results in similar

experiments using non-ruminants (Yan et al., 2004) or reflect the physiological level and albumin-bound state of LPC in circulation (Law et al., 2019). The cellular suspensions were then incubated for 1 h at 37℃ with gentle shaking, then serially diluted and plated on Luria Broth (LB) agar (Thermo Fisher Scientific, Waltham, MA) plates to be incubated overnight at 37℃ and counted the following morning. Bacterial concentrations were determined and confirmed by diluting bacterial cell suspensions to an OD_{600} of 1 and enumerating bacterial colony forming units (CFUs) on LB agar plates. Following a co-incubation period of 60 min, the neutrophil-*E. coli* cellular suspension was then serially diluted, and the dilutions were plated in triplicate within quadrants on LB agar plates, dried, and cultured overnight at 37°C. Viable CFUs were then counted the following morning. The percentage of bacteria killed was calculated as a percentage of viable CFUs present the following morning relative to *E. coli*-only controls.

3.2.f. Statistical Analyses

An analysis of variance was used to examine differences among groups. Data were analyzed under the MIXED procedure in SAS (version 9.4; SAS Institute Inc., Cary, N.C.). The statistical model included the random effect of calf, and the fixed effects of treatment and time (when applicable), as well as their interaction and the random effect of calf and replicate nested within treatment. The model included post hoc adjustment for multiple comparisons, using Tukey's adjustment. The method of Kenward–Rogers was used for calculation of denominator degrees of freedom. Normality of the residuals was evaluated with normal probability, box plots, and homogeneity of variances with plots of residual versus predicted values.

3.3. Results

Neutrophil purity was quantified by calculating the relative percentage of Diff-Quik-stained neutrophils to peripheral blood mononuclear cells per 100 cells under a compound light microscope in duplicate. Viability was determined via Trypan Blue exclusion by incubating 10 µL of the cell suspension in an equal volume of 0.4% Trypan for 5 minutes prior to enumeration on a hemacytometer. Percentages of Trypan-positive cells were calculated relative to total cells and were run in duplicate. The mean ± standard deviation for neutrophil purity and viability were 92% (± 4%) and 93% (± 4%), respectively.

Addition of PMA to the neutrophil suspension induced a rapid increase in H_2O_2 production relative to non-stimulated cells ($P < 0.001$; **Figure 3.1**).

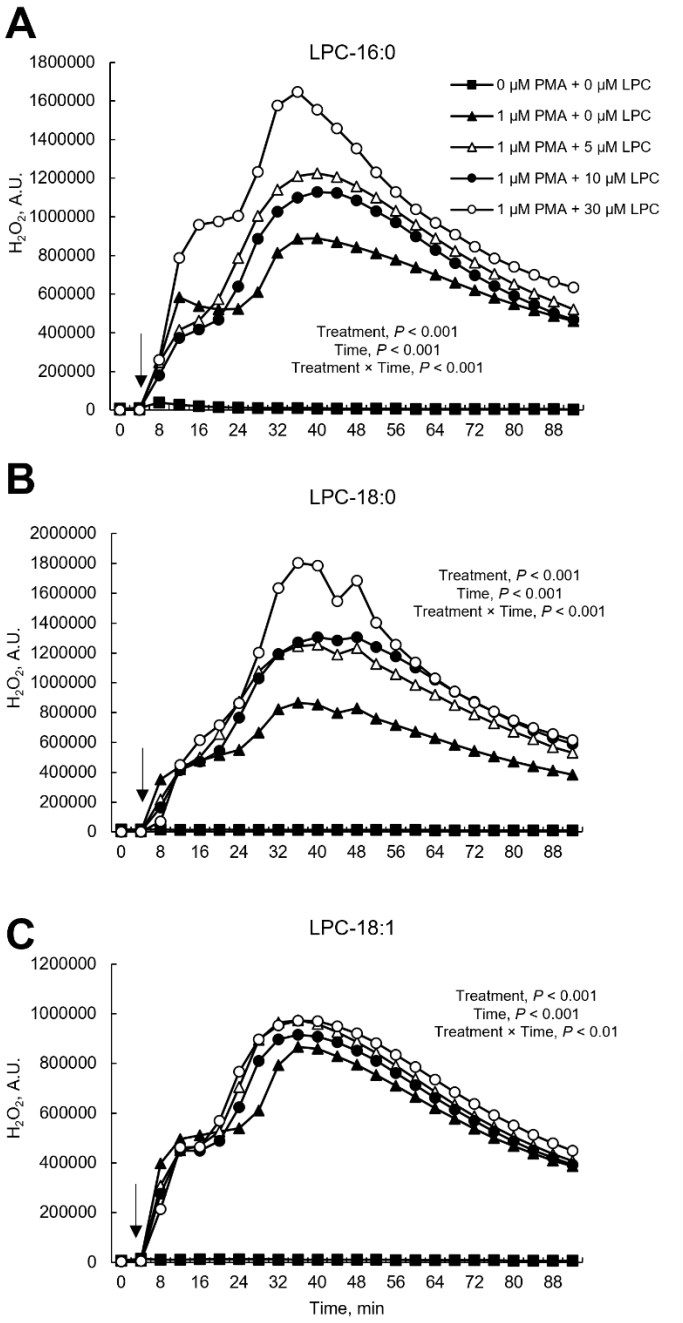

Figure 3.1. Pre-treatment with unbound LPC elicits variable effects on neutrophil hydrogen peroxide production in a dose-dependent manner in neutrophils isolated from Holstein heifer calves. Neutrophils were pre-incubated with either vehicle (HBSS without Ca^{2+} and Mg^{2+}) or increasing concentrations of unbound LPC for 5 min prior to stimulation with PMA. Saturated LPC (A, B) and unsaturated LPC-18:1 (C) increased the production of H_2O_2 in a dose-dependent manner. Note that the y-axis scale is different for LPC-18:1 (C), but that PMA-only control values are similar. Arrow indicates when PMA was added. For each experiment, 3 calves were used with biological and technical replicates performed in duplicate.

The effects of LPC on PMA-induced H_2O_2 production were influenced by the species of LPC. Neutrophils pre-incubated with LPC-16:0, -18:0, and -18:1 exhibited a significant increase in H_2O_2 production over the course of 90 min ($P < 0.01$; Figure 3.1) in a dose-dependent manner, though the magnitude of change was lower in cells treated with LPC-18:1. Interestingly, the marked increase in H_2O_2 production by neutrophils in the saturated LPC-treated groups came at the expense of reduced cellular viability ($P < 0.01$; **Figure 3.2**).

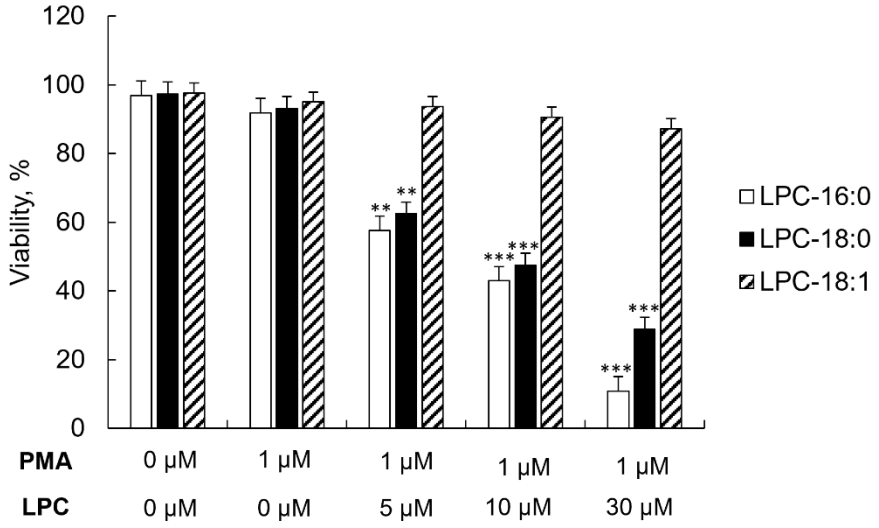

Figure 3.2. Administration of increasing concentrations of unbound saturated LPC negatively impacted neutrophil viability in neutrophils isolated from Holstein heifer calves. Neutrophils were pre-incubated with either vehicle (HBSS without Ca^{2+} and Mg^{2+}) or various concentrations of unbound LPC and stimulated with PMA prior to being incubated for 6 hours at 37°C, 5% CO_2. For each experiment, 3 calves were used with biological and technical replicates performed in duplicate. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, relative to control.

This was especially pronounced in the cells treated with 100 µM free LPC for which viable cells were undetectable (data not shown). However, neutrophil viability was not compromised by treatment with LPC-18:1 ($P > 0.05$). To further explore the effects of LPC on PMA-induced H_2O_2 production and viability, we cultured neutrophils with 100 µM LPC prepared with either 5% FBS

or 50 µM BSA to closely reflect physiological concentrations and potentially mitigate the cytotoxic effects of unbound LPC. Although FBS- or albumin-treated LPC diminished the overall H_2O_2 production response as compared to the prior experimental test, neutrophils treated with LPC-16:0 and LPC-18:0, as well as LPC-18:1, exhibited significantly greater H_2O_2 production rates than neutrophils cultured in the absence of LPC ($P < 0.05$; **Figure 3.3**).

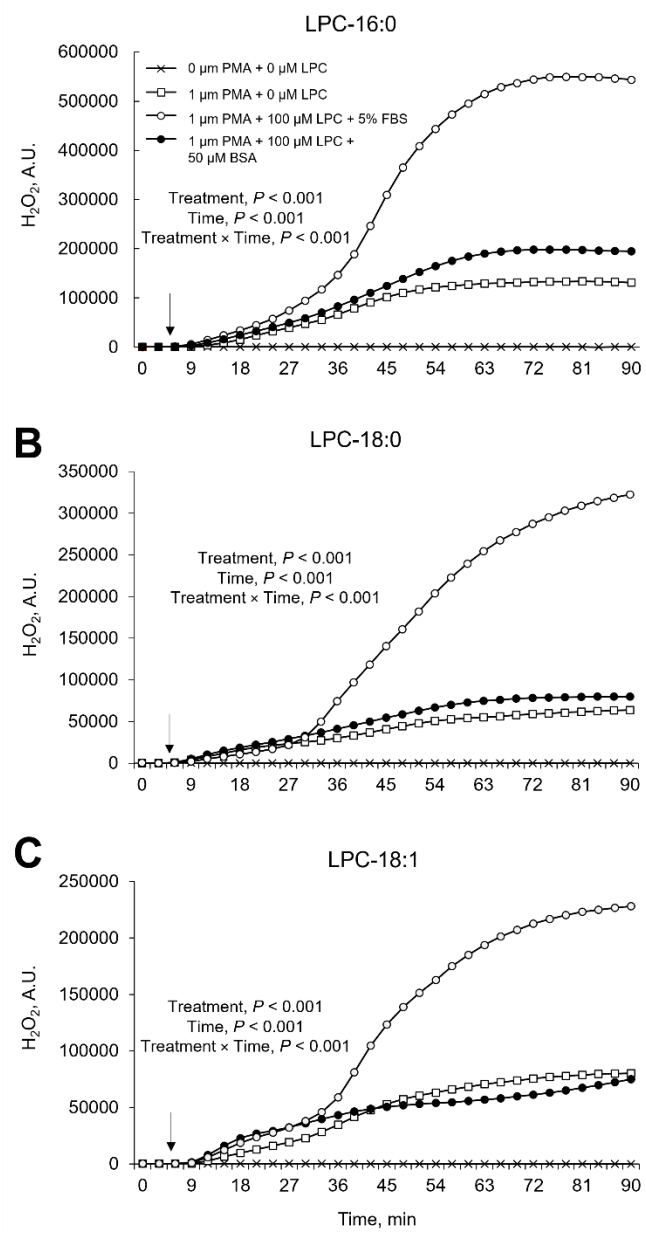

Figure 3.3. LPC preparation with either BSA or FBS increases overall production of hydrogen peroxide in neutrophils isolated from Holstein heifer calves. Neutrophils were pre-

incubated with either vehicle (HBSS without Ca^{2+} and Mg^{2+}) or increasing concentrations of LPC prepared in either BSA or FBS for 5 min prior to stimulation with PMA. Saturated LPC-16:0 (A), LPC-18:0 (B), and LPC-18:1 (C) increased the production of H_2O_2. Arrows indicate when PMA was added. Cells treated with LPC prepared in FBS exhibited marked increases in H_2O_2 production over the course of the experiment (A-C; $P < 0.001$). Saturated LPC prepared in albumin increased H_2O_2 production at a lower magnitude relative to LPC prepared in FBS, albeit at a still significantly higher rate than non-LPC treated cells stimulated with PMA alone ($P < 0.05$). Unsaturated LPC-18:1 did not significantly increase H_2O_2 production over time relative to the PMA-stimulated control ($P > 0.05$). For each experiment, 3 calves were used with biological and technical replicates performed in duplicate.

Viability was found to be salvaged by the LPC treatment with 50 µM BSA ($P > 0.05$) but not 5% FBS ($P < 0.01$; **Figure 3.4**).

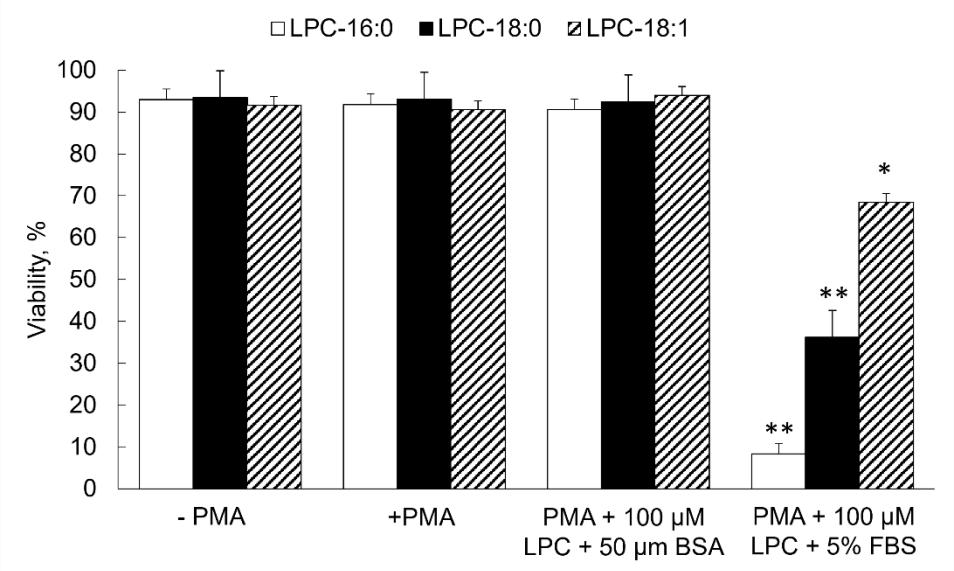

Figure 3.4. Administration of LPC supplemented with either BSA or FBS maintained viability in neutrophils isolated from Holstein heifer calves. Neutrophils were pre-incubated with vehicle and LPC supplemented with either 50 µM BSA or 5% FBS for 5 min then stimulated with PMA prior to being incubated for 6 h at 37°C, 5% CO_2. For each experiment, 3 calves were used with biological and technical replicates performed in duplicate. * = $P < 0.05$, ** = $P < 0.01$; ** = $P < 0.001$, relative to PMA-control.

The effects of LPC treatment on inflammatory TNFα and IL-6 secretions are presented in **Figure 3.5**.

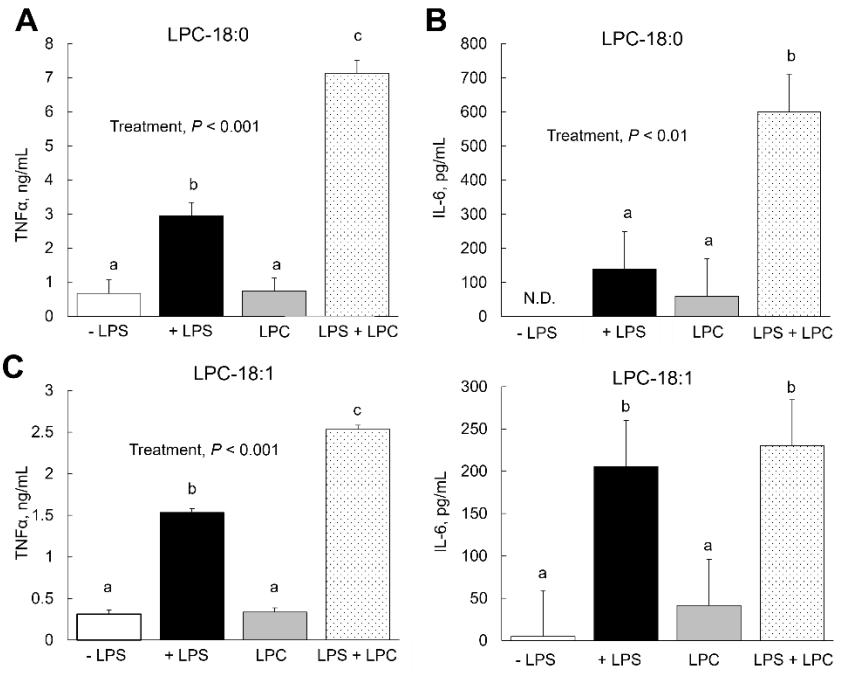

Figure 3.5. Pre-treatment with LPC-18:0 enhances LPS-stimulated TNFα and IL-6 secretion from neutrophils isolated from Holstein heifer calves. Neutrophils were pre-incubated with either vehicle (HBSS without Ca^{2+} and Mg^{2+} + 50 µM BSA), 100 µM LPC-18:0 + 50 µM BSA, or 100 µM LPC-18:1 + 50 µM BSA for 30 min prior to being stimulated with 100 µg/mL of O55:B5 LPS and incubated for 6 h at 37 °C, 5% CO_2. Concentrations of TNFα (A & C) and IL-6 (B & D) were quantified via ELISA. For each experiment, 3 calves were used with biological and technical replicates performed in duplicate. N.D. = not detected (value set at 0).

As expected, LPS stimulation significantly increased TNFα and IL-6 cytokine secretion from bovine neutrophils, as compared to the unsupplemented control ($P < 0.01$). Neutrophils treated with BSA-bound LPC-18:0 or -18:1, in the absence of LPC, did not elicit a cytokine response; however, BSA-bound LPC-18:0 and -18:1 potentiated LPS-stimulated TNFα secretion from bovine neutrophils ($P < 0.001$). Neutrophils treated with LPC-18:0 also potentiated the production of IL-6 in the presence of LPS ($P < 0.01$); however, this effect was not observed for LPC-18:1.

The effects of LPC-18:0 and -18:1 on *E. coli* killing are presented in **Figure 3.6**.

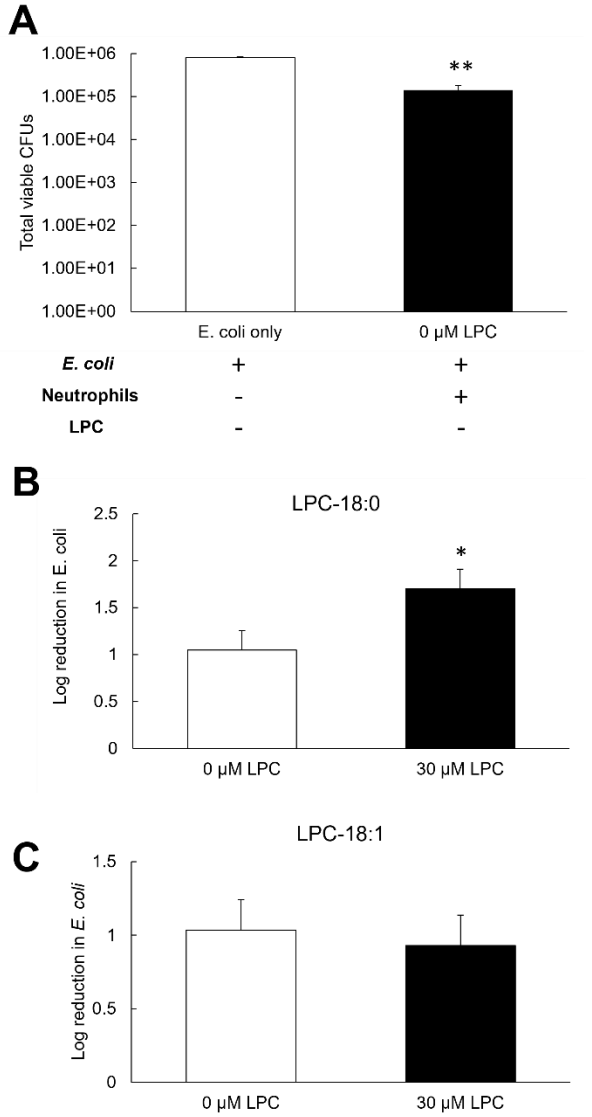

Figure 3.6. Pre-treatment with LPC-18:0, enhances neutrophil-mediated *E. coli* killing in neutrophils isolated from Holstein heifer calves. Basal *E. coli* killing was determined by quantification of CFUs following neutrophil-*E. coli* co-incubation without LPC treatment (A). The open bar represents *E. coli* cultured in the absence of both neutrophils and LPC. The closed bar represents *E. coli* cultured in a 1:10 ratio with neutrophils without LPC. In order to test the effects of LPC on neutrophil-mediated *E. coli* killing, neutrophils were pre-incubated with either vehicle (HBSS without Ca^{2+} and Mg^{2+}) or 30 µM unbound LPC-18:0 or LPC-18:1 for 30 min prior to being co-incubated with *E. coli* in a 1:10 ratio for 1 h. The effects of LPC-18:0 and LPC-18:1 on *E. coli* killing relative to untreated neutrophil controls are shown in panel B and C respectively. Effects of albumin-bound LPC-18:0 and LPC-18:1 (100 µM + 50 µM BSA) alone on *E. coli* killing in the absence of neutrophils were also tested, though no significant effects were detected (data not shown). Log reduction values were calculated relative to *E. coli* only controls. For each

experiment, 3 calves were used with biological and technical replicates performed in duplicate. * = $P < 0.05$, ** = $P < 0.01$.

Neutrophils were pre-treated with either vehicle HBSS or 30 µM unbound LPC for 30 min prior to co-culture with *E. coli* at a 1:10 ratio. As expected, co-culturing with neutrophils significantly reduced the number of viable *E. coli* CFUs compared to the *E. coli*-only treatment group (**Figure 3.6A**), even in the absence of unbound LPC treatment ($P < 0.01$). However, when unbound LPC-18:0 was added, the reduction in viable *E. coli* CFUs was over a half log-scale higher ($P < 0.05$). There were no significant effects of unbound LPC-18:1 on neutrophil-mediated *E. coli* killing ($P > 0.05$). Effects of albumin-bound LPC-18:0 and LPC-18:1 (100 µM + 50 µM BSA) alone on E. coli killing in the absence of neutrophils were also tested, though no significant effects were detected (data not shown).

3.4. Discussion

Lysophosphatidylcholine (LPC), formed via the cyclical synthesis and degradation of phosphatidylcholine (i.e., the Lands Cycle), has immunomodulatory effects in a variety of human and murine models of disease (Drobnik et al., 2003b; Yan et al., 2004; Law et al., 2019). Recent evidence points to the role of LPC as an important ligand and initiator of immune cell activation and bactericidal mechanisms (Hong et al., 2010). For example, the effects of LPC on neutrophilic mechanisms have received increased attention due to the important role of this cell type in pathogen clearance and the documented immunomodulatory effects LPC enacts via G-protein coupled receptor activation (Yan et al., 2004; Kabarowski, 2009; Hong et al., 2010). Lysophosphatidylcholines are able to stimulate intracellular cascades that result in the propagation of bactericidal functions (e.g., granule release) and the production of antibacterial compounds (e.g., superoxide) that ultimately aid in the clearance of an invading pathogen (Yan et al., 2004;

Ojala et al., 2007; Hong et al., 2010). Whether LPC modulates neutrophil functionality in the immunologically susceptible pre-ruminant dairy calf has yet to be explored. Therefore, neutrophils isolated from neonatal dairy calves were treated with both saturated and unsaturated LPC and various measures of neutrophil functionality were evaluated. Palmitoyl, stearoyl, and oleoyl-LPC were studied because these LPC species are the most abundant species of LPC in dairy cattle. We tested 5, 10, 30, and 100 μM concentrations of LPC in order to recapitulate treatment levels used in prior studies (Ojala et al., 2007) but also mimic physiological concentrations observed in cattle.

In neutrophils, assembly and activation of the NADPH oxidase complex results in the production of ROS (Kuwabara et al., 2015). One particular ROS, H_2O_2, is highly potent in the eradication of bacterial cells by activating granular proteases and crossing the membranes of bacterial pathogens to damage their nucleic acids, proteins, and cell membranes (Silliman et al., 2003). In our study, saturated palmitoyl- and stearoyl-LPC were found to enhance PMA-induced production of H_2O_2 in a dose-dependent manner. This is similar to findings from other studies where saturated LPC were found to enhance the oxidative burst response in murine neutrophils following cecal ligation puncture (Yan et al., 2004). These observations were modulated in part by the action of LPC on the G protein coupled receptor G2A, which elevates cyclic AMP concentrations and intracellular calcium flux when activated (Yan et al., 2004; Lin et al., 2005; Hong et al., 2010); however, this requires confirmation in cattle. In human neutrophils, unsaturated LPC increased the superoxide production more than saturated LPC (Ojala et al., 2007). Although the vehicles in which LPC treatments were prepared and administered differed between our study and Ojala et al. (2007) (i.e., Hepes buffer and ethanol versus HBSS), the discrepancy could also be attributed to differences in the animal model or physiological state (i.e., neonatal vs. adults). Similarly to our findings, Silliman et al. (Silliman et al., 2003) observed significant increases in

calcium flux, a precursor of the oxidative burst response, following saturated LPC administration in human neutrophils, but a more suppressed response following LPC-18:1 administration. Kuplez et al. (2020) suggested that the conical shape and higher spontaneous curvature values of saturated LPC-16:0 and -18:0, in contrast to unsaturated LPC-18:1, could explain variable modes of action. This is possible considering that membrane lipid curvature plays an important role in immune cell polarization and activation (Kamal et al., 2009; Ren et al., 2019).

The observed ability of unbound LPC to compromise neutrophil viability could have been due to direct cytotoxic effects caused by LPC. But cellular viability was not found to be compromised when LPC-18:0 was administered to neutrophils in the absence of PMA (data not shown). It is plausible that LPC-mediated H_2O_2 production induced cellular damage. Hydrogen peroxide released during neutrophil oxidative burst has been shown to impact cellular membrane integrity and induce cytotoxicity (Jaganjac et al., 2016). Therefore, robust H_2O_2 responses that result in its extracellular release typically result in the death of the effector cell as well as surrounding cells (Gujral et al., 2004). As a result, enhanced bactericidal mechanisms can come at the expense of cell survival, as has been shown in *in vivo* models. It is therefore important to be cognizant of the tradeoff of inducing strong oxidative burst responses on cell viability and integrity. Binding of albumin to LPC in circulation has been demonstrated to mitigate the cytotoxic effects it would otherwise have on surrounding cells (Kim et al., 2007). We were able to confirm that albumin co-supplementation was able to maintain bovine neutrophil viability in the presence of a saturated LPC-mediated H_2O_2 production. This was likely due to a muted H_2O_2 response but could also be attributed to the antioxidant and scavenging abilities of albumin (Kouoh et al., 1999). Interestingly, H_2O_2 production increased when neutrophils were treated with albumin-bound LPC-18:1. This is analogous to the observations of Ojala et al. (22) where LPC-18:1 and 18:2

significantly increased superoxide production when administered in the absence of albumin; these effects were attenuated by the addition of albumin in a 1:2 molar ratio with LPC (Ojala et al., 2007). A large proportion (~ 80%) of circulating LPC is associated with proteins such as albumin and this binding has been found to inhibit the effects of LPC on biochemical pathways that control modulate endothelial and immune cell activation (Kabarowski et al., 2002; Law et al., 2019). It has been hypothesized that albumin-binding may make LPC inaccessible to degradation via extracellular enzymes into inflammatory effector molecules such as lysophosphatidic acid or block its incorporation into cell plasma membranes (Vuong et al., 2001; Kabarowski et al., 2002; Kim et al., 2007). Therefore, albumin could potentially serve as a regulatory element in LPC-mediated immunomodulation. Fetal bovine serum incorporation at a level of 5% of volume was not observed to have similar effects. Even though FBS does contain albumin, it is likely that the amount in the experimental environment was not enough to bind LPC to the extent that would mitigate its cytotoxic effects. Further optimization of FBS inclusion rate for the successful ablation of LPC's cytotoxic effects is needed.

Cytokine production is a key hallmark of an effective immune response. When neutrophils become activated, they secrete inflammatory cytokines TNFα and IL-6 into the extracellular environment to promote cellular proliferation, upregulate production of antimicrobial effector molecules (e.g., defensins), and recruit immune cells such as macrophages and neutrophils to the site of infection (Fielding et al., 2008; Kolls et al., 2008; Wright et al., 2010; Selders et al., 2017). These processes are coordinated to facilitate the successful clearance of an invading pathogen. Although cytokine production is a sign of an effective immune system, production of inflammatory cytokines in excess can potentially induce cellular and tissue damage by overactive immune cells (Yamamura et al., 1992). Therefore, a fine-tuned balance of both pro- and anti-inflammatory

cytokine levels must be maintained in order to ensure pathogen clearance without damage to the host. While LPC has been observed to have modulatory direct effects on cytokine production in neutrophils (Yan et al., 2004), LPC-18:0 or -18:1 was not able to modify TNFα and IL-6 secretion alone but was able to potentiate the ability of LPS to stimulate the secretion of TNFα. It is possible that LPC primed the bovine neutrophils, thus allowing for a more robust response to the LPS stimulus. Priming is a process neutrophils can undergo whenever they encounter certain types of stimuli in their environment that renders their bactericidal mechanisms, such as oxidative burst and cytokine production, to be more responsive (Miralda et al., 2017). While the mechanisms by which this phenomenon takes place are still being explored, it is believed that this process is mediated by increased membrane trafficking and cell surface receptor expression (i.e., selectins) (Miralda et al., 2017). If in fact LPC primed neutrophils, this effect could potentially be utilized to preemptively bolster the innate immune response of calves prior to the onset of an infection; however, this requires confirmation in vivo. Further research must also determine whether phenotypic markers of priming are modulated in bovine neutrophils in the presence of LPC and how these responses are related to pathogen clearance.

The *E. coli* strain K99 was studied because this strain is known for inducing scours in dairy calves (Shams et al., 2012). Infections with *E.* coli are especially dangerous due to the bacterial strain's high resistance and detrimental immunologic effects. Indeed, *E. coli* infections are a most prevalent cause of calf mortality (Acres, 1985). Clearance of this bacterium in the instance of infection is paramount to minimize tissue injury and sickness of the host (Kieckens et al., 2015). As the first line of defense in the immune response, neutrophils must effectively engage and neutralize infiltrating pathogens such as *E. coli* for the host to efficiently eliminate the infection. The effects of LPC on neutrophil-mediated *E. coli* killing were quantified using an *E. coli* killing

assay. Neutrophils co-cultured with *E. coli* showed significant reductions in viable bacterial CFUs following incubation. This response is expected because neutrophils utilize an arsenal of specialized bactericidal mechanisms to kill bacteria (Selders et al., 2017). Interestingly, when neutrophils were pre-treated with unbound LPC-18:0, the log reduction in *E. coli* CFUs was significantly reduced. Similar effects have been documented when murine and human neutrophils have been treated with LPC (Yan et al., 2004; Hong et al., 2010). These findings suggest that LPC-18:0 therapy could potentially be utilized to clear an *E. coli* infection in a susceptible host, such as the neonatal dairy calf. Perhaps LPC can be incorporated into early-life disease prevention programs and strategies (e.g., dietary milk replacers enriched in LPC) to prevent or reduce calfhood disease and mortality.

Our collective findings indicate that saturated LPC-18:0 act upon bovine neutrophils to enhance oxidative burst and inflammatory cytokine secretion in the presence of endotoxin, two mechanisms that could provoke neutrophil-mediated *E. coli* killing by saturated LPC. The orchestrated execution of these key neutrophilic processes has been documented to work in tandem to facilitate an efficacious clearance of an invading pathogen (Acuff et al., 2017). Engulfment of a bacterium by a neutrophil facilitates the assembly of the NADPH oxidase complex, thus promoting the production of ROS (Kobayashi et al., 2018). Reactive oxygen species may be released into the extracellular environment or incorporated intracellularly into a phagolysosome to neutralize the engulfed bacterium (Nguyen et al., 2017). Additionally, ROS can stimulate the production of proinflammatory cytokines such as TNFα (Nguyen et al., 2017). Increases in inflammatory cytokines can then enhance pathogen clearance, as evidenced by the doubled killing rate of phagocytosed *Mycobacterium tuberculosis* in human neutrophils treated with exogenous TNFα (Kisich et al., 2002). Therefore, an immunomodulatory intervention that enhances these

cellular processes, such as LPC administration, provides a potential therapeutic mode of action to mitigate or prevent bacterial infection in an immunodeficient host.

3.5. Conclusion

In conclusion, we have discovered the ability of LPC-16:0, -18:0, and -18:1 to amplify the oxidative burst, the ability of LPC-18:0 and -18:1 to potentiate the ability of endotoxin to stimulate TNFα and IL-6 secretion, and the ability of LPC-18:0 to enhance *E. coli* killing in neutrophils isolated from neonatal Holstein dairy calves. Our findings suggest a potentially therapeutic role of stearoyl LPC to enhance pathogen clearance and mitigate disease in young ruminants. Future research will need to determine whether changes in dietary, gastrointestinal, or endogenous LPC status and therapies impact bacterial disease progression in calves.

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Chapter 4: Lysophosphatidylcholine administration promotes a febrile and immune response in Holstein heifer calves

4.1. Introduction

The immune system of the dairy calf is classified as “naïve” or “deficient” during the first few weeks of life (Chase et al., 2008). This is due in part to the physiology of the ruminant placenta that prevents exchange of immunoglobulins between the dam and fetus in utero. As a consequence, dairy calves are agammaglobulinemic at birth and key effector mechanisms of their adaptive and innate immune cells are underdeveloped (Chase et al., 2008; Lombard et al., 2020). For example, neutrophils, “first responder” innate immune cells that traffic to an infiltrating pathogen and neutralize it using bactericidal mechanisms (i.e., the oxidative burst and phagocytosis) have reduced phagocytic capabilities in neonatal dairy claves. This is likely due to reduced expression of key receptors necessary for successful recognition and phagocytosis of bacteria, such as the fragment crystallizable (Fc) receptors, in comparison to neutrophils isolated from older animals (Zwahlen et al., 1992; Barrington and Parish, 2001; Chase et al., 2008). They also have reduced antimicrobial peptide (i.e., myeloperoxidase) production (Zwahlen et al., 1992), and impaired adaptive immune function (i.e., reduced lymphocyte population, lack of memory B and T cells, and lower antibody production) (Chase et al., 2008).

The calf is reliant on passive immunity acquired from consuming colostral secretions from the dam, which include immunoglobulins and cytokines. The industry standard to bolster calf immune protection is to feed quality colostrum shortly after birth. However, failure of passive transfer (FPT), or insufficient concentrations of circulating colostrum-derived antibodies, is a prevalent issue in regard to colostrum management. Failure of passive transfer can occur as often as in 20% of calves born on dairy operations and the ramifications of this can be perceived well

into adulthood (e.g., reduced milk production) (Raboisson et al., 2016). Due to the high prevalence of FPT, it is imperative that alternative interventions are implemented that bolster calf immunity. Although antibiotics are one tool for disease prevention, their use can potentially result in the emergence of antibiotic resistant strains of bacteria and are becoming progressively discouraged by veterinarians and consumers alike (Langford et al., 2003; Berge et al., 2005; Walker et al., 2012). Therefore, the development of efficacious, non-antibiotic therapies that effectively boost immune function and mitigate disease in calves is essential.

Lysophosphatidylcholine (LPC) has been scrutinized as a potential immunomodulator and corollary for disease outcomes in non-ruminants (Hong and Song, 2008). The lysophospholipid LPC is derived from the enzymatic activity of phospholipase A_2 (PLA_2) on phosphatidylcholine (PC) in circulation or by the transfer of fatty acids to cholesterol via lecithin-cholesterol acyltransferase (LCAT) (Law et al., 2019). Circulating concentrations of LPC are significantly lower in patients with sepsis as compared to healthy individuals, and specific types of LPC have been suggested to be potential markers for disease severity and mortality in septic patients (Drobnik et al., 2003; Park et al., 2014). In a murine model of sepsis, mice that received stearoyl-LPC (i.e., LPC-18:0) subcutaneously, either before or after cecal ligation puncture, had a dose-dependent increased rate of survivorship than mice that received only vehicle (Yan et al., 2004). Additionally, supplementing media with LPC was found to increase the oxidative burst response of murine neutrophils in a dose-dependent manner (Yan et al., 2004). Moreover, phagocytic activity is enhanced following stearoyl-LPC treatment in murine neutrophils (Quan et al., 2016), and LPC enhances antibody production and interferon-γ secretion from isolated human peripheral blood mononuclear cells (Huang et al., 1999). These findings highlight the extensive immunomodulatory capabilities of LPC at both the systemic and cellular level.

Although the effects of LPC have not been previously explored in the neonatal dairy calf, given their high susceptibility to disease due to immunodeficiency, the calf has much to gain from the potential observed protective immunomodulatory benefits of LPC. Therefore, the objective of this study was to determine the effects of LPC on parameters of growth as well as markers of inflammation and immunity in pre-weaned dairy calves. To test our objective, we carried out a longitudinal study utilizing forty-six heifer Holstein calves to test the effects of either s.c. or dietary lysolecithin enriched in LPC on measures of growth, and immune and liver and health. We hypothesized that LPC administration would enhance measures of calf health and, in turn, promote calf growth.

4.2. Materials and Methods

4.2.a. Experimental design and treatments

All experimental procedures were approved by the Cornell University Institutional Animal Care and Use Committee (protocol #2018-0110). Forty-six heifer calves (4.2 ± 0.8 d of age [mean ± SD] and 38 ± 3.91 kg of BW) were enrolled in a completely randomized design to study the effects of LPC. Calves were housed in individual pens bedded with sawdust in the Cornell University Block Barn (Ithaca, NY). Following 7 d of acclimation, calves were assigned to one of four treatment groups: a milk replacer diet unsupplemented with lecithin in the absence (CON; n = 11) or presence of s.c. administered mixed (mLPC; 69% LPC-16:0, 25% LPC-18:0, 6% other; n = 11) or pure (pLPC; 99% LPC-18:0; n = 11) LPC, or a milk replacer diet supplemented with 3% lecithin enriched in lysophospholipids containing LPC in the absence of s.c. administered LPC (LYSO; n = 10) for a 5-wk experimental period. The LPC content of the enriched milk replacer was approximately 6%, meaning that for every kg of milk replacer consumed, approximately 60 g

of LPC is ingested. Exclusion criteria for calf enrollment included adequate hydration as assessed by hematocrit, a serum Brix value ≥ 8.3 (Deelen et al., 2014), and visual confirmation of sound clinical health by assessing mobility, fecal score, and respiration rate. **Figure 4.1** illustrates the treatment and sampling timeline.

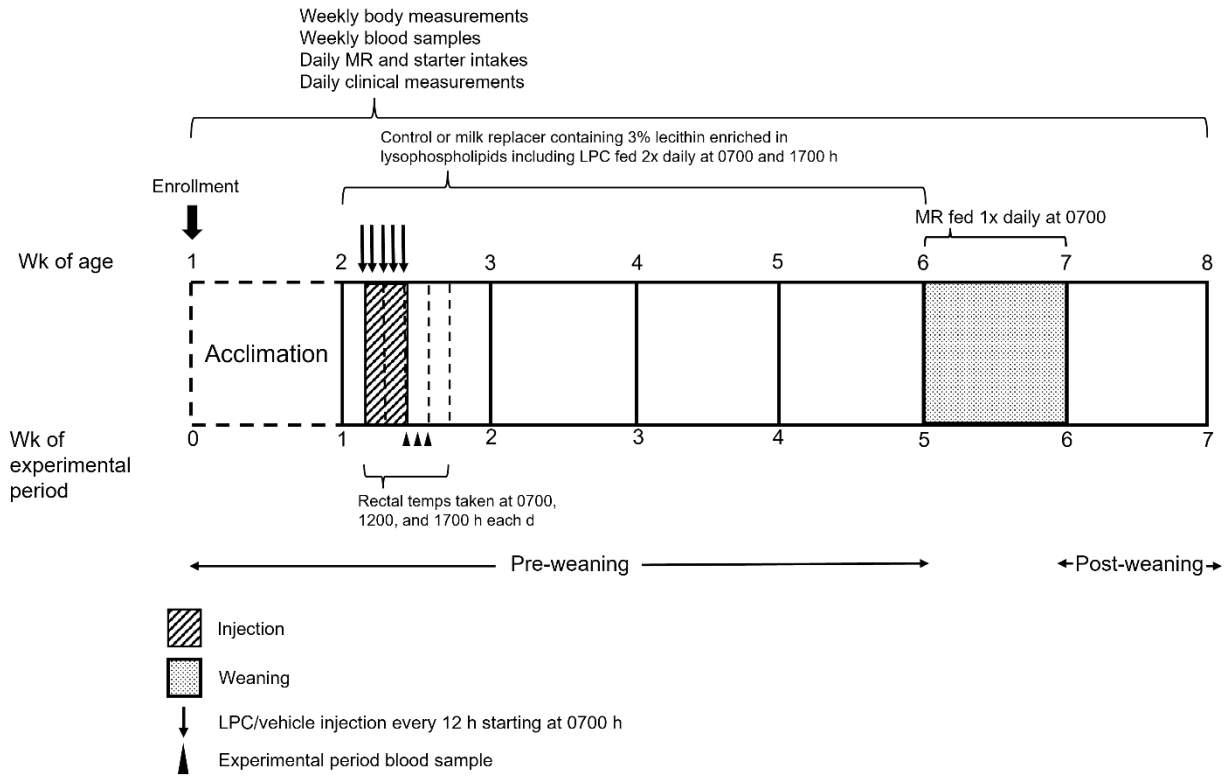

Figure 4.1. Experimental timeline. Following 7 d of acclimation, forty-three clinically healthy Holstein heifer calves (age 7 ± 3 d) were randomly assigned to 1 of 4 treatments: a milk replacer diet unsupplemented with lecithin in the absence (CON; n=11) or presence of s.c. administered mixed (mLPC; 69% LPC-16:0, 25% LPC-18:0, 6% other; n = 11) or pure (pLPC; 99% LPC-18:0; n = 11) LPC, or a milk replacer diet supplemented with 3% lecithin enriched in lysophospholipids containing LPC in the absence of s.c. administered LPC (LYSO; n = 10) for a 5-wk experimental period. Calves were fed twice daily (0700 and 1400 h) a milk replacer containing 27% crude protein, 24% fat at 1.75% of BW per d (dry matter basis) until wk 6 of life (start of weaning). At wk 6 of life, milk replacer intake was reduced by half and terminated by wk 7. Starter (22% CP) and water were provided ad libitum throughout study. Body measurements were recorded weekly and intakes and clinical observations were recorded daily. Blood samples were collected weekly as well as 0, 5, and 10 h following the final injection.

During acclimation, calves were fed a common milk replacer (24% CP and 17% fat) at 1.75% of BW on a DM basis twice daily at 0700 and 1600 h. At the start of the experimental period, the provision of milk replacer unsupplemented or supplemented with lecithin was initiated (**Table 4.1**).

Item	Unsupplemented MR	Lysolecithin-enriched MR	Starter
DM, %	96.6 ± 0.11	94.9 ± 1.3	84.8 ± 1.1
CP	28.6 ± 0.26	27.3 ± 0.36	26.0 ± 0.85
Soluble protein	27.6 ± 0.15	26.2 ± 0.68	6.37 ± 0.25
NDF	0.60 ± 0.26	0.467 ± 0.06	15.2 ± 0.85
ADF	51.6 ± 0.10	46.7 ± 0.10	6.07 ± 0.12
TDN	93.2 ± 0.21	99.1 ± 0.57	79.1 ± 0.44
Ash	8.87 ± 0.34	8.76 ± 0.67	7.74 ± 0.56
Crude fat (EE)	10.1 ± 0.44	14.7 ± 0.16	3.62 ± 0.09
Lignin	0.037 ± 0.01	0.03 ± 0.00	1.48 ± 0.05
ME (Mcal/kg of DM)	1.68 ± 0.01	1.8 ± 0.02	1.39 ± 0.01
NE_M (Mcal/kg of DM)	1.17 ± 0.01	1.27 ± 0.01	0.95 ± 0.01
NE_G (Mcal/kg of DM)	0.833 ± 0.01	0.91 ± 0.01	0.643 ± 0.01

Table 4.1. Nutrient composition (% of DM unless otherwise noted) of experimental milk replacer and starter (mean ± SD) fed to calves[1].

[1]During the experimental period, calves were fed twice daily (0700 h and 1700 h) either an unsupplemented control milk replacer or a milk replacer containing 3% lysolecithin enriched in lysophospholipids including LPC at 1.75% of BW per d on a dry matter basis until wk 6 of life (start of weaning). At wk 6 of life, milk replacer intake was reduced by half and terminated by wk 7. Starter and water were provided ad libitum throughout study. CP = crude protein, NDF = neutral detergent fiber, ADF = acid detergent fiber, TDN = total digestible nutrients, EE = ether extract.

During wk 2 of the experimental period, calves received 5 s.c. injections of either vehicle (PBS with 20 mg of BSA/mL; CON and LYSO) or LPC in vehicle (10 mg/kg of BW; mLPC and pLPC) every 12 h over the course of 48 h beginning at 0700 h on d 1 and terminating at 0700 h on d 3 of the experimental period. In order to facilitate weaning, milk replacer intake was reduced by

half beginning wk 6 and terminated by wk 7 of the experimental period. Starter grain and water were provided *ad libitum* for the duration of the study.

4.2.b. Treatment preparation and administration

L-α-lysophosphatidylcholine derived from egg yolk (cat. #: 830071; 69% LPC-16:0, 25% LPC-18:0, 6% other) and stearoyl-LPC (>99% LPC-18:0; cat. #: 855775) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL) and Cayman Chemical Company (Ann Arbor, MI), respectively. Treatments were prepared by dissolving the necessary amount of LPC (10 mg/kg of BW/injection), and bovine serum albumin (BSA; 200 mg/injection) for 5 injections (plus 5% extra) into 52.5 mL of PBS. Treatments were sonicated for 3, 5-sec intervals then heated on a hot plate with intermittent gentle swirling until clear. Control injections containing vehicle were prepared in a similar manner with the omission of LPC. Single-dose treatments were then aliquoted into sterile 50-mL conical tubes and frozen at -20 ℃ until use. Immediately prior to administration, injections were prepared by thawing aliquots in a heated water bath until clear and drawn into 12-mL syringes using 18-gauge needles. Injections were administered s.c. near the scapula.

4.2.c. Sample and data collection

Milk replacer and starter grain intakes were recorded daily. Samples of each milk replacer, in addition to starter grain, were collected weekly, composited by month, and stored at −20°C. Clinical assessments were performed daily at morning feedings (0700 h). During these assessments, rectal temperatures, respiration rates, and fecal scores were recorded for the duration of the study. In addition, rectal temperatures were recorded at h 0, 5, and 10, relative to each injection of either vehicle or LPC beginning at the first s.c. injection during wk 2 of the experimental period. Daily respiration rates were determined by counting flank movements for a

15-s duration, then multiplied by 4 to obtain movements per minute. Body weights were recorded weekly.

Blood samples (10 mL) were collected once weekly before morning feeding and at h 0, 5, and 10, relative to the final injection of vehicle or LPC during the experimental period (**Figure 1**). Blood was collected via jugular venipuncture into evacuated blood tubes containing potassium ethylenediaminetetraacetic acid (EDTA) as an anticoagulant when blood was collected for plasma or whole blood collection. Plasma and serum samples were separated using centrifugation (3,400 × *g* for 20 min). Separated plasma or serum was samples were initially stored at -20°C and then transferred to -80°C for long-term storage within 2 wk of collection.

4.2.d. Sample analyses

Starter grain was ground through a 1-mm sieve in a Wiley mill (Thomas-Wiley, Swedesboro, NJ) and, along with milk replacer samples, were submitted to a commercial laboratory for determination of nutrient composition by near-infrared spectroscopy (Cumberland Valley, Cumberland, MD; AOAC International, 1995; method 989.03). Plasma tumor necrosis factor α (TNFα) was quantified using a validated enzyme-linked immunoassay (ELISA) approach (Farney et al., 2011). Plasma glucose and total fatty acid concentrations (#997-03001 Autokit Glucose and #999-34691, #995-34791, #991-34891, and #993-35191 HR Series NEFA-HR (2), respectively; Wako Chemicals, Inc., Richmond, VA), and serum amyloid A (SAA) concentrations (#TP-802; Tridelta Development Limited Ireland Co., Maynooth, Kildare) were quantified according to manufacturer's instructions. All spectrophotometric measurements were conducted using a SpectraMax Plus 384 Microplate Reader (Molecular Devices, Sunnyvale, CA). Serum insulin concentrations were determined using radioimmunoassay (#PI-12K Porcine Insulin RIA Kit; EMD Millipore Corporation, St. Charles, MI) on an LKB-Wallac CliniGamma Counter

(Beckman Coulter, Fullerton, CA) as described previously (Krumm et al., 2019). Inter- and intra-assay CVs were 7.96% and 3.37%, 11.77% and 5.19%, 5.97% and 5.36%, and 9.17% and 1.55% for glucose, total fatty acid, SAA, and insulin, respectively.

Serum samples were submitted to the University of Missouri Veterinary Medical Diagnostic Laboratory for quantification of liver health markers (i.e., albumin, globulin, total protein, total bilirubin, direct bilirubin, aspartate aminotransferase (AST), glutamate dehydrogenase (GLDH), gamma-glutamyl transferase (GGT)) as well as cortisol. Plasma samples were submitted to the Johns Hopkins University School of Medicine for quantification of LPC using liquid chromatography and tandem mass spectrometry (LC-MS/MS). Whole blood samples collected into potassium EDTA tubes were immediately transported on ice to the Cornell University Animal Health Diagnostic Center (Ithaca, NY) for white blood cell count and profile analyses via automated hemogram.

4.2.e. Calculations and statistical analyses

Average daily gains (ADG) were calculated by dividing the differences in weight between each week by 7 d. Feed to gain ratios (F:G) were computed by dividing ADG by overall DMI within week. Statistical analyses were carried out using the mixed model procedure of SAS (v9.4, SAS Institute Inc., Cary, NC, USA). The model included the random effects of calf, block, and baseline measurements, and the fixed effects of treatment, time (i.e., h, d, wk), and their interaction. The pre-weaning period is defined as wk 1 to wk 5 of the experimental period. The post-weaning period is defined as wk 6 to wk 7 of the study. The SLICE effect was utilized to analyze treatment differences by time. The covariance structures used to test fit statistics included variance components, compound symmetry, autoregressive one, and unstructured. Smaller fit values (BIC) were always selected. The model was used to evaluate body measurements, clinical parameters,

and blood metabolites. Normality of the residuals was checked with normal probability and box plots and homogeneity of variances with plots of residuals versus predicted values in order ensure no violation of model assumptions. Studentized residual values > 3.0 or < −3.0 were considered outliers and removed from the analysis (typically 1 per response variable). A Dunnett's multiple comparisons test was performed between treatments (i.e., mLPC, pLPC, and LYSO) and reference control (i.e., CON). Main effects and interactions were declared significant at $P \leq 0.05$ and trending towards significance at $0.05 > P \leq 0.10$. Results are expressed as least squares means ± SEM, unless otherwise noted.

4.3. Results

Three calves developed serious complications during the study to warrant veterinary intervention during wk 3 of the experimental period. One LYSO calf and one mLPC calf developed pneumonia. One mLPC calf developed vasculitis and skin lesions at the site of injection. These calves received veterinary treatment (i.e., flunixin meglumine); however, they were euthanized by captive bolt within 1 wk of initial diagnosis. For these calves, all data collected were utilized in our statistical analyses.

4.3.a. Effects of LPC administration on acute changes in plasma LPC concentrations

Changes in plasma concentrations of LPC, relative to the final injection of either vehicle or LPC, are shown in **Table 4.2**.

Table 4.2. Changes in plasma lysophosphatidylcholine concentrations relative to the final injection of s.c. vehicle or LPC in pre-weaned Holstein heifer calves.

		Treatment[1]					*P*-value		
LPC[2]	Hour[3]	CON	mLPC	pLPC	LYSO	SEM	Treatment	Hour	Treatment × Hour
16:0	0	39.4	40.1	40.0	36.2	1.98	<0.001	<0.001	<0.001
	5	35.6	54.5***	38.5	47.0***	2.07			
	10	39.0	53.1***	38.6	48.6**	1.99			
16:1	0	7.77	7.32	7.86	7.15	0.739	0.712	0.018	0.688
	5	7.71	8.23	9.03	8.36	0.774			
	10	9.72	8.21	8.73	9.83	0.741			
18:0	0	25.1	24.3	25.5	25.4	1.45	<0.001	<0.001	<0.001
	5	23.3	32.9***	24.4	36.7***	1.51			
	10	25.5	31.3*	25.3	37.7***	1.45			
18:1	0	30.5	26.3	27.2	31.1	1.73	<0.001	<0.001	<0.001
	5	26.7	37.5***	29.7	43.8***	1.81			
	10	29.2	33.9	29.1	38.9***	1.74			
18:2	0	10.4	10.7	10.8	10.6	0.727	0.753	0.395	0.821
	5	9.94	10.1	10.0	9.67	0.760			
	10	9.28	10.3	10.3	11.3	0.730			
18:3	0	7.57	9.87	9.31	7.79	0.598	0.194	0.001	0.372
	5	7.44	7.23	7.15	6.80	0.625			
	10	7.00	7.33	7.61	7.23	0.600			
20:0	0	2.47	2.68	2.48	2.58	0.231	0.857	<0.001	0.955
	5	1.94	1.95	1.65	1.76	0.241			
	10	2.01	1.86	1.86	2.05	0.231			
20:3	0	4.38	5.11	4.84	4.09	0.349	0.668	0.003	0.066
	5	4.22	3.51	3.57	3.63	0.365			
	10	4.58	3.68	3.74	4.48	0.351			
20:4	0	4.41	5.40	5.14	4.49	0.382	0.742	<0.001	0.265
	5	4.22	3.79	3.54	3.88	0.399			
	10	3.09	3.32	3.78	3.69	0.384			
20:5	0	5.82	6.61	6.53	5.79	0.508	0.882	0.052	0.846
	5	5.42	5.41	5.32	5.35	0.529			
	10	5.67	5.59	5.80	6.07	0.510			
22:1	0	1.25	1.47	1.36	1.20	0.113	0.462	<0.001	0.490
	5	1.04	1.17	0.956	0.850	0.119			
	10	0.988	0.968	1.01	1.10	0.114			
22:5	0	3.84	5.15	4.69	4.06	0.486	0.338	0.205	0.649
	5	3.90	4.19	3.70	3.87	0.502			
	10	4.02	4.23	4.12	4.42	0.488			
22:6	0	4.88	6.04	5.83	5.10	0.528	0.159	0.019	0.768
	5	4.47	4.47	4.63	4.85	0.546			
	10	4.36	4.95	5.16	4.78	0.530			
24:1	0	0.985	1.13	1.13	0.943	0.080	0.377	<0.001	0.609
	5	0.797	0.857	0.785	0.654	0.083			
	10	0.750	0.710	0.759	0.777	0.080			
24:5	0	2.18	2.47	2.36	2.25	0.182	0.638	<0.001	0.259
	5	1.71	1.84	1.57	1.39	0.190			
	10	2.12	1.81	1.65	1.92	0.183			
26:1	0	1.00	0.956	1.05	1.00	0.090	0.731	<0.001	0.712
	5	0.723	0.723	0.719	0.595	0.093			
	10	0.976	0.830	0.774	0.819	0.090			
Total	0	152	156	156	149	5.35	<0.001	0.021	<0.001
	5	139	178***	145	179***	5.35			
	10	148	172**	148	184***	5.35			

[1]Calves were randomly assigned to 1 of 4 treatments: a milk replacer diet unsupplemented with lecithin in the absence (CON; n=11) or presence of s.c. administered mixed (mLPC; 69% LPC-16:0, 25% LPC-18:0, 6% other; n = 11) or pure (pLPC; 99% LPC-18:0; n = 11) LPC, or a milk replacer diet supplemented with 3% lecithin enriched in lysophospholipids containing LPC in the absence of s.c. administered LPC (LYSO; n = 10) for a 5-wk experimental period. Data are presented as LS Means ± SEM. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$, relative to CON.

[2]Values represent normalized intensities i.e. the ratio of the analyte peak area under the curve (AUC) to the corresponding internal standard AUC in each sample run.

[3]Blood was collected immediately prior (0 h) as well as 5 h and 10 h following administration of the final of 5 serial s.c. injection of either LPC or vehicle.

For plasma total LPC concentrations, we observed a significant effect of treatment, hour, and treatment × hour ($P < 0.05$). Specifically, plasma total LPC concentrations were greater for calves administered mLPC and LYSO at h 5 and 10, relative to CON ($P < 0.05$). Similar observations were observed for plasma LPC-16:0, -18:0, and -18:1. Significant time effects were observed for LPC-18:3, -20:0, -20:3, -20:4, -22:1, -22:6, -24:1, -24:5, and -26:1 with their concentrations greatest at 0 h and progressively declining until h 10 ($P < 0.05$). We did not observe an effect of treatment, hour, or treatment × hour for LPC-18:3. -20:5, and -22:5.

4.3.b. Effects of LPC administration on rectal temperatures, respiration rates, and fecal scores

Changes in daily and hourly rectal temperatures, respiration rates, and fecal scores in response to LPC administration are shown in **Table 4.3.** and **Figure 4.2**.

Table 4.3. Changes in clinical measurements in pre-weaned Holstein heifer calves receiving injections of s.c. vehicle or LPC.

		Treatment[1]					*P*-value		
	Period[2]	CON	mLPC	pLPC	LYSO	SEM	Treatment	Week	Treatment × Week
Rectal temperature, °C	Pre-weaning	38.7	38.9	38.7	38.8	0.051	0.100	<0.001	0.005
	Post-weaning	38.8	38.9	38.7	38.8	0.112	0.747	<0.001	0.835
	Overall	38.7	38.9	38.7	38.8	0.047	0.076	<0.001	0.200
Respiration rate, bpm	Pre-weaning	40.1	41.5	40.0	41.0	1.86	0.913	<0.001	0.915
	Post-weaning	38.2	37.3	37.9	38.4	2.18	0.935	<0.001	0.978
	Overall	39.7	39.9	39.5	40.3	1.30	0.975	<0.001	0.996
Fecal score	Pre-weaning	1.21	1.24	1.32	1.26	0.184	0.894	<0.001	0.922
	Post-weaning	1.34	1.53	1.28	1.24	0.199	0.187	0.015	0.248
	Overall	1.22	1.30	1.28	1.26	0.145	0.497	<0.001	0.744

[1] Calves were randomly assigned to 1 of 4 treatments: a milk replacer diet unsupplemented with lecithin in the absence (CON; n=11) or presence of s.c. administered mixed (mLPC; 69% LPC-16:0, 25% LPC-18:0, 6% other; n = 11) or pure (pLPC; 99% LPC-18:0; n = 11) LPC, or a milk replacer diet supplemented with 3% lecithin enriched in lysophospholipids containing LPC in the absence of s.c. administered LPC (LYSO; n = 10) for a 5-wk experimental period. Clinical measurements were recorded daily at each morning feeding. Data are presented as LS Means ± maximum SEM. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$, relative to CON.

[2]The pre-weaning period is defined as wk 1 to wk 5 of the experimental period. The post-weaning period is defined as wk 6 to wk 7 of the study.

For acute changes in rectal temperatures, we observed a significant effect of treatment, treatment × day, and treatment × hour (P <0.05, **Figure 4.2A & 4.2B**).

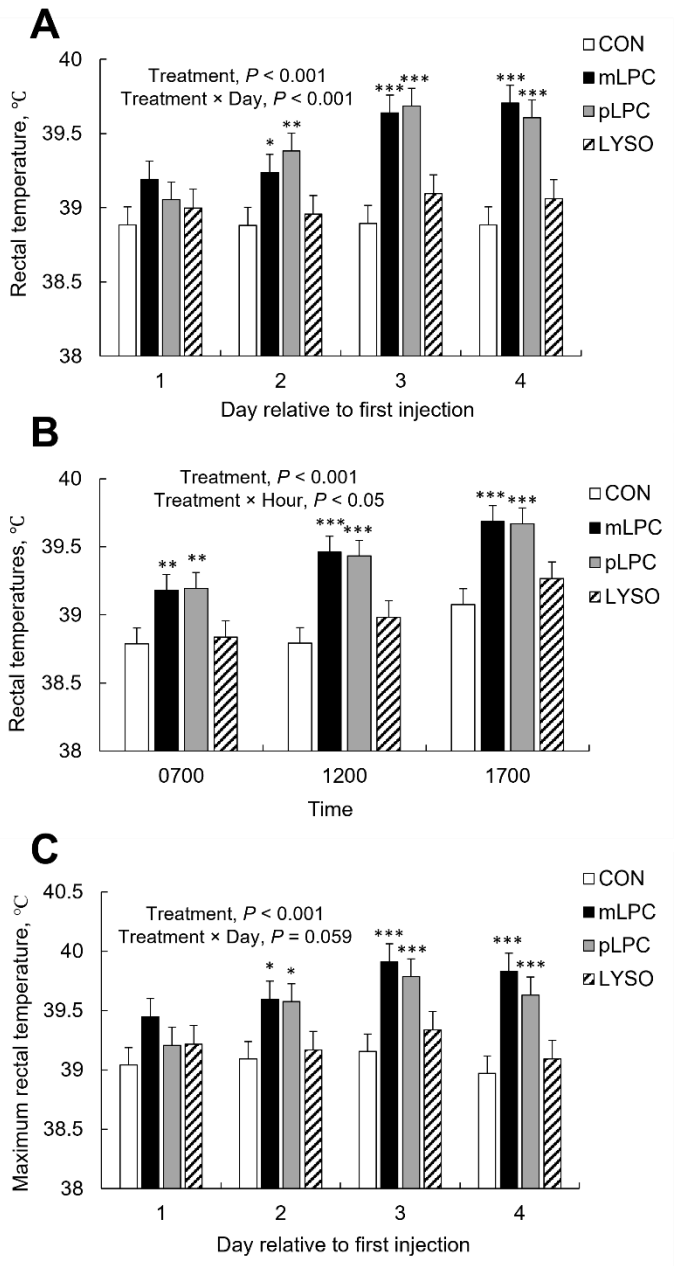

Figure 4.2. Effects of s.c. vehicle (CON & LYSO) or LPC (mLPC & pLPC) on mean (A & B) and maximum rectal temperatures taken over the course of the 3 d experimental period as well as 24 h post-final injection. Calves were randomly assigned to 1 of 4 treatments: a milk replacer diet unsupplemented with lecithin in the absence (CON; n=11) or presence of s.c. administered mixed (mLPC; 69% LPC-16:0, 25% LPC-18:0, 6% other; n = 11) or pure (pLPC; 99% LPC-18:0; n = 11) LPC, or a milk replacer diet supplemented with 3% lecithin enriched in lysophospholipids containing LPC in the absence of s.c. administered LPC (LYSO; n = 10) for a 5-wk experimental period. Rectal temperatures were taken immediately before (0700 h) and 5 h after (1200 h) the first injection and immediately before the second injection (1700 h) of LPC or

vehicle every day during the experimental period (d 1 to 3) and 24 h after the final injection (d 4 of wk 2). Data are presented as LSM ± maximum SEM. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$, relative to CON.

Calves receiving mLPC or pLPC treatment experienced marked increases in mean rectal temperatures beginning d 2 of injection, relative to CON ($P < 0.05$). The rectal temperatures of mLPC and pLPC calves remained elevated until d 4 following following the initial injection, relative to CON ($P < 0.001$). For chronic rectal temperatures, we observed a significant effect of week and treatment × week during the pre-weaning period ($P < 0.01$). Rectal temperatures were greater for mLPC and pLPC calves at h 5 and 10, relative to CON ($P < 0.001$). Calves provided the LYSO treatment did not experience a change in acute or chronic rectal temperatures, relative to CON. We also did not observe an effect of treatment for respiration rates or fecal scores.

4.3.c. Effects of LPC administration on dry matter intake and growth

Changes in DMI and growth parameters in response to LPC administration are provided in **Table 4.4.**

Table 4.4. Changes in plasma body measurements and dry matter intake in pre-weaned Holstein heifer calves receiving injections of s.c. vehicle or LPC

		Treatment[1]					*P*-value		
	Period[2]	CON	mLPC	pLPC	LYSO	SEM	Treatment	Week	Treatment × Week
DMI, kg/d	Pre-weaning	0.913	0.799	0.888	0.891	0.04	0.058	<0.001	0.143
	Post-weaning	1.47	1.17	1.51	1.41	0.10	0.058	<0.001	0.902
	Overall	1.07	0.884*	1.07	1.04	0.06	0.021	<0.001	0.277
Body weight, kg	Pre-weaning	47.2	46.5	47.0	47.1	0.42	0.372	<0.001	0.723
	Post-weaning	68.2	63.1*	67.0	67.9	1.75	0.031	<0.001	0.005
	Overall	55.1	52.8*	54.5	54.9	0.04	0.030	<0.001	0.705
ADG, kg/d	Pre-weaning	0.674	0.616	0.634	0.671	0.07	0.189	0.001	0.921
	Post-weaning	0.927	0.554***	0.978	0.92	0.07	0.012	<0.001	0.463
	Overall	0.747	0.605***	0.730	0.741	0.04	0.010	<0.001	0.266
Heart girth, cm	Pre-weaning	82.3	82.5	81.7	82.2	0.56	0.723	<0.001	0.506
	Post-weaning	93.4	91.2	92.5	93.1	0.92	0.071	<0.001	0.013
	Overall	86.5	85.0	85.8	86.3	0.62	0.752	<0.001	0.013
Mid girth, cm	Pre-weaning	83.7	82.9	82.7	82.5	0.62	0.250	<0.001	0.982
	Post-weaning	99.7	96.3	97.8	98.4	1.39	0.230	<0.001	0.169
	Overall	89.7	87.9	88.4	88.5	0.64	0.174	<0.001	0.452
Flank girth, cm	Pre-weaning	79.9	79.3	78.0	79.6	0.64	0.080	<0.001	0.653
	Post-weaning	93.6	89.6	91.6	92.9	1.49	0.070	<0.001	0.533
	Overall	85.0	83.2	83.1	84.6	0.71	0.070	<0.001	0.297
Body length, cm	Pre-weaning	66.7	67.0	66.1	66.9	0.72	0.544	<0.001	0.748
	Post-weaning	76.6	75.4	76.4	76.7	0.69	0.491	<0.001	0.497
	Overall	70.4	70.3	69.9	70.5	0.54	0.772	<0.001	0.872
Hip height, cm	Pre-weaning	85.7	85.7	85.5	86.0	0.73	0.963	<0.001	0.499
	Post-weaning	92.6	91.5	92.0	92.6	1.02	0.654	<0.001	0.518
	Overall	88.3	88.0	87.9	88.5	0.73	0.933	<0.001	0.705
Hip width, cm	Pre-weaning	22.9	23.0	22.9	23.1	0.24	0.702	<0.001	0.35
	Post-weaning	26.2	25.4	26.3	26.1	0.43	0.051	<0.001	0.774
	Overall	24.2	23.9	24.2	24.2	0.26	0.392	<0.001	0.065
Feed:Gain	Pre-weaning	1.48	1.89	1.56	1.59	0.21	0.391	<0.001	0.658
	Post-weaning	1.84	1.18	1.62	1.66	0.42	0.621	0.077	0.684
	Overall	1.59	1.89	1.58	1.61	0.20	0.584	<0.001	0.913

[1] Calves were randomly assigned to 1 of 4 treatments: a milk replacer diet unsupplemented with lecithin in the absence (CON; n=11) or presence of s.c. administered mixed (mLPC; 69% LPC-16:0, 25% LPC-18:0, 6% other; n = 11) or pure (pLPC; 99% LPC-18:0; n = 11) LPC, or a milk replacer diet supplemented with 3% lecithin enriched in lysophospholipids containing LPC in the absence of s.c. administered LPC (LYSO; n = 10) for a 5-wk experimental period. Body measurements were recorded weekly, and intakes were recorded daily. Data are presented as LS Means ± SEM. * = P <0.05; ** = P < 0.01; *** = P < 0.001, relative to CON.

[2]The pre-weaning period is defined as wk 1 to wk 5 of the study. The post-weaning period is defined as wk 6 to wk 7 of the study.

During the pre-weaning period (wk 1 to 5), no significant differences were detected among treatment groups for DMI, BW, ADG, heart girth, mid girth, flank girth, hip height, hip width, body length, or feed:gain. During the post-weaning period (wk 6 to 7), mLPC calves had significantly lower BW ($P < 0.05$) and ADG ($P < 0.001$), relative to CON calves. We also observed a significant treatment × week effect for post-weaning and overall heart girth. Specifically, mLPC calves had significantly lower heart girth measurements on wk 8, relative to CON calves ($P < 0.05$). Additionally, mLPC calves had significantly lower overall DMI, relative to CON calves ($P < 0.05$). No significant differences in pre-weaning, post-weaning, or overall BW, ADG, heart girth, mid girth, flank girth, hip height, hip width, body length, DMI, and feed:gain were detected for pLPC and LYSO calves, relative to CON calves.

4.3.d. Effects of LPC administration on plasma glucose, total fatty acid, and insulin concentrations

Changes in plasma glucose, total fatty acids, and insulin concentrations in response to LPC administration are provided in **Table 4.5**.

Table 4.5. Changes in plasma glucose, total fatty acid, and insulin in pre-weaned Holstein heifer calves receiving injections of s.c. vehicle or LPC

		Treatment[1]					*P*-value		
	Period[2]	CON	mLPC	pLPC	LYSO	SEM	Treatment	Week	Treatment × Week
Glucose, mg/dL	Pre-weaning	110	109	115	111	5.40	0.407	0.578	0.124
	Post-weaning	106	95.9	107	105	4.84	0.084	<0.001	0.194
	Overall	108	104	112	109	4.86	0.162	<0.001	0.081
Total fatty acids, µmol/L	Pre-weaning	367	304	349	287	16.7	0.525	0.014	0.303
	Post-weaning	444	367	245	291	18.3	0.716	0.026	0.644
	Overall	394	313	314	280	19.7	0.302	0.011	0.289
Insulin, ng/mL	Pre-weaning	0.276	0.354	0.316	0.289	0.06	0.283	0.013	0.959
	Post-weaning	0.396	0.464	0.488	0.440	0.11	0.181	0.064	0.534
	Overall	0.321	0.404	0.382	0.349	0.08	0.428	<0.001	0.938

[1]Calves were randomly assigned to 1 of 4 treatments: a milk replacer diet unsupplemented with lecithin in the absence (CON; n=11) or presence of s.c. administered mixed (mLPC; 69% LPC-16:0, 25% LPC-18:0, 6% other; n = 11) or pure (pLPC; 99% LPC-18:0; n = 11) LPC, or a milk replacer diet supplemented with 3% lecithin enriched in lysophospholipids containing LPC in the absence of s.c. administered LPC (LYSO; n = 10) for a 5-wk experimental period. Pre-prandial blood samples were collected weekly. Data are presented as LS Means ± SEM.

[2]The pre-weaning period is defined as wk 1 to wk 5 of the experimental period. The post-weaning period is defined as wk 6 to wk 7 of the experimental period.

Treatment did not modify plasma glucose, total fatty acid, or insulin concentrations. There was a tendency for mLPC calves to have lower plasma glucose concentrations during the post-weaning period, relative to CON calves ($P = 0.084$).

3.3.e. Effects of LPC administration on circulating markers of immune and liver health

Changes in circulating concentrations of immune and liver health markers in response to LPC administration are provided in **Table 4.6**.

Table 4.6. Changes in concentrations of serum markers of liver health relative to the final injection of s.c. vehicle or LPC in pre-weaned Holstein heifer calves.

		Treatment[1]					*P*-value		
	Hour[2]	CON	mLPC	pLPC	LYSO	SEM	Treatment	Hour	Treatment × Hour
Albumin, g/dL	0	2.95	2.71***	2.73***	2.94	0.049	<0.001	<0.001	0.007
	10	2.93	2.57***	2.56***	2.88	0.049			
Total protein, g/dL	0	6.24	5.77***	5.97*	6.14	0.084	<0.001	<0.001	0.007
	10	6.18	5.46***	5.60***	6.01	0.084			
Globulin, g/dL	0	3.31	3.09	3.25	3.21	0.098	0.063	<0.001	0.069
	10	3.25	2.90	3.03	3.13	0.098			
Total bilirubin, mg/dL	0	0.346	0.442	0.383	0.393	0.056	0.183	0.009	0.767
	10	0.255	0.406	0.283	0.343	0.056			
Direct bilirubin, mg/dL	0	0.081	0.120	0.080	0.111	0.015	0.061	0.350	0.778
	10	0.081	0.129	0.098	0.111	0.015			
Aspartate transaminase, U/L	0	30.7	51.9***	41.1*	34.9	2.83	<0.001	0.002	0.250
	10	31.0	54.4***	43.9**	35.8	2.83			
γ-glutamyl transferase, U/L	0	152	163	110	148	21.2	0.304	<0.001	0.044
	10	141	140	94.1	136	21.2			
Glutamate dehydrogenase, U/L	0	13.1	20.8	14.4	13.3	2.75	0.075	0.082	0.516
	10	11.8	19.2	13.2	13.6	2.75			
Cortisol, µg/dL	0	0.759	1.64	1.39	0.952	0.322	0.075	<0.001	0.631
	10	0.450	0.743	0.643	0.392	0.322			
TNFα, pg/mL	0	21.6	21.4	22.5	16.5	5.36	0.500	0.812	0.942
	10	23.2	24.5	20.8	16.3	5.36			
IgG, mg/dL	0	2038	2111	2056	1946	99.9	0.685	0.836	0.342
	10	2179	1985	1942	2105	99.9			

[1]Calves were randomly assigned to 1 of 4 treatments: a milk replacer diet unsupplemented with lecithin in the absence (CON; n=11) or presence of s.c. administered mixed (mLPC; 69% LPC-16:0, 25% LPC-18:0, 6% other; n = 11) or pure (pLPC; 99% LPC-18:0; n = 11) LPC, or a milk replacer diet supplemented with 3% lecithin enriched in lysophospholipids containing LPC in the absence of s.c. administered LPC (LYSO; n = 10) for a 5-wk experimental period. Data are presented as LSM ± SEM. * = P <0.05; ** = P < 0.01; *** = P < 0.001, relative to CON.

[2]Blood was collected immediately prior (0 h) and 10 h following the final of 5 serial s.c. injections of either LPC or vehicle.

We observed a significant effect of treatment, hour, and treatment × hour for albumin and total protein (P < 0.01). For instance, mLPC and pLPC calves had lower circulating albumin and total protein concentrations at h 0 and h 10, relative to the final injection, as compared to CON (P < 0.05). Serum albumin, total protein, globulin, total bilirubin, AST, GGT, and cortisol concentrations decreased by 10 h post final injection (P < 0.01). Alternatively, serum concentrations of AST significantly decreased (P < 0.001) while GLDH tended to decrease (P = 0.082). Serum albumin and total protein concentrations were significantly reduced in mLPC and pLPC calves, relative to CON calves (P < 0.001). Serum globulin concentrations tended to be

modified by treatment (P = 0.06). Specifically, serum globulin concentrations were lowest for calves that received the mLPC treatment. Relative to CON calves, at 0 h mLPC calves had significantly higher concentrations of serum cortisol (P < 0.05). Aspartate transaminase was significantly higher in mLPC (P < 0.001) and pLPC (P < 0.05) calves relative to CON calves. Calves receiving mLPC tended to have higher serum concentrations of total (P = 0.076) and direct (P = 0.052) bilirubin at 10 h. Serum GLDH was significantly higher in mLPC calves at h 0 (P < 0.05) and h 10 (P < 0.05) relative to CON calves. No significant differences were detected between LYSO and CON calves in concentrations of serum markers of liver health during the experimental period. Plasma concentrations of TNFα and serum IgG were not found to be modified by LPC administration.

Changes in plasma SAA relative to the final s.c. injection of vehicle or LPC are shown in **Figure 4.3**.

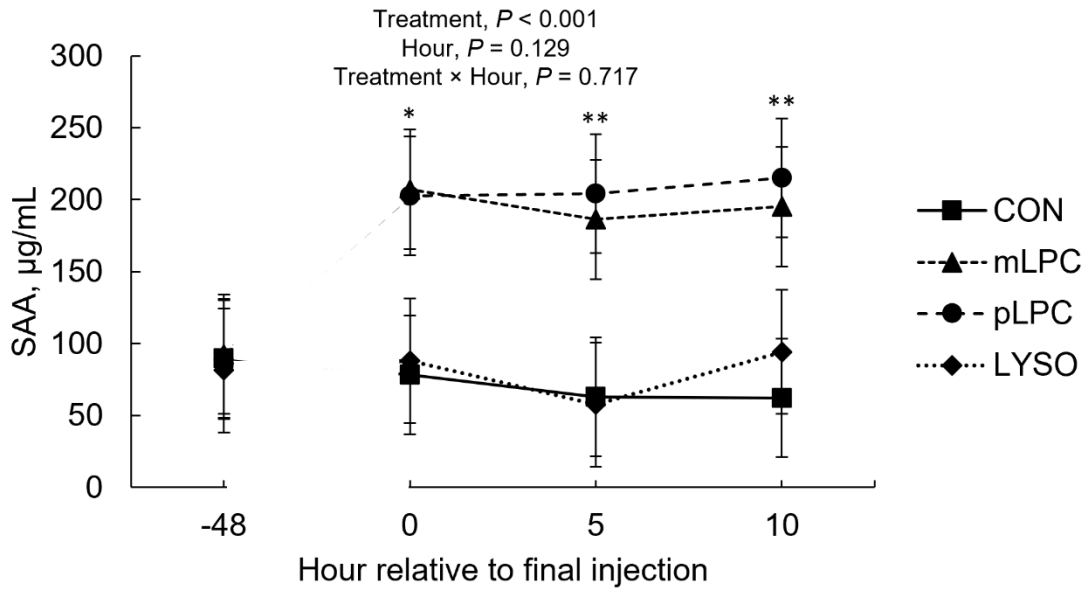

Figure 4.3. Effects of s.c. vehicle (CON & LYSO) or LPC (mLPC & pLPC) on plasma serum amyloid A at -48, 0, 5, and 10 h relative to final injection. Calves were randomly assigned to 1 of 4 treatments: a milk replacer diet unsupplemented with lecithin in the absence (CON; n=11) or presence of s.c. administered mixed (mLPC; 69% LPC-16:0, 25% LPC-18:0, 6% other; n = 11) or pure (pLPC; 99% LPC-18:0; n = 11) LPC, or a milk replacer diet supplemented with 3% lecithin enriched in lysophospholipids containing LPC in the absence of s.c. administered LPC (LYSO; n = 10) for a 5-wk experimental period. Data are presented as LS Means ± SEM. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$, relative to CON. Blood was taken 48 h before the first injection of either LPC or vehicle as well as 0, 5, and 10 h after the final injection. Data are presented as LSM ± SEM.

Significant treatment effects were observed for plasma SAA concentrations ($P < 0.05$). Specifically, mLPC and pLPC calves had greater circulating SAA concentrations, relative to CON ($P < 0.05$). Plasma SAA concentrations were not different between LYSO and CON.

3.3.f. Effects of LPC on calf white blood cell profiles

Changes in acute and long-term white blood cell profiles in response to LPC administration are displayed in **Table 4.7**.

Table 4.7. Changes in the white blood cell profiles of pre-weaned Holstein heifer calves receiving injections of s.c. vehicle or LPC.

		Treatment[1]					*P*-value		
	Week[2]	CON	mLPC	pLPC	LYSO	SEM	Treatment	Week	Treatment × Week
WBC, 10^3/μL	2	8.96	11.1	9.81	10.2	0.826	0.073	0.263	0.823
	6	8.49	10.9	9.69	9.00	0.921			
Segmented neutrophils, 10^3/μL	2	4.01	5.81	4.64	5.13	0.62	0.043	<0.001	0.740
	6	2.97	4.66	3.50	3.10	0.70			
Lymphocytes, 10^3/μL	2	0.396	0.800	0.799	0.460	0.178	0.073	0.263	0.823
	6	0.560	0.976	0.581	0.560	0.197			
Monocytes, 10^3/μL	2	4.52	4.07	4.31	4.58	0.405	0.805	<0.001	0.373
	6	4.81	4.94	5.45	5.19	0.440			
Eosinophils, 10^3/μL	2	0.036	0.036	0.055	0.040	0.030	0.614	0.006	0.882
	6	0.091	0.112	0.137	0.078	0.034			
Basophils, 10^3/μL	2	0.028	0.018	0.027	0.029	0.020	0.681	0.024	0.682
	6	0.055	0.074	0.036	0.077	0.022			
Segmented neutrophils, %	2	45.3	50.2	46.6	49.2	3.92	0.712	<0.001	0.688
	6	34.7	40.5	35.1	33.2	4.42			
Lymphocytes, %	2	49.8	39.7	44.6	45.5	4.29	0.135	<0.001	0.719
	6	56.8	48.9	57.1	58.5	4.72			
Monocytes, %	2	4.36	6.63	7.97	4.50	1.31	0.236	0.931	0.360
	6	6.52	4.72	6.04	6.57	1.46			
Eosinophils, %	2	0.453	0.316	0.580	0.473	0.580	0.666	0.005	0.917
	6	1.07	0.957	1.43	0.902	1.43			
Basophils, %	2	0.330	0.235	0.271	0.286	0.236	0.691	0.019	0.751
	6	0.672	0.877	0.436	0.896	0.249			

[1] Calves were randomly assigned to 1 of 4 treatments: a milk replacer diet unsupplemented with lecithin in the absence (CON; n=11) or presence of s.c. administered mixed (mLPC; 69% LPC-16:0, 25% LPC-18:0, 6% other; n = 11) or pure (pLPC; 99% LPC-18:0; n = 11) LPC, or a milk replacer diet supplemented with 3% lecithin enriched in lysophospholipids containing LPC in the absence of s.c. administered LPC (LYSO; n = 10) for a 5-wk experimental period. Data are presented as LS Means ± SEM.

[2] Blood was collected 5 h (wk 2) and 4 wk (wk 6) following the final of 5 serial s.c. injections of either LPC or vehicle.

Significant time effects were observed for segmented neutrophils, monocytes, eosinophils and basophils: the absolute counts of segmented neutrophils significantly decreased ($P < 0.001$) while absolute counts of monocytes, eosinophils, and basophils significantly increased over time ($P < 0.05$). Relative percentages of segmented neutrophils significantly decreased over time ($P <$

0.001) while relative percentages of lymphocytes, eosinophils, and basophils significantly increased over time ($P < 0.05$). Treatment tended to modify WBC ($P = 0.073$). Specifically, WBC was greatest in cows provided mLPC and lowest in CON. Additionally, while relative percentage of segmented neutrophils was not significantly higher in this group, absolute numbers of segmented neutrophils was significantly higher in mLPC calves than CON calves (5.81 vs 4.01 × 10^3 cells/μL; $P < 0.05$). No significant treatment or treatment × week effects were detected in segmented neutrophil, monocyte, lymphocyte, eosinophil or basophil profiles at any time point amongst treatment groups.

4.4. Discussion

Plasma concentrations of total and individual species of LPC were found to be significantly elevated in the hours following LPC administration in both mLPC calves and LYSO calves, suggesting that these forms of LPC are able to remain intact and detectable within the animal long after their introduction into circulatory and digestive systems, respectively. In the case of the lysolecithin supplemented LYSO calves, their continuous consumption of the lysolipid-enriched milk replacer over the course of the experimental period most likely contributed to their elevated LPC concentrations in circulation, especially at the 10 h timepoint which corresponded to a post-prandial sample, hence the observed increase in circulating LPC concentrations at this time. Calves receiving mixed-LPC showed an initial spike in LPC concentrations at 5 h, followed by a decline at 10 h, indicative of the progressive degradation of circulating LPC by enzymes such as lysophosphatidylcholine acyltransferase (LPCAT) and PLA_2 overtime (Law et al., 2019). Conversely, although pLPC calves also received s.c. injection of refined stearoyl-LPC, this species of LPC was not found to be significantly elevated within these animals. Physiologically, in circulation, LPC is relatively short-lived, being rapidly degraded or converted into different

metabolites within lipoproteins or upon its secretion from the liver (Law et al., 2019). The absence of an LPC response in pLPC calves could insinuate that the purity of the s.c. administered LPC may modulate its susceptibility to degradation within the liver relative to the mixed form of LPC provided to the mLPC calves. The LPC profile of the mixed-LPC product contained primarily LPC-16:0 (69%) with the second most abundant LPC species being LPC-18:0 (24.6%), followed by LPC-18:1 (3.4%) and LPC-16:1 (1.4%) whereas pLPC calves received highly refined (99% purity) LPC-18:0. The incorporation of unsaturated LPCs within the mixed-LPC product coupled with a predominately LPC-16:0 composition may also potentially reduce its ability to be modified by enzymes such as PLA_2 and LPCAT which have been demonstrated to show preferential activity on LPCs based on their acyl chain position, saturation, and length (Kazachkov et al., 2008; Mouchlis et al., 2019). Saturated and pure LPC sources may present a more available substrate for enzymatic activity within circulation and the liver. Determination of the LPC/PC ratio or quantification of downstream markers of LPC degradation such as free fatty acids or arachidonic acid (AA) concentrations during and following acute LPC therapy would allow for more resolution to determine whether this is so. Additionally, the species of LPC that is modified by may determine the phenotypic response to LPC administration. The acyl chain length and saturation of the LPC molecule has been shown to modulate its effector mechanisms. For example, Yan et al. (2004) documented that unsaturated, long chain LPC (i.e., LPC-18:0) improved survival in mice with experimentally induced sepsis while short chain (i.e., LPC-6:0) and unsaturated LPC (i.e., LPC-18:1) did not have this effect. Interestingly, however, it has been documented that LPC-16:0, LPC-18:0, LPC-18:1, and LPC-18:2 are all significantly reduced in septic patients (Drobnik et al., 2003). Therefore, due to the fact that marked increases in these species (except for LPC-18:2) were observed in mLPC and LYSO calves, it can be postulated that these changes may have modified

immune responses and conferred immune protection in calves presented with an immunogenic challenge.

We discovered that acute s.c. or dietary LPC administration induced a febrile response and modifies physiological parameters of liver, immune, and metabolic health in calves. Most notably, acute s.c. mLPC or pLPC administration increased rectal temperatures of calves. As this response was absent in LYSO calves, it is clear that the route of LPC administration, in addition to the concentration and profile of LPC administered, plays a substantial role in determining the physiological effects of LPC. The increases in rectal temperatures were also accompanied by significant increases in plasma SAA concentrations. Acute phase protein SAA is a potent marker for inflammation and an increase in its circulating concentrations suggests a systemic inflammatory response to s.c. LPC. Interestingly, this response was observed in the absence of changes in TNFα, a proinflammatory cytokine. Typically, inflammatory cytokines such as TNFα stimulate the production of SAA, and SAA and TNFα concentrations are observed to increase in tandem during instances of systemic inflammation and disease (Lassen et al., 2015). However, in the case of s.c. LPC-treated calves, marked increases in SAA were not accompanied by increases in TNFα. This could suggest that SAA is being upregulated independently of mechanisms that would also upregulate TNFα concentrations. Due to the fact that SAA is primarily produced and secreted from the liver in instances of liver damage and inflammation, coupled with the significant increase in markers of liver damage observed in mLPC and pLPC calves, it is possible that the increase in SAA is due to liver-specific mechanisms that do not modulate plasma TNFα. Alternatively, the lack of a TNFα response may also be in part explained by the physiological effect of continuously elevated body temperatures on TNFα production. It has been previously reported that recurrent exposure to febrile temperatures (40°C) can reduce TNFα production in

macrophages (Ensor et al., 1995). In fact, this phenomenon has been demonstrated across multiple studies and has been attributed to reduced stability of TNFα mRNA and premature deactivation of TNFα transcription (Fouqueray et al., 1992; Snyder et al., 1992; Ensor et al., 1994). Due to the prolonged increase in rectal temperatures observed in s.c. LPC-treated calves over the course of the 3 d experimental period, it is possible that, upon multiple exposures to LPC and the resulting febrile response, transcription and production of TNFα within the cells in the liver (i.e., tissue-resident macrophages like Kupffer cells) may have been ablated, resulting in lower circulating TNFα. Further exploration into how repeated and/or continuous febrile temperatures affect cytokine mRNA expression and production in young bovine Kupffer cells would need to be carried out in order to determine if this is indeed the case.

Liver markers of health and functionality were significantly modified as a result of s.c. LPC (both mLPC and pLPC). Marked differences in serum albumin, total protein, and AST were detected among these treatment groups relative to CON calves. The significant decrease in serum albumin and total protein is indicative of an acute inflammatory response during which albumin metabolism has been demonstrated to become attenuated due to decreased hepatic protein synthesis (Liao et al., 1986). Indeed, previous studies have shown a similar drop in albumin during instances of systemic inflammation (Jacobsen et al., 2004; Joshi et al., 2018). Interestingly, while both mLPC and pLPC calves displayed elevated concentrations of AST relative to CON calves, these concentrations were higher in mLPC calves than in pLPC calves. Additionally, mLPC calves did display significantly lower concentrations of serum globulin at h 10 and significantly higher concentrations of cortisol at h 0 relative to CON calves while pLPC calves did not. These findings suggest that s.c. injections of mLPC induced more severe liver damage than pLPC. This is underpinned by the finding that mLPC, but not pLPC, calves had significantly higher

concentrations of GLDH 0 and 10 h relative to the final injection. These calves also tended to have higher concentrations of total and direct bilirubin at h 10. Because the liver is an important site of LPC metabolism, changes in its functional integrity could have profound impact on the production and catabolism of other lipid species and intermediates. It could be considered that the observed changes in markers of liver health could be a result of enhanced LPC clearance from circulation or modulation of lipoprotein metabolism in order to mitigate the significant increases in circulating LPC, creating an increased physiological demand on the liver. Measurements of the expression of key liver enzymes involved in LPC and lipoprotein metabolism such are LPCAT or hepatic lipases are needed in order to determine whether this is indeed the case. Radiolabeling of the administered LPC and quantifying its levels within the liver could also determine whether the LPC is indeed localizing here and carrying out its effector mechanisms.

One possibility is that the difference in the acute liver response to LPC therapy may be attributed to differences observed in WBC profile. As mentioned before, SAA concentrations were significantly increased as a result of s.c. LPC therapy and SAA has been shown to be a potent stimulus for the chemotaxis and activation of neutrophils (Ye and Sun, 2015) as well as a priming agent for the oxidative burst reaction (Hatanaka et al., 2003). Additionally, liver damage has been observed to be induced by neutrophilic migration to and activation within the liver during instances of inflammation has been observed (Gujral et al., 2004). Therefore, because WBC levels tended to be higher in mLPC calves and not pLPC relative to CON calves 5 h post-final injection, it is feasible that the observed damage to the liver in mLPC calves could be a result of the elevated numbers of neutrophils or other immune cells infiltrating and carrying out their effector mechanisms such as oxidative burst within the organ. It is interesting to note that studies have found WBC count to positively correlate with the risk of developing incidental non-alcoholic fatty

liver disease independently of metabolic disorders, citing the possibility of increased production of inflammatory mediators and cytokines by WBC cells as a possible explanation (Chung et al., 2016; Zhang et al., 2019). It is also important to note that, unlike pLPC calves, mLPC calves did have elevated concentrations of circulating LPC, which has been shown to enhance the oxidative burst response in neutrophils (Yan et al., 2004; Hong et al., 2010). This may have played a role in the more pronounced liver damage observed in these animals, perhaps acting synergistically with SAA's neutrophilic priming effects. Although, because elevated plasma LPC did not correlate with increased SAA and liver damage in LYSO calves, it is possible that dietary consumption rather than s.c. injection of LPC may attenuate LPC's effects on immune cells and, consequently, liver health in these animals. Additionally, one could consider that LPC could directly be enacting deleterious effects on liver health and function, and LPC has been observed to promote apoptosis of hepatic cells (Kakisaka et al., 2012). However, due to the lack of an observable increase in markers of liver injury in LYSO calves and the lack of treatment-induced changes in concentrations of circulating LPC in pLPC calves, it is likely that the gastral modifications to dietary LPC as well as the potential rapid clearance of refined LPC from circulation may have attenuated their potential hepatic effects and underpins the notion that the form and profile of the administered LPC plays a profound role in regard to its physiological effects. Further experimentation elucidating the role of WBCs on liver damage during the acute phase response in dairy calves and how dietary and s.c. refined LPC may differentially modulate this activity is needed.

Significant reductions in parameters of growth were observed post-weaning in calves given mLPC, more specifically lower ADG and body weights. Heart girth, flank girth, and hip width also tended to be reduced in this treatment group. These changes could be attributed to the

concurrent reduction in DMI observed in these animals as well. Due to the fact that the most significant differences in growth were detected in the post-weaning period, it is unlikely that these changes are directly due to concentrations of LPC in circulation as a result of s.c. LPC administration but more so due to the long-term effects this therapy had on metabolic function. While circulating insulin and total fatty acid concentrations were not found to be significantly affected by LPC treatment, blood glucose in mLPC calves did show a tendency to be lower than the other treatment groups during the post-weaning period as well, suggesting a reduction in the pool of available nutrients from which the calf can utilize as substrate for growth. While this could likely be due to the aforementioned reduced DMI, it is also possible that glucose stores could be diverted to other processes within the calf that were upregulated as a result of LPC treatment. However, due to the fact that other circulating biomarkers of liver or cardiometabolic health were not quantified during the post-weaning period, it is difficult to attribute a definitive cause to this tendency for mLPC calves to have lower levels of circulating glucose. Interestingly, WBC counts in mLPC calves during the post-weaning period were significantly higher than the other treatment groups, with a higher relative percentage of segmented neutrophils. It is possible that LPC could be having an injurious effect within the animal, causing increases in white blood cell populations. Immune cells are known consumers of glucose, especially in instances of immune activation where they upregulate their glucose uptake substantially in order to accommodate increased intracellular metabolic activity (Wolowczuk et al., 2008). Neutrophils are especially dependent on glucose in order to fuel their many extra- and intracellular bactericidal mechanisms such as oxidative burst and phagocytosis (Kummer et al., 2007). Increased numbers of WBCs and neutrophils could create an increased requirement for glucose which can redirect these stores away from being used for anabolic processes such as growth (Maratou et al., 2007; Schuster et al., 2007). Indeed, increased

immune activation in instances of systemic inflammation or disease has been shown to negatively impact body weights and growth in animals (Hiss and Sauerwein, 2003; Sauerwein et al., 2013). Therefore, elevated counts of immune cells and, in turn, increased basal glucose consumption in mLPC calves could likely attribute to the reduced growth observed in these calves. However, without confirmatory experiments to determine the activation status of these circulating white blood cells and where in the animal these cells could be localizing, it is difficult to determine whether this is indeed the case.

Our findings in total highlight the differential effects of dietary and s.c. LPC on physiological parameters. It is important to note and understand the fundamental differences in the ways that LPC is processed when administered under these two conditions. When LPC is directly administered under the skin, it is able to diffuse into circulation in a relatively short time compared to when it is ingested. Alternatively, consumption of LPC requires that it is digested and processed as it moves through the gastrointestinal tract where it is ultimately either absorbed by enterocytes or incorporated into chylomicrons (Komoda et al, 2009). While further studies would need to confirm this, the LPC found in circulation following s.c. vesus dietary administration are likely different from one another as a result of the differing routes of modification that they undergo. While acute s.c. injections of LPC seem to elicit a more rapid, pronounced physiological response, this route is more labor intensive and stressful for the animal, raising questions as to the translatability of this method into commercial practice. On the other hand, dietary consumption of LPC via its incorporation into milk replacer is a much more manageable system, allowing for a more stable, long-term usage. This method would also be more congruous with conferring long-term immune protection in the dairy calf over an extended amount of time. The feeding of LPC also negates the observed inflammatory response and observed drops in intake and growth found

to be induced by s.c. LPC (i.e., mLPC). Therefore, further refinement of an optimal feeding strategy for LPC-enriched milk replacers is a promising prospect in the development of novel, non-antibiotic therapies and interventions in dairy calves.

While this study was novel in scope in terms of determining the immunomodulatory effects of LPC within the dairy calf, certain limitations of the study are present. Firstly, due to the gap in knowledge regarding LPC as an exogenously supplied immunomodulator in dairy calves, optimal concentrations for treatment could not be determined. Therefore, LPC treatment doses had to be extrapolated from murine studies. This presents a potential source of uncertainty regarding whether the chosen dosages were sufficient, inadequate, or in excess in order to achieve the desired response. The apparent paucity in LPC immunomodulatory mechanistic studies within ruminants as well as the lack of utilization of LPC as a potential intervention or disease preventative is likely due to LPC's prolific roles in many metabolic processes. Due to the numerous cascades and pathways that LPC has been reported to affect in circulation and tissues, it is difficult to achieve an isolated, targeted effect of LPC when it is administered to an organism. Indeed, LPC has been observed to either correlate with or affect many physiological states and processes such as the development and progression of atherosclerosis, inflammation, and cardiometabolic health. Upon introduction into circulation, LPC is readily incorporated into either lipoproteins or transport proteins, modulating is bioactive capabilities and consequently is physiological effects. Therefore, LPC presents a difficult target for use as a therapeutic unless refined experiments are carried out in order to determine ways to directly induce the desired effects of LPC without impacting other important cascades in a potentially detrimental manner.

We also recognize that a further limitation of our study was that we did not test an unsaturated refined form of s.c. LPC in order to determine whether the treatment effects we

observed were modulated by the degree of saturation of the LPC molecule. An additional treatment group consisting of calves administered LPC-18:1 and/or LPC-18:2 would have provided more resolution in order to explore this. Lastly, we were unable to test the effects of LPC in the presence of an immune challenge. Our own work has shown that LPC administered in tandem with a pathogenic stimulus such as LPS potentiates antimicrobial responses (i.e., proinflammatory cytokine production); however, LPC on its own was not shown to have this effect. Therefore, it is possible that we did not observe significant changes in certain measurements and parameters of immune and overall health and function due to the lack of an immunogenic stimulus. Future studies elucidating the effects of LPC on calves experiencing disease or experimentally induced inflammatory status (i.e., LPS challenge) are needed to determine the synergistic relationship between LPC, immune cells, and pathogens.

4.5. Conclusion

Our experimental findings merge to tell a very interesting and complex story highlighting the interplay of LPC therapy and its effects on the acute phase response, immune cell profile, liver health, and overall growth in Holstein heifer calves. Five serial s.c. injections of LPC every 12 h of the course of 3 d was found to induce an acute febrile response, as evidenced by significant increases in SAA and rectal temperatures. This was accompanied by marked increases in measures of liver damage. In the case of mLPC calves, acute LPC therapy also had long-term effects, demonstrated by significant reductions in post-weaning ADG and body weights as well as overall DMI. Interestingly, s.c. LPC therapy only increased circulating acute LPC concentrations in mLPC calves and not pLPC calves while lysolecithin-fed LYSO calves also showed marked increases in circulating LPC. Taken together, our results suggest that mixed-LPC administered s.c. to our calves at 10mg/kg BW induces deleterious effects on liver health and overall growth. Refined

LPC-18:0 also induced liver damage at this concentration. Dietary LPC was not found to modify immune cell or liver health parameters. It is clear that further experiments are needed in order to determine the optimal route and concentration of LPC to administer to calves in order to enhance immune health and functionality without compromising liver health and overall growth.

In light of the documented short half-life of neutrophils in circulation, it is important to note that acute administration of LPC may be inappropriate in order to confer long-term immune protection within the calves. As, prolonged s.c. administration of LPC to calves is costly, labor intensive, and presents a potential risk to animal welfare, the continuous feeding of dietary lysolecithin enriched in LPC appears a more feasible and translatable method by which LPC can be effectively administered and utilized as an immunomodulator. This route also mitigates the observed deleterious physiological effects that resulted from s.c. LPC administration. Therefore, special emphasis should be placed on experiments further optimizing and refining the conditions under which dietary LPC can be administered in order to confer continuous immune protection and improve immune responses while negating any adverse outcomes that may result from an overactivated and unregulated immune response.

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4.6. References

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Chapter 5: Conclusion

The immunomodulatory effects of the lysophospholipid LPC were investigated in bovine neutrophils and the neonatal dairy calf. We hypothesized that direct administration of LPC would enhance the bactericidal mechanisms of neutrophils and confer immune protection for the benefit of the pre-weaned dairy calf. In our primary experiments, we observed that administration of exogenous stearoyl LPC to isolated bovine neutrophils prior to their stimulation with pathogenic stimuli significantly increased the concentrations of H_2O_2 and inflammatory cytokines produced. Additionally, we observed enhanced neutrophil-mediated bacterial killing, as evidenced by significant increases in log reductions of *E. coli* CFUs in LPC-treated neutrophils, relative to non LPC-treated controls. For Study 1, I conclude that saturated LPC (i.e., LPC-18:0) promotes the oxidative burst response, potentiates the ability of LPS to enhance cytokine production, and accelerates *E. coli* killing in neutrophils isolated from pre-weaned Holstein heifer calves. I further conclude that unsaturated LPC (i.e., LPC-18:1) does not overtly modify bovine neutrophil functionality. In Study 2, I conclude that s.c. saturated LPC administration was able to immediately induce a febrile and acute phase response in pre-weaned Holstein heifer calves. This was evident by an increase in rectal temperatures and circulating SAA concentrations with 24 h of s.c. LPC administration. This was observed in tandem with increases in circulating markers of liver damage such as aspartate transaminase (AST). Moreover, the administration of LPC (i.e., mLPC) was able to compromise growth performance. These observations suggest that the delivery of LPC at 10 mg/kg of BW every 12 h for 48 h during wk 3 of life may induce physiological changes (i.e., liver injury) that necessitate the reprioritization of nutrient use toward the immune system. Our study approach was unable to determine whether s.c. administration would protect against pathogen exposure. Further research is needed to examine

this possibility. It is interesting to note that dietary lysolecithin supplementation did not elicit a febrile or acute phase response; albeit, circulating LPC concentrations were increased with this dietary intervention, relative to unsupplemented control calves. These findings would suggest that mode of administration, and likely dose, impacts the immunomodulatory outcomes of LPC in the calf. Future research will need to discern whether the calves fed lysolecithin are provided a higher degree of immune protection when exposed to a pathogen. Based on our findings, dietary incorporation of lysolecithin presents the most promising route for administration of LPC due to the observed increases in circulating LPC in the absence of an acute febrile response and no observable effects on growth and production. If the role of LPC as a potential immunomodulator is to be further explored within dairy calves, emphasis on the optimization of the incorporation rate and LPC profile of fed lysolecithin as well as the elucidation of the digestive modifications to LPC that take place in the gut are needed. Future experiments in which pre-weaned calves are administered differing compositions and feeding rates of dietary lysolecithin incorporated within their milk replacer throughout weaning and monitoring the progressive resulting changes in innate (i.e., phagocytic capabilities) and adaptive (i.e., antibody production) immune responses over time would be a logical next step in addressing this paucity in knowledge. Additionally, perhaps developing and utilizing a method of labeling and tracking fed LPC as it moves and changes throughout digestion would yield important information regarding how LPC is processed during this time and how these changes in turn modify its bioactive immunomodulatory capabilities. Perhaps if the final post-digestive form of LPC that is found in circulation could be determined, isolated, and recapitulated in an artificial setting, its use in *in vitro* experiments could provide additional mechanistic insight into if and how dietary LPC drives changes in immune responses. Indeed, such findings would provide much-needed

information toward the development and refinement of LPC as a potential immunomodulator in dairy calves.