LACTOFERRIN IN NEONATAL DEVELOPMENT: EFFECTS ON INTESTINAL STRUCTURE AND INTESTINAL FUNCTION AND IMMUNE RESPONSE IN A PIGLET MODEL OF SYSTEMIC STAPHYLOCOCCUS AUREUS INFECTION

BY
ELIZABETH A. REZNIKOV

DISSERTATION
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Doctoral Committee:
Associate Professor Michael J. Miller, Chair
Professor Sharon M. Donovan, Director of Research
Professor H. Rex Gaskins
Assistant Professor Ryan Dilger
ABSTRACT

Human milk is the best source of nutrition for the human infant. Breast-fed infants are more resistant to disease and infection early in life than formula-fed children. Breast-fed infants have decreased incidence of diarrhea and gastroenteritis, necrotizing enterocolitis, respiratory infection, urinary tract infections and neonatal sepsis (Stuebe 2009; Golding et al 1997). The difference in health outcomes observed between breast-fed and formula-fed infants is in part explained by immune factors present in human milk that provide specific and innate immune factors that protect against and directly interact with pathogens (Stuebe 2009). The consumption of human milk also impacts the development of the intestinal microbiota in infants, which has implications for immunological programming (Kainonen et al 2013; Jacobi and Odle, 2012).

Lactoferrin (Lf), a bioactive protein found in high concentrations in human milk, when fed orally, has bacteriostatic activity against S. aureus in vivo (Bhimani et al 1999) and has been proven efficacious against neonatal septicemia when supplemented in infant formula (Manzoni et al 2009). In addition to its antioxidant, immune modulating and antibacterial properties, Lf promotes the growth of bifidobacteria, while inhibiting pathogenic bacteria. Bifidobacteria is the predominant fecal bacterium in breast-fed infants (Trebichavsky et al 2009), and contributes to development of the mucosal immune system, regulation of the systemic immune response, and decreased bacterial sepsis in preterm infants (Dong et al 2010; Cohen-Wolkowiez et al 2009). Lastly, Lf has a role on intestinal maturation and has been shown to have a proliferative effect at the intestine (Nguyen et al 2014; Buccigrossi et al 2007; Nichols et al 1987). It has been
suggested intestinal maturation leads to improved local immunity that in turn affects the systemic immune system and overall health.

Treatment of Staphylococcus aureus (S. aureus) infections is complicated by an increasing number of antibiotic resistant strains. Antibiotic resistant strains are a significant cause of morbidity and mortality in neonatal intensive care units, disproportionately affecting infants less than three months old (Zervou et al 2014; Maraqa et al 2011). One example of an antibiotic resistant strain is methicillin-resistant S. aureus, or MRSA, which results in more deaths annually than any other single infectious agent in the United States (Miller and Cho, 2011). Colonization of S. aureus is incompletely understood, but involves virulence factors which are structural to and secreted by S. aureus. The pathogen is able to adhere to host cells, evade the immune response, and persist intracellularly within epithelial cells and cells of the host immune system (Liu 2009). Newborn infants are unable to mount an effective immune response, making them a highly susceptible population in the hospital setting.

The proposed research in this dissertation was to understand how Lf can modulate intestinal maturation and immune response. In addition, we looked at how oral intake of Lf and bifidobacteria attenuate a systemic S. aureus infection. While these bioactive ingredients are found naturally in human milk, and have been shown to be clinically efficacious in blood-born S. aureus infection, there is a lack of understanding of their biological activities in order to justify their addition to infant formulas. Thus, the findings in this research are a significant step in identifying the potential role of Lf to improve gastrointestinal maturation and immune response in newborn infants. Herein, we have
three aims to investigate the effects of supplementing Lf on neonatal intestinal and immune development.

Proliferative properties of bovine Lf (bLf) were explored through 5-ethynyl-2'-deoxyuridine (EdU) incorporation in vivo for aim one. Colostrum-deprived pigs were fed formula containing 0.4 (CON), 1 (LF1) or 3.6 (LF3) g bLf/L. On d7 or 14, pigs were intraperitoneally injected with 10mM EdU (1ml/kg body weight) 2 h prior to euthanasia. Proliferation was assessed ex vivo in formalin-fixed jejunal sections using Click-iT EdU Alexa Fluor 555 Kit. Sections were imaged with a confocal microscope. Staining was quantified with AxioVision, where area positive for EdU (proliferation) was normalized by the area positive for DAPI (total nuclei). Formalin-fixed jejenum sections were stained with haematoxylin and eosin for villus and crypt measurements. LF1 and LF3 pigs had a 1.5-fold increase in crypt cell proliferation compared to CON pigs. In LF3 pigs, crypt depth and area were greater (1.5-fold on d 7, 1.3-fold on d 14) than in CON pigs. This study supports in vitro studies that showed LF could stimulate proliferation and was the first to show that physiologically-relevant concentrations of milk-borne bLf stimulate crypt cell proliferation in pigs suggesting Lf plays a role in healthy GI development.

The effect of bLf alone or in combination with B. infantis on the course of S. aureus infection was assessed. Colostrum-deprived pigs had umbilical catheters placed at birth and were fed formula with 4g/L bLf (LF) or whey protein (CON); half of the piglets in each group were further randomized to receive B. infantis (10⁹ CFU/day, ATCC 15697). On d 7, all piglets were infected intravenously with S. aureus (10⁵ CFU/kg BW, S54F9) and euthanized on d 12. The same study design was used to investigate aims two and
three. In aim two, LF piglets had decreased (p<0.05) *S. aureus* load at the kidney, and LF tended to decrease *S. aureus* load at the lung and heart compared to CON, with no effect of *B. infantis*. LF tended to decrease the incidence of infection at the kidney, and when combined with *B. infantis* (COMB; LF + *B. infantis*) had the lowest incidence of infection at the lung. Furthermore, LF-fed animals had significantly increased IFNγ expression at the lung, significantly decreased IL-10 expression at the kidney, and when combined with *B. infantis* (COMB) ameliorated TLR2 expression at the kidney. Lastly, *B. infantis* significantly increased IFNγ expression at the spleen. Overall, bLf decreases translocation of *S. aureus* to tissues, which could potentially reduce organ dysfunction.

In aim three, piglets had elevated (p<0.05) rectal temperature beginning at 36 h post-infection. LF piglets had elevated rectal temperatures and improved weight gain on d10, 72 h post-infection. LF piglets also had decreased serum IL-10 and increased lymphocyte percent in whole blood prior to infection. *B. infantis* increased serum IL-10 following infection, with a peak IL-10 concentration at 96 and 108 h post-infection. Despite these results, Foxp3 expression was actually depressed in Lf and *B. infantis* animals, and Foxp3 expression was restored in COMB animals relative to CON. Lastly, Lf decreased relative proportions of monocytes and B cells in the PBMC population, whereas *B. infantis* decreased memory T cells at 120 hours post-infection. Overall, *B. infantis* decreased inflammatory immune responses. The combination of Lf and *B. infantis* may improve the neonatal immune response, while decreasing potentially harmful over-inflammatory responses.
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LIST OF ABBREVIATIONS

BW, body weight
bLF, bovine lactoferrin
CCK, cholecystokinin
COMB, combined
CON, control
DAPI, diamidino-2-phenylindole
DC, dendritic cells
DPP IV, dipeptidylpeptidase IV
Duo, duodenum
EdU, 5-ethynyl-2'-deoxyuridine
EEC, enteroendocrine cells
ELISA, enzyme-linked immunoabsorbent assay
GALT, gut-associated lymphoid tissue
GI, gastrointestinal
H&E, hematoxylin and eosin
hLF, human lactoferrin
HMO, human milk oligosaccharides
IFNγ, interferon gamma
Ile, ileum
IL, interleukin
Jej, jejunum
LF, lactoferrin
LFR, lactoferrin receptor
LPS, lipopolysaccharide
Mø, macrophage
MAMPs, molecular-associated molecular patterns
MAPK, map kinase
MHC, major histocompatibility complex
MLN, mesenteric lymph nodes
mRNA, messenger RNA
MRSA, methicillin-resistant S. aureus
MSCRAMMs, microbial surface components recognizing adhesive matrix molecules
NF-κβ, Nuclear Factor-KappaB
NK cell, natural killer cell
NLRs, NOD-like receptors
OCT, Optimal Cutting Temperature
PBMCs, peripheral blood mononuclear cells
PMN, polymorphonuclear leukocytes
PRRs, pattern recognition receptors
PP, Peyer’s patches
RLRs, RIG-I-like receptor
Staphylococcus aureus, S. aureus
TCR, T-cell receptor
Th1, T helper cell 1
Th2, T helper cell 2
Th17, interleukin-17 producing cells
TLRs, toll-like receptors

TNFα, tumor necrosis factor α

Tregs, regulatory T cells
CHAPTER 1

LITERATURE REVIEW

I. Neonatal Immune System

A. Innate Immunity

The innate immune system evolved as a host-defense mechanism that is triggered by pattern recognition to pathogens to protect the host as a first line of defense. Host-pathogen interactions are governed by recognition of conserved structures on microorganisms called molecular-associated molecular patterns (MAMPs) (Janeway and Medzhitov, 2002). MAMPs are broadly recognized by the innate immune system via germ-line encoded receptors called pattern recognition receptors (PRRs) expressed on innate immune cells (Medzhitov, 2007). PRRs activate signaling pathways to clear pathogens by inducing an inflammatory response mediated by cytokines and chemokines. There are three major classes of PRRs involved in microbial sensing: Toll-like receptors (TLRs), NOD-like receptors (NLRs), and the RIG-I-like receptor (RLRs) family, comprised of retinoid acid-inducible gene-1 (RIG-I), melanoma-differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology-2 (LGP2) (Kumar et al 2011). RLRs constitute a family of cytoplasmic RNA helicases critical for host antiviral responses (Chen et al 2009) and will not be discussed in detail.

TLRs and NLRs are important for bacterial infections. TLRs are transmembrane receptors capable of activating tissue-resident macrophages (Mφ) and dendritic cells (DC) to produce pro-inflammatory cytokines, and activate the complement system allowing for phagocytosis of pathogens by macrophages and neutrophils (Kumar et al 2011; Medzhitov, 2007). TLRs trigger innate immune response through recruitment of adaptor
molecules which activate transcription factors such as Nuclear Factor-KappaB (NF-κβ). TLRs are expressed at the cell surface, on the membrane of endocytic vesicles or other intracellular organelles and they recognize viral nucleic acids and several bacterial products. There are seven TLR (TLR1-TLR7 and TLR9), with two being heterodimers (TLR1/2 and TLR 6/2) (Takeda and Akira, 2004). Lipopolysaccharide (LPS), a major component found in the outer membrane of Gram-negative bacteria, is able to elicit strong immune responses when bound to TLR4 (Akira et al 2006). Components of Gram-positive bacterial cell walls contain lipoteichoic acid (LTA), lipoproteins and peptidoglycan which are potent stimulators of TLR2 (Akira et al 2006). Gram negative bacteria also stimulate TLR2 due to the presence of lipoproteins and peptidoglycan. TLR2 is important in host defense against S. aureus (Echchannaoui et al 2002; Takeuchi et al 2000). Humans with a polymorphism in TLR2 gene have reduced response to bacterial lipoproteins and are more susceptible to septic shock after infection by Gram-positive bacteria, especially staphylococcal septic shock (Cook et al 2004).

Similar to TLRs, NLRs activate downstream signaling pathways for production of proinflammatory mediators. NLRs are a special group of intracellular receptors which contain a nucleotide-binding oligomerization domain (NOD). There are 22 known NLRs in humans that can be divided into four subfamilies based on their effect domain; these include: NLRA, NLRB, NLRC and NLRP (Chen et al 2009). The best characterized NLRs are of the subfamily NLRC, which include NOD1 (CARD4) and NOD2 (CARD15). NOD 1 and 2 are involved in sensing bacterial peptidoglycans, which in turn upregulates NF-κβ and MAPK transcription and triggers production of pro-inflammatory cytokines, chemokines, and additional effector cells of the immune system (Chen et al...
2009; Kanneganti et al 2007). NLRC4, another member of the NLRC subfamily, is a sensor of extracellular and cytosolic flagellin, independent of TLR5 (Chen et al 2009). NLRC4 is one of three known multiprotein complexes called the inflammasome which play a role in regulation of caspase-1. The activation of caspase-1 results in cleaving pro-IL-1β and pro-IL-18 into their mature and biologically active forms (Stutz et al 2009). The other two inflammasomes are part of the NLRP subfamilies, and these include the NLRP1 and NLRP3 inflammasomes. NLRP3 inflammasomes mediate caspases-1 activation in response to various bacterial stimuli, including whole bacteria such as Staphylococcus aureus, bacterial RNA and toxins.

B. Adaptive Immunity and T cell Subsets

The innate immune system is also responsible for effective initiation of adaptive immunity, which is long-lasting, provides immunological memory for the host and is antigen specific (Medzhitov 2007). The adaptive immune system evolved only in vertebrates and consists of two types of lymphocytes, T cells and B cells, in contrast to the innate immune cells, which include DC, Mø and neutrophils as shown in Figure 1.1 (Iwasaki and Medzhitov, 2010). The PRRs of the innate immune system have broad specificity, while the receptors responsible for immune recognition of the adaptive immune system are mediated by antigen receptors that undergo somatic gene rearrangement and are then clonally distributed on T and B lymphocytes (Medzhitov 2007, Schatz et al 1992). Due to the specificity of the adaptive response, this is a slower response than the innate immune system as demonstrated in Figure 1.2. The T cell and B cell receptors both undergo a variable (V), diversity (D) and joining (J) or V(D)J
recombination, where multiple gene segments undergo a physical rearrangement at the loci known as somatic gene rearrangement (Market and Papavasiliou, 2003). While this creates a diverse set of highly-variable receptors, this may also create some self-reacting cells and therefore the host has evolved a means of eliminating those cells, known as central tolerance (Schwartz, 2012). T cell lymphoid progenitors arise in the bone marrow and migrate to the thymus where they undergo a selection process to delete T cells that have an affinity for self and following this process thymocytes can become circulating mature naïve T cells. Similarly, B cells also undergo a central tolerance process in the bone marrow where immature B cells that interact with self-antigens are deleted. Due to the specific nature of the receptors in the adaptive immune response, this is known to be a slower response than the innate immune system which relies on germline-encoded immune responses (Medzhitov, 2007).

In the thymus, T cells are initially double-negative and give rise to either γδ or αβ T-cell receptor (TCR)-expressing cell lineages. The αβ T cell is the dominating type in lymphoid organs and is characterized by how the T cell reacts to peptides presented by Major Histocompatibility Complex (MHC) class I or II expressed on antigen presenting cells (Carpenter and Bosselut, 2010). The αβ TCR pathway first requires the formation of a pre-T-cell receptor (pre-TCR) at the thymocyte. The pre-TCR consists of a β chain which undergoes somatic DNA rearrangement and forms a heterodimer with a pre-TCRα (pTα) resulting in the pTαβ-CD3 complex (von Boehmer 2005). Thymocytes that successfully pass through this β selection process will express pre-TCR complex and can enter the thymus for further αβ T-cell differentiation (Carpenter and Bosselut, 2010). Thymocytes express co-receptor proteins CD8 and CD4 to form an immature αβ T cell
that is CD4+CD8+ double positive (DP). DP thymocytes undergo a positive selection process based on their ability to bind MHC class I or II molecules. Those αβ T cells with an affinity to MHC I or II move on to undergo a negative selection process where those cells with too high an affinity for MHC or an affinity for self-peptides (autoimmunity) are eliminated at the thymus, a process called central tolerance (Xing and Hogquist, 2012). Those double positive thymocytes that survive go on to differentiate into single-positive thymocytes with either an effector potential of helper (CD4+) for those T cells interacting with MHC class II or cytotoxic (CD8+) T cell for those T cells interacting with MHC class I (Carpenter and Bosselut, 2010).

Naïve T cells require a co-stimulatory signal from professional antigen-presenting cells, bridging innate and adaptive immune response. Adaptive immune responses are initiated at organized peripheral lymphoid tissues through which naïve T cells are continually migrating. Pathogens infecting mucosal surfaces accumulate in lymphoid tissues such as the Peyer’s patch (PP) of the gut or the tonsils; those that enter the blood are trapped in the spleen and those infecting peripheral sites are trapped in lymph nodes near the site of infection (Murphy et al 2008). All these lymphoid organs contain cells that present antigens to T cells, the most important of these are the DC. DC’s are stimulated at the site of infection by MAMPs and cytokines, leading these cells to migrate to downstream lymph nodes where they express co-stimulatory molecules required for the activation of naïve T cells (Murphy et al 2008). After encountering an antigen presented by an MHC molecule, in the context of co-stimulation, antigen specific populations of lymphocytes are selected to expand in response to a pathogen which serves as the basis for immunological memory. Naïve B cells require accessory signals
and these signals can come from helper T cells or microbial antigens. When stimulated with the peptide MHC complex from helper T cells, B cells proliferate and differentiate into antigen-specific antibody secreting cells, thus driving the humoral immune response (Murphy et al 2008).

The TCR and cytokines are responsible for T-cell differentiation by initiating the T-cell response, clonal expansion and for maintaining T-cell memory generation (Schluns and Lefrançois, 2003). Naïve helper (CD4+) T cells differentiate into several subtypes of effector cells, including T helper cell type 1 (Th1), T helper cell type 2 (Th2), follicular helper T cells (Tfh), regulatory T cells (Treg), and interleukin-17 producing cells (Th17) (Nakayamada et al 2012). These subtypes are classified according to their pattern of cytokine secretion and expression of surface antigens, and several transcription factors responsible for T-cell differentiation, such as T-Bet for Th1 cells, GATA3 for Th2 cells, RORyt for Th17 cells and FOXP3 for Treg cells. Recent studies have shown the flexibility and plasticity of helper T cells, wherein helper cells can change their phenotype and can be reprogrammed to express a different transcription factor, or co-express more than one (Oestreich and Weinmann, 2012). Additionally, while subtypes are classified by their signature cytokine, these cytokines are broadly expressed by other subtypes and made by a variety of innate immune cells (Nakayamada et al 2012).

C. The Developing Infant Immune System

The neonate is highly susceptible to infections at birth due to deficiencies in both the innate and adaptive immune systems. The neonate is not deficient in immune cells; rather, it is inexperienced, lacking antigenic stimulation, immunological memory thereof, as well as immune effector functions such as low cytolytic activity. For example: the
numbers of natural killer (NK) cells in young children are high, however, the cytotoxicity is much lower likely due to a reduced capacity to produce cytokines such as IL-12 and IFNγ (Nguyen et al 1998). The NK cell cytotoxicity was found to be further impaired with prematurity, sepsis and recurrent infections (Georgeson et al 2001). Neonates experience a rapid expansion in lymphocyte count in the first week of life (Walker et al 2011). B and T lymphocytes of the neonatal immune system, including cytotoxic and helper T cells, are primarily naïve at birth (Walker et al 2011). The number of naïve cells and total T- and B-lymphocyte counts are much lower in children and adults, when compared with neonates. However, memory and effector cell counts are much higher in adults. Additionally, term and preterm newborns do not differ in total numbers of naïve cells, with the exception of preterm infants having lower counts of naïve helper T lymphocytes (Walker et al 2011).

Neutrophils are sparse before 32 wks of gestation, after which they increase exponentially to become the dominant white blood cell by term (Davies et al 1992). Neonatal neutrophils have deficiencies in their antibacterial function that contribute to neonatal sepsis (Melvan et al 2010). Several of these deficiencies include reduced ability to recognize pathogenic bacteria, ability to migrate to the site of infection, and the ability to form neutrophil extracellular traps (NETs). In addition, neonatal neutrophils are further impaired in that they are less able to generate bactericidal substances such as lactoferrin (Ambruso et al 1981). Polymorphonuclear leukocytes (PMN) in term and preterm infants fail to form neutrophil extracellular traps (NETs), which leads to impaired extracellular bacterial killing (Yost et al 2009). Additionally, term neonatal PMNs did not form NETs in response to incubation with live Escherichia coli (E. coli) or S. aureus while adult
PMNs successfully formed NET lattices. Preterm and term infants have impaired neutrophil adhesion and chemotaxis compared with adult neutrophils due to decreased surface expression of glycoproteins related to neutrophil adhesion function (McEvoy et al 1996).

When compared with adult cells, healthy neonatal cells have decreased phagocytic capacity, and neonatal monocytes and granulocytes ingest significantly fewer bacteria (Strunk et al 2004). Another study found decreased phagocytic activity by premature infant PMNs compared with full term neonates in response to group B streptococci, but no difference between full term neonates and adult PMNs (Kallman et al 1998). In contrast, no difference was observed in phagocytic activity by premature infant, term infant or adult monocytes and lymphocytes in response to group B streptococci (GBS) (Currie et al 2011). Neutrophils from preterm infants were less able to phagocytose Candida albicans than those from term infants and adults; however, in this same study, phagocytosis of S. aureus was identical in neutrophils from preterm infant, term infant or adult neutrophils (Bektas et al 1990). However, another study found peripheral blood neutrophil uptake of S. aureus was lower for preterm infants than term infants (Fujiwara et al 1997).

D. Cytokines in the Neonatal Immune System

Activation of TLR pathways leads to secretion of proinflammatory and immunoregulatory cytokines and chemokines, leading to the recruitment of neutrophils and monocytes. The number of IL-6 producing monocytes following LPS or GBS stimulation is significantly lower in the preterm infant at <32 wks (Dembinski et al 2003;
Schultz et al 2002), while the number of IL-6 positive monocytes in the term neonate is elevated above adult monocytes with LPS stimulation (Angelone et al 2006; Schultz et al 2002) and live GBS stimulation (Mohamed et al 2007; Currie et al 2011). In contrast, other studies found neonatal production of IL-6 was significantly reduced compared to adults (Sadeghi et al 2007; Schibler et al 1992). The number of TNFα producing monocytes is significantly impaired in the fetus and preterm infant (Strunk et al 2004), and term neonate (Angelone et al 2006; Hodge et al 2001). TNFα production from full term neonatal blood monocytes was shown to be impaired with most TLR agonists, while IL-6 was enhanced in newborns (Levy et al 2004). IL-6 and TNFα production is predominantly confined to monocytes with no detectable production in granulocytes or lymphocytes (Levy et al 2006). These studies demonstrate that the neonatal innate immune response varies with gestational age; however, the ratio of IL-6 to TNFα is higher for newborns.

One possible explanation for reduced IL-6 in preterm infants compared with term infants is decreased surface expression of TLR4 on monocytes (Forster-Waldl et al 2005). Following recognition of pathogens by TLRs the signaling pathways activated lead to effector functions such as maturation and differentiation of monocytes into macrophages leading to increased phagocytic activity. An effect of developmental age has been shown where infants <30 wks gestational age had significantly less TLR4 expression on monocytes than term infants, and term infants 38-42 weeks (wks) gestational age had significantly less TLR4 expression on monocytes than adult monocytes (Sadeghi et al 2007). In contrast, others have shown no difference in basal expression of TLRs and TLR-related molecules in newborn and adult monocytes (Levy et
A possible explanation for the deficient TLR-induced TNFα synthesis from neonatal blood monocytes may be related to modulatory factors in neonatal plasma. One potential contributor is the production of adenosine found in high levels in cord blood compared to adult peripheral blood (Levy et al 2006). Adenosine can act through adenosine receptors on neonatal mononuclear cells to increase the accumulation of cyclic adenosine monophosphate (cAMP), which specifically inhibits TNFα and IL-12 production, while increasing expression of IL-6 and IL-10 (Haskó et al 2000; Levy et al 2006).

Studies have suggested neonatal immunity may be Th2-biased to avoid Th1-type inflammation. While this is generally true, it depends on the stimulant and the cells involved in these experiments. Th2-associated cytokine production of IL-4, IL-5 and IL-10 were similar in neonate and adult PBMC stimulated with Phytohaemagglutinin (PHA), a stimulant that primarily activates CD4+ T cells (Trivedi et al 1997). However, IL-10 was significantly lower in neonate relative to adult PBMC when stimulated with GBS (Currie et al 2011). Infants have impaired production of inflammatory cytokines such as: IL-1α and IL-1β in LPS stimulated monocytes (Hodge et al 2001); impaired IL-18 in GBS stimulated PBMC (La Pine et al 2003); impaired IFNγ production in PHA stimulated (Trivedi et al 1997; Currie et al 2011) and LPS stimulated (Lee et al 1996) PBMC. Impaired IFNγ and IL-12 production in neonate PBMC has been identified as a cause of Group B streptococcal septicemia in the newborn (La Pine et al 2003; Joyner et al 2000). Age differences are observed in IL-12 production by IFNγ-primed PBMC stimulated with heat-killed S. aureus. Infants were most significantly impaired, followed by 5 year olds, who were still impaired relative to adult PBMC (Upham et al 2002).
It has been suggested that neonates have impaired antigen-presenting-cell function necessary to induce protective Th1 immune responses which requires IFNγ and IL-12. Inability of neonatal cells to synthesize IL-12 can be overcome with the addition of cytokine signals, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4, that induce dendritic cell differentiation (Upham et al 2002). There is a similar percentage of plasmacytoid DCs (pDC) in adult and neonatal PBMC samples however adult PBMC samples contained more myeloid/conventional DC (cDCs) (Kollmann et al 2009). cDCs are highly phagocytic, and known for their capacity to stimulate naïve T cells. Neonatal pDCs are also less responsive to TLR stimulation and have diminished production of type 1 IFNs, IL-12 and IFNγ (Kollmann et al 2009). The Th1 response in the neonate may be impaired in part due to a reduced production of IL-12 and type I IFNs by DCs that would normally stimulate T cells to produce IFNγ, facilitating Th1 while suppressing Th2 development. Furthermore, the reduced capacity of neonatal T cells to produce IFN-γ is markedly improved when these T cells are cultured with adult antigen presenting cells, suggesting that neonatal antigen presenting cells lack the capacity to deliver Th1 polarizing signals to T cells (Trivedi et al 1997).

II. Neonatal Intestinal Microbiota

A. Microbial Colonization Differs Between Breast- and Formula-fed Infants

Many factors have been found to contribute to the microbial makeup of the mammalian gastrointestinal tract; including: maternal colonization, birth environment, diet, antimicrobial therapies and genetic factors. The gastrointestinal tract becomes colonized immediately at birth with organisms from the environment. The initial
colonization consists predominantly of aerobic bacteria, including potential pathogens; beneficial anaerobic bacteria appear a few days later. It is proposed that initial aerobic microorganisms consume the oxygen in the intestine and set the stage for anaerobic bacteria to establish by as early as the third day (Orrhage and Nord, 1999). Infants delivered by cesarean section have different microbial composition than vaginally-delivered infants (Huurre et al 2008; Biasucci et al 2008). Studies show vaginally delivered babies have a large number of bifidobacterial species relative to cesarean delivered. Cesarean delivered infants have delayed acquisition of anaerobes Bacteroides and bifidobacteria (Adlerberth et al 2006; Penders et al 2006; Grönlund et al 1999). Prophylactic IV antibiotic administration to the mother may cause some of these differences in primary colonization of bacteria in cesarean delivered infants.

Diet plays a major role in determining microbiota composition in a second phase of colonization. The intestinal microbiota of exclusively breast-fed infants and their mothers show similar bifidobacteria colonization rates, suggesting breast feeding is another mode of maternal colonization (Grönlund et al 2011) and breast-milk is a source of bifidobacteria (Grönlund et al 2007). Infants fed solely human breast milk have a microbiota colonized with predominantly bifidobacteria as early as the third day of life. Formula-fed infants over the span of a month show development of a more diverse microbiota generally lower in bifidobacteria and with more Staphylococci, Clostridia, E. coli, and other bacterial species, with large interindividual variability (Harmsen et al 2000). Others have shown a diverse and dominant bifidobacteria population that generally persists for several months with the cessation of breast-feeding (Roger et al 2010). In contrast, studies have found total bifidobacteria colonization did not differ
between breastfed and formula-fed, but exclusively formula-fed infants were more often colonized with *E. coli* and *C. difficile* at one month (Penders et al 2005). In another study, exclusively formula-fed infants were more often colonized with *E. coli, C. difficile, Bacteroides fragilis* and *Lactobacilli*, compared with breast-fed infants at one month of age (Penders et al 2006).

Preterm infants are initially colonized similarly to term infants at birth. However, several studies have shown delayed colonization with bifidobacteria and *Lactobacilli* (Schwiertz et al 2003; Gewolb et al 1999; Sakata et al 1985; Blakey et al 1982). Introduction of human milk in preterm infants favors development of a diverse microbiota over time with significant effects by one month (Gewolb et al 1999). Delayed introduction of non-pasteurized human milk, a common practice in the preterm population, may cause delayed colonization (Björkström et al 2009). Increased hygienic procedures, use of antiseptics and antibiotic prophylaxis are also a cause of delayed gut colonization in newborns in industrialized countries (Jauréguy et al 2004). Delayed establishment of anaerobic bacteria in the preterm infant may also be related to antibiotic treatments to the infant, prolonged hospitalization and exposure to nosocomial microbes in the neonatal intensive care unit. Oral administration of antibiotics to infants during the first month of life results in decreased numbers of bifidobacteria and *Bacteroides* (Penders et al 2006). Generally, human milk feeding favors more diverse microbiota over time, while antibiotic treatments reduce microbial diversity, with more days on antibiotic leading to a more significant reduction (Gewolb et al 1999; Bennet et al 1986).
B. Mucosal Immune System

Appropriate microbial colonization of the gastrointestinal tract plays an important role in the postnatal development of the gut-associated lymphoid tissue (GALT), which comprise of lymphoid structures throughout the mucosa and submucosa of the gastrointestinal tract. GALT is comprised of Peyer’s patch (PP) structures which contain follicular B-cell and interfollicular T cell areas in the small intestine, other organized tissues such as the mesenteric lymph nodes (MLN), and scattered lymphocytes in the intestinal lamina propria and epithelium (Forchielli et al 2005). PP contains microfold cells (M cells) on their surface that have the unique ability to internalize microorganisms and macromolecules for transport to underlying lymphoid tissue and antigen presenting cells, such as MØ and DC (Man et al 2004). Antigens are presented using PRRs to local T lymphocytes in the PP or migrate through the lymphatic system to present antigens at the MLN.

The fetal human small intestine contains an appreciable number of PP by wk 30 gestation, however the germinal centers develop after birth when intestines are exposed to commensal bacteria and antigens (Cornes 1965). Human PP follicles contain primarily CD4+ T cells and when cultured with anti-CD3/CD28 promoted Th1 cytokines, suggesting developed PP are components of the Th1 response (Junker et al 2009). Infants are born with a Th2 bias making them especially vulnerable to pathogens at birth. The early neonatal microbiota interacts with the immature mucosal immune system as a first step toward immune development at the GALT. Infants with delayed microbial colonization, such as premature infants or infants receiving antibiotic treatment, are at a
disadvantage in response to pathogens due a skewed commensal microbiota which may adversely affect immune function and development (Dimmitt et al 2010).

Microbe interactions with the intestinal epithelium occur primarily with the surface sensory enteroendocrine cells (EEC) in the small intestine. EEC have endocrine functions, such as secretion of cholecystokinin (CCK), and a paracrine effect on vagal afferent nerve fibers. Human and mouse EEC express TLRs that release CCK when stimulated, enabling EEC to serve as innate immunity sensors in the GI (Selleri et al 2008; Bogunovic et al 2007; Palazzo et al 2007). Additionally, CCK plays a role in clearing pathogens from the intestine by increasing gastric emptying in the distal small intestine, and TLR stimulated EEC also release antimicrobial peptides, such as defensins, involved in neutralizing intestinal bacteria thus microbe-EEC interactions play a valuable role in intestinal homeostasis (Palazzo et al 2007). TLR-stimulated EEC in vitro activate NF-κβ and MAPK pathways, leading to an upregulation of downstream inflammatory cytokines, such as TNFα, and chemokines, such as IL-8 (Bogunovic et al 2007). TLR activated EEC also secrete significant amounts of TGF-β, thought to inhibit the immune response and maintain mucosal homeostasis. Therefore, stimulated EEC exert a regulatory role at the intestine (Bogunovic et al 2007).

III. Bioactive Milk Components

A. Lactoferrin

Bifidobacteria are the predominant microorganism in breast-fed infant gut. At weaning, the introduction of solid food and the withdrawal of human milk shift the infant to a more diverse microbiota that resembles that of formula-fed infants (Favier et al
There are several bifidogenic factors in human milk, rather than one contributing factor; these include lactoferrin (Lf), prebiotics, such as human milk oligosaccharides (HMO) and probiotics. High concentrations of lactoferrin, an iron-binding glycoprotein (Figure 1.3; Vogel et al 2012) and the major whey protein in human milk, is present in early milk (<28 days lactation) at approximately 6g/L and at 2g/L in mature milk (Hirai et al 1990). Human and bovine Lf share a high sequence homology (Figure 1.3; Table 1.1), allowing bLf to be used in infant formula. Lf is also present in various secretions in addition to breast milk, including tears, saliva, and degranulated neutrophils (Adlerova et al, 2008). The concentration of Lf in different biological fluids is shown in Table 1.2. Lf promotes the growth of bifidobacterium spp. in vitro, especially B. longum subsp. infantis (Rahman et al 2008; Kim et al 2004; Liepke et al 2002; Petschow et al 1999). In addition, Lf inhibits the growth of gram positive and gram negative pathogens in vitro, such as Listeria monocytogenes, S. aureus, Salmonella Typhimurium and E. coli (Tian et al 2010). In vitro studies have shown that bovine Lf (bLf) interacts with B. infantis by way of Lf binding proteins in the cell wall of bifidobacteria, and this binding promotes the growth of bifidobacteria in vitro (Rahman et al 2007). Lf supplementation in weanling pigs led to increased colonic Lactobacillus and Bifidobacterium, and reduced counts of E. coli and Salmonella (Wang et al 2007). In vivo human studies to date have shown a modest beneficial effect of bLf alone in promoting bifidobacteria, however a low dose Lf was used (0.1-1g/L), which is lower than that in human milk (Roberts et al 1992). The ability of bifidobacteria to colonize the intestine of breast-fed infants likely depends on the interaction of synergistic factors found in human milk, including bifidobacteria itself.
It has been hypothesized that Lf may play a role in gastrointestinal development, by stimulating cell division, as evidenced by increased thymidine incorporation (Nichols et al 1990; Nichols et al 1987; Amouric et al 1984; Buccigrossi et al 2007). Thus far, only in vitro studies have been used to study Lf as a potential mitogenic factor. One of the more well cited studies looked at rat crypt cells (Nichols et al, 1987). Crypt cells incubated with colostrum, Lf standards and Lf isolated from human milk showed an increased percentage of $^3$H-thymidine incorporation into DNA. In another study by Nichols et al, rat crypt cells showed increased $^3$H-thymidine incorporation in those cells incubated with human milk and Lf supplementation relative to commercial cow milk and soy formulas (Nichols et al 1989). This study was inspired by a clinical study which had looked at babies with acquired monosaccharide intolerance. Based on biopsy results, the infants had villus atrophy leading to reduced surface area for absorption of essential nutrients. The formulas used for re-feeding suppressed DNA synthesis and this was overcome by a degree with the supplementation of Lf (Nichols et al 1989).

In a recent in vivo study, calves supplemented with Lf showed a significant increase in villus height and area, which has been correlated with an increase in crypt cell proliferation and decreased apoptosis (Prgomet et al 2007). In vivo studies have also shown lactoferrin is rapidly cleared from circulation via a specific receptor-dependent mechanism by intestinal enterocytes. These specific receptors have been identified in the pig (Gislason, 1993). Given the potential for Lf to enter the enterocyte, it may be a contributing factor to increased cell proliferation leading to gut maturation in colostrum-fed animals. An in vitro study was conducted by Buccigrossi et al that showed bLf may
be an even more potent additive than human Lf. Their study concluded that the use of
bLF could be used as a functional component of infant formula (Buccigrossi et al 2007).

Lf’s structure makes it resistant to proteolytic degradation, which allows it to pass
through the gastrointestinal tract undegraded and substantial quantities of Lf are found in
the stool and urine of breast fed infants (Hutchens et al 1991; Davidson and Lönnerdal,
1987). Lf is transported into systemic circulation from the intestinal epithelium in vivo in
the rat (Takeuchi et al 2004), neonatal pig (Harada et al, 1999), and newborn calf
(Talukder et al 2002). Orally-administered bLf was transported into the blood and
excreted into bile intact in neonatal piglets, likely through a receptor-mediated
transcytosis (Harada et al 1999). These intestinal Lf receptors (LfR) have been
characterized in the pig and are expressed at the enterocyte brush border membrane (Liao
et al 2007). The LfR is homologous to a protein called intelectin-1, which is also
expressed in mice, cows and humans (Blease et al 2009; Wrackmeyer et al 2006). The
LfR has also been characterized in human fetal intestinal brush-border membranes
(Kawakami and Lönnerdal, 1991). Intelectin is also expressed on intestinal paneth and
goblet cells in pigs, where it is likely involved in the development of local host-defense
mechanisms (Wrackmeyer et al 2006).

The density of LfRs and their binding affinity at the small intestine and colon was
highest in the epithelium overlying PP in the jejunum and ileum (Talukder and Harada,
2006). LfR has also been identified on T and B-cell lymphocytes, NK cells, Mø, and DC
(Actor et al 2009). Lf uptake by CD3+ T cells in the lamina propria has been
demonstrated in vitro, suggesting one mechanism by which Lf activates underlying T
cells of the small intestine (Nielsen et al 2010). A clinical study in preterm (~30 weeks)
very low birth weight (<1500g) infants was conducted at eleven tertiary Italian neonatal intensive care units. Infants supplemented with bLf had decreased incidence of blood-borne infection of bacterial, viral and fungal origin, and significantly reduced incidence of late-onset sepsis. (Manzoni et al 2009). bLf administered at 100mg/day decreased blood cultures for common pathogens, including gram-positive Staphylococcus spp. The study also found lower weight infants (<1000g) derived the most protective effect from bLf. The dose used in this study was effective for low weight infants, however, an adjusted dose would likely be used in larger infants (Manzoni et al 2009). This study supports the safe and efficacious use of bLf in the neonate population.

**B. B. infantis**

*Bifidobacterium* is the predominant genera in the intestinal microbiota of infants, and the most common species reported in breast-fed infants include *B. infantis, B. longum, B. bifidum* and *B. breve* (Rinne et al 2005; Matsuki et al 1999). Breast milk itself contains probiotic bifidobacteria, with *B. longum* being the predominant species, including specifically *B. longum* subsp. *longum* and *B. longum* subsp. *infantis* (Grönlund et al 2007; Gueimonde et al 2007). *B. bifidum, B. breve* and *B. infantis* have demonstrated anti-inflammatory properties at the intestinal epithelium and have been shown to induce the anti-inflammatory cytokine IL-10 *in vitro* (Chichlowski et al 2012; He et al 2002). Oral consumption of *B. infantis* in human subjects for eight wks increased IL-10 secretion and induced Foxp3 regulatory CD4+ T cell expression in human PBMC stimulated *ex vivo* with anti-CD3/CD28, suggesting the possibility of cross talk between the gastrointestinal mucosa and the immune system (Konieczna et al 2012). These results
have been previously observed in mice supplemented with *B. infantis* in a systemic LPS model. Mice showed reduced NF-kβ activation *in vivo* and increased expression of Foxp3 regulatory CD4+ T cells locally and systemically (O’Mahony et al 2008).

IV. *Staphylococcus aureus*

A. *S. aureus* colonization and Emergence of Resistant Strains

*S. aureus* is an opportunistic bacterial pathogen and has survival strategies which allow the pathogen to colonize skin and mucous membranes to persist. The pathogen may become virulent at any time upon entering the blood stream or when a susceptible individual is exposed to *S. aureus*; i.e., recent hospitalization or surgery, residence in a long-term-care facility, or use of injected drugs. While most of the time *S. aureus* is a commensal organism, its ability to persist provides a source from which bacteria can be introduced when host defenses are breached, whether by shaving, insertion of a catheter or surgery. Those with *S. aureus* infections are generally infected with their colonizing strain. In a multicenter study of *S. aureus*, bacteremia blood isolates were found to be identical to nasal isolates in 82% of patients (von Eiff et al 2001). The colonization of *S. aureus* presents a complication in the health care and community settings where treatment is challenging due to the evolution of antibiotic resistant strains and therefore exposure to this pathogen becomes a health threat in susceptible populations.

*S. aureus* has developed resistance to a range of antimicrobial drugs, such as beta-lactams, aminoglycosides and macrolides, which complicates treatment and control of staphylococcal infections. Methicillin was introduced in 1959 to treat infections caused by penicillin-resistant *S. aureus* and within two years there were reported cases of
methicillin resistance, known as methicillin-resistant *S. aureus* (MRSA). *S. aureus* becomes MRSA through the acquisition of a DNA fragment known as staphylococcal chromosome cassette mec (SCCmec) containing methicillin resistance determinant mecA. MRSA strains are a major concern and the prevention of staphylococcal infections to reduce the spread and emergence of MRSA is essential. Vancomycin is one of the last therapeutic options available for MRSA infections; however, clinical isolates of vancomycin-resistant *S. aureus* have emerged (CDC 2011) and this further complicates *S. aureus* in nosocomial infections and community-acquired infections. Reduced susceptibility to vancomycin would render the pathogen potentially resistant to all available antimicrobials.

**B. *S. aureus* evasion of the Immune System and Virulence Factors**

Interference of *S. aureus* with wound-healing mechanisms is thought to depend on virulence factors. Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) are surface proteins that mediate adherence to host tissue. MSCRAMMs play a key role in initiation of *S. aureus* infections including endovascular infections, bone and joint infections, and prosthetic-device infections. Once *S. aureus* adheres to host tissues or prosthetic materials, it is able to grow and persist by forming biofilms which enable *S. aureus* to evade the host defense. *S. aureus* biofilms have been shown to evade TLR2 and TLR9 activation *in vivo* in a mouse model, and in-dwelling devices such as catheters are especially susceptible to pathogen protective biofilms (Thurlow et al 2011). Studies also show that *S. aureus* can invade and survive inside neonatal endothelial cells *in vitro* (Ogawa et al 1985). *S. aureus* forms small-colony variants
(SCVs) that have been shown to hide in host cells, without causing damage or signs of infection while remaining protected from antibiotics and host defenses. SCVs have been observed in patients with relapsed *S. aureus* infection, indicating dormant *S. aureus* can become virulent and cause recurrent infections (Proctor et al 1995). MSCRAMMs are under the control of accessory gene regulator (agr) (Novick et al 1993); and up-or-down regulating agr leads to secretion of proteins, including toxins, hemolysins and surface proteins, such as protein A (Traber et al 2008).

IgG-binding regions of protein A have been identified (Uhlén et al 1984). One way *S. aureus* evades the host immune system is when protein A binds the Fc portion of immunoglobulins, prevents opsonization and phagocytosis (Palmqvist et al 2002). Most clinical isolates of *S. aureus* also produce antiphagocytic microcapsules that protect *S. aureus* from opsonization by complement and phagocytic uptake by human neutrophils (O’Riordan and Lee, 2004). *S. aureus* also secretes chemotaxis inhibitory protein of *S. aureus* (CHIPS) and inhibits chemoattractants C5a- and formylated peptides, such as fMLP, which lead to phagocyte activation of neutrophils and monocytes (de Haas et al 2004). *S. aureus* secretes leukotoxins, such as γ-hemolysins, Panton-Valentine (PVL), and leukocidins (LukE/LukD and M/F-PV-like) that are pore-forming toxins and damage leukocyte membranes (Menestrina et al 2003). Lastly, *S. aureus* has evolved mechanism for scavenging iron, essential to bacterial survival. *S. aureus* has iron surface determinant (Isd) systems that allow for heme-uptake from iron-binding proteins. Hemoglobin is released from red blood cells by staphylococcal hemolysins, bound to Isd staphylococcal surface receptors and heme is transported via Isd proteins into the bacterial cytoplasm to degrade and release free iron (Lowy 2011; Skaar and Schneewind, 2004).
V. Piglet as a Model for the Proposed Research

The piglet is considered the best model for the human infant in terms of physiology, immune response and anatomy (Odle et al 2014; Meurens et al 2012). Like humans, the pig is a large, monogastric omnivore with highly comparable gastrointestinal anatomy. Furthermore, piglets are a heterogeneous genetic population which is more representative of the human condition than laboratory rodents. At birth, the expansion of lymphocytes in the piglet secondary lymphoid organs is related to colonization of the gastrointestinal tract which drives the development of adaptive immune response and production of immunoglobulins (Butler et al 2009). Piglets are capable of reproducing the gradual pathophysiologic changes and mimic clinical characteristics of neonatal sepsis (Kato et al 2004). *S. aureus* is a dominant cause of widespread septicemia in pigs and humans (Jensen et al 2010). Strains of *S. aureus* have been known to cross-over from pigs to pig farmers. These community strains complicate hospital care (Morcillo et al 2012). *S. aureus* (strain: S54F9), the strain we will be using in our studies, has been used in an intravenous inoculation in 8-wk-old pigs at $10^8$ CFU/ml at 1ml per kg body weight (Leifsson et al 2010; Nielsen et al 2009). We have established a novel model of systemic *S. aureus* infection in piglets which will be presented in our preliminary work.
Figure 1.1 The two branches of the immune system and the cells associated with each branch (Schiffrin et al 2014).
Figure 1.2 The two branches of the immune system and the timing of the response (Abbas et al 2007).
Figure 1.3 Crystal structures of bovine and human Lf, an 80kDa iron-binding glycoprotein of the transferrin family (Vogel et al 2012).
Table 1.1 Number of amino acid residues in bovine and human Lf

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Bovine milk</th>
<th>Human milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>67</td>
<td>63</td>
</tr>
<tr>
<td>Proline</td>
<td>30</td>
<td>35</td>
</tr>
<tr>
<td>Arginine</td>
<td>39</td>
<td>43</td>
</tr>
<tr>
<td>Lysine</td>
<td>54</td>
<td>46</td>
</tr>
<tr>
<td>Asparagine</td>
<td>29</td>
<td>33</td>
</tr>
<tr>
<td>Valine</td>
<td>47</td>
<td>48</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>Cysteine</td>
<td>34</td>
<td>32</td>
</tr>
<tr>
<td>Threonine</td>
<td>36</td>
<td>31</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>Serine</td>
<td>45</td>
<td>50</td>
</tr>
<tr>
<td>Glutamine</td>
<td>29</td>
<td>27</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>40</td>
<td>42</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>27</td>
<td>30</td>
</tr>
<tr>
<td>Methionine</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Leucine</td>
<td>65</td>
<td>58</td>
</tr>
<tr>
<td>Glycine</td>
<td>48</td>
<td>54</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>22</td>
<td>21</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>36</td>
<td>38</td>
</tr>
<tr>
<td>Histidine</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td><strong>Tot # of residues</strong></td>
<td><strong>689</strong></td>
<td><strong>691</strong></td>
</tr>
</tbody>
</table>

Table 1.2 Occurrence of Lf in biological fluids and cow’s milk.

<table>
<thead>
<tr>
<th>Biological fluid</th>
<th>Amounts reported</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colostral breast milk</td>
<td>&gt;7 g/L</td>
</tr>
<tr>
<td>Mature breast milk</td>
<td>&gt;1-2 g/L</td>
</tr>
<tr>
<td>Tear fluid</td>
<td>&gt;2.2 g/L</td>
</tr>
<tr>
<td>Seminal plasma</td>
<td>&gt;0.4-1.9 g/L</td>
</tr>
<tr>
<td>Synovial fluid</td>
<td>&gt;10-80 mg/L</td>
</tr>
<tr>
<td>Saliva</td>
<td>&gt;7-10 mg/ml</td>
</tr>
<tr>
<td>Cow’s colostral whey</td>
<td>&gt;1.5 g/L</td>
</tr>
<tr>
<td>Cow’s milk</td>
<td>&gt;20-200 mg/L</td>
</tr>
</tbody>
</table>

CHAPTER 2

RESEARCH OBJECTIVES, SPECIFIC AIMS, AND HYPOTHESES

The **overall objective** of this research was to investigate the impact of bovine Lf (bLf) supplementation on gastrointestinal development and how bLf and *B. infantis* combined mediate the immune response during a systemic *Staphylococcus aureus* (*S. aureus*) infection to promote bacterial clearance. *S. aureus* is the leading cause of bacterial infections worldwide, including skin and soft tissue infections, lower respiratory tract infections, blood-stream infections, osteomyelitis and endocarditis (Graves et al 2010). Methicillin-resistant *S. aureus* (MRSA) is a gram-positive bacterium resistant to first-line antibiotic treatment. MRSA is a major pathogen in neonatal intensive care units (NICU) leading to septicemia (Maraqa et al 2011; Carey et al 2010). There is a growing need for non-antibiotic means of treating *S. aureus* infection.

A recent clinical trial and subsequent meta-analysis showed that Lf, a bioactive protein found in human milk, reduced septicemia to *Staphylococcus* species in premature infants when supplemented in infant formula (Manzoni et al 2009). Lf strengthens host immune defense against bacterial infections and is known for its anti-microbial properties (Tomita et al 2009; Wakabayashi et al 2006). When added to the diet, Lf reduced systemic *S. aureus* load in *S. aureus* challenged mice (Bhimani et al 1999). Lf-transgenic mice had enhanced Th1 response to *S. aureus* infection and these mice show enhanced IFN-γ response with increased ability to clear bacteria (Guillen et al 2002). *In vitro* work supports Lf’s role in recruiting neutrophils and macrophages, activating dendritic cells, and promoting a Th1 immune response during infection (de la Rosa et al 2008). Th1
response against *S. aureus* is protective through improved bacterial clearance (Miller and Cho, 2011).

Lf also promotes the growth of bifidobacteria spp., especially *B. infantis* (Rahman et al 2008; Kim et al 2004); and inhibits the growth of pathogenic bacteria *in vitro* (Tian et al 2010). *B. infantis* is a predominant bifidobacteria species in breast milk, and in the breast-fed infant intestine (Grönlund et al 2007), and is influential in reducing inflammatory markers and activating underlying immune cells of the gut (Corthesy et al 2007; Picard et al 2005). While the Th1 response leads to early inflammation which improves bacterial clearance, excessive inflammation over time becomes detrimental to the host. Supplementing with *B. infantis* could potentially reduce detrimental inflammatory processes. *B. infantis* supplemented mice in a systemic LPS model show reduced NF-kβ activation *in vivo* and increased Foxp3 T regulatory expressing cells locally and systemically (O’Mahony et al 2008). Oral *B. infantis* increased IL-10 secretion and induced Foxp3 regulatory CD4+ T cell expression in human PBMC (Konieczna et al 2012). Overall, beneficial bacteria that inhabit the intestine have a role in local immune cell homeostasis and regulation of systemic inflammatory responses (Hill et al 2011). Regulatory T-cell derived IL-10 is also essential for limiting inflammation activated during early infection (Delgoffe et al 2011).

In addition to Lf’s effects on the intestinal microbiota, research suggests Lf also has a trophic effect on the intestine. Diet is the most important factor modulating structure and function of the intestine during the postnatal period. The structural and functional development of the neonatal intestine has implications in improved digestive and absorptive capacity and host-defense (Barszcz and Skomial, 2011; Donovan 2006).
Previous *in vitro* studies have shown that Lf increased proliferation in porcine intestinal epithelial cells (Nguyen et al 2014), in rat crypt cells (Nichols et al 1987), and in Caco-2 cells (Buccigrossi et al 2007). Thus, in addition to its anti-microbial effects, Lf may impact host immunity through improved intestinal maturation.

Despite these known benefits, Lf and bifidobacteria are not currently added to infant formulas. Our *central hypothesis* was that Lf will improve intestinal maturation in the piglet and potentiate an early inflammatory response to a systemic *S. aureus* infection leading to resolution of infection, whereas *B. infantis* will promote a systemic T regulatory response to decrease sustained elevated inflammatory responses during *S. aureus* infection. **To test this hypothesis, three specific aims were undertaken.**

**Specific Aim 1.** Determine the effect of orally administered, supplemental bLf on gastrointestinal structural and functional development. The **objective** of this aim was to determine the effect of orally-administered bLf on the morphological and functional development of the neonatal piglet intestine. Our **working hypothesis** was that piglets fed bLf would have increased intestinal cell proliferation *in vivo*. We further hypothesized that increased proliferation would translate into improved villus morphology and digestive enzyme activity. In this study, piglets were divided into three dietary groups which included control formula (0.4g bLf/L), or formula with added bLf at either 1g bLf/L or 3.6 g bLf/L. All three diets were formulated to be isocaloric and isonitrogenous and the data is reported in Chapter 3 of this dissertation.
**Specific Aim 2.** Dietary bLf and *B. infantis* supplementation on *S. aureus* load and tissue cytokine expression in a systemic *S. aureus* infection in the neonatal piglet. The *objective* of this aim was to understand how bLf and *B. infantis* affect *S. aureus* load and tissue expression following *S. aureus* infection. Our *working hypothesis* was that piglets fed bLf would have decreased *S. aureus* load due to increased expression of IFNγ, and decreased expression of IL-10. We also hypothesized that *B. infantis* would increase expression of IL-10, which may be potentially detrimental. However, when combined with Lf, the interaction would provide the optimal balance of the inflammatory and anti-inflammatory responses. In this study, piglets were infected IV with *S. aureus* via an umbilical catheter using an established systemic *S. aureus* infection model as reported in Chapter 4. Piglets were randomized to receive formula containing 4g/L bLf or 4g/L whey supplemental protein; with half the piglets in each group further randomized to receive *B. infantis* (10⁹ CFU/day) by oral gavage. The data are reported in Chapter 5 of this dissertation.

**Specific Aim 3.** Dietary bLf and *B. infantis* supplementation on systemic immune response in an *S. aureus* infection in the neonatal piglet. The *objective* of this aim was to characterize the effect of dietary bLf and *B. infantis* on the early systemic immune response to *S. aureus* infection. Our *working hypothesis* was that piglets fed bLf would have an early inflammatory response followed by initiation of an early adaptive response with increased Th1 cytokine, IFNγ, and increased expression of Th1 differentiation marker expression, T-bet. We also hypothesized animals fed *B. infantis* would have increased Treg cytokine, IL-10, and increased Treg differentiation marker expression,
Foxp3, leading to a dampened immune response. Further, animals receiving both would have a balanced immune response, demonstrating both inflammatory and regulatory responses. The same experimental set-up was used for Aim’s 2 and 3, and the data for Aim 3 is reported in Chapter 6 of this dissertation.
CHAPTER 3:
DIETARY bLF INCREASES INTESTINAL CELL PROLIFERATION IN NEONATAL PIGLETS

Abstract
LF is a bioactive milk protein that stimulates cell proliferation \textit{in vitro}. However, limited \textit{in vivo} evidence exists to allow LF to be incorporated into infant formula. Herein, the effect of dietary bLF on neonatal intestinal growth and maturation was investigated. Colostrum-deprived pigs were fed formula containing 0.4 (CON), 1.0 (LF1) or 3.6 (LF3) g bLF/L for the first 7 or 14 days of life. To provide passive immunity, sow-serum was provided orally during the first 36h of life. Intestinal cell proliferation, histomorphology, mucosal DNA concentration, enzyme activity, gene expression and fecal bLF content were measured. Intestinal enzyme activity, DNA concentration and villus length were unaffected by bLF. However, crypt proliferation was 1.6-fold greater in LF1 and LF3 than CON pigs, and crypt depth and area were 1.2-fold greater in LF3 than CON pigs. Crypt cells from LF3 pigs had 3.5-fold higher beta-catenin (\( \beta\)-catenin) mRNA expression than crypt cells from CON pigs. Lastly, feces of pigs fed bLF contained intact bLF suggesting that some bLF was resistant to digestion and could potentially affect intestinal proliferation through direct interaction with intestinal epithelial cells. This study is the first to show that dietary bLF stimulates crypt cell proliferation \textit{in vivo}. The increased \( \beta\)-catenin expression indicates that Wnt signaling may in part mediate the stimulatory effect of bLF on intestinal cell proliferation.
Introduction

The early postnatal period is a critical time for neonatal gastrointestinal (GI) structural and functional development. This process is induced by feeding and in turn promotes improved digestive and absorptive capacity and host-defense (Donovan 2006). Animals fed their own mother’s colostrum or milk have more rapid GI growth and development than those artificially-reared on formula, as evidenced by increased intestinal mucosa mass, DNA synthesis and digestive enzyme activity (Donovan 2006; Xu et al 2000; Heird et al 1984). Human milk contains many bioactive components that may contribute to its trophic effects on intestinal growth (Donovan 2006). One such component is LF, an iron-binding glycoprotein. LF is one of the major proteins in human milk with concentrations ranging from 6 g/L in early milk (<28 d lactation) to 2 g/L in mature milk (Rai et al 2014). LF is also present in bovine and sow milk at a substantially lower concentration with approximately 0.8 to 0.1 g LF/L in bovine (Sánchez et al 1988) and 1.6 to 0.4 g LF/L in porcine (Yang et al 2000) colostrum and mature milk, respectively. Nucleotide sequence analysis showed that porcine LF shares 72.6% and 70.7% overall amino acid sequence identity with bovine and human LF, respectively (Lydon et al 1992).

Previous in vitro studies have shown that human LF (hLF) increased enterocyte proliferation in rat crypt cells (Nichols et al 1987). More recently, hLF and bLF were shown to dose-dependently increase proliferation in Caco-2 cells (Buccigrossi et al 2007) and porcine intestinal epithelial cells (Nguyen et al 2014). Limited in vivo evidence exists supporting biological effects of orally administered hLF at high concentrations (12 g/L) in mice (Zhang et al 2001) and in weanling pigs (Wang et al 2006). Whether orally
administered bLF increases intestinal epithelial cell proliferation or intestinal structure or function has not been assessed in neonatal animals at physiologically-relevant doses. This is an important question as LF is one of the most predominant whey proteins in human milk (Lönnerdal et al 2014).

Therefore, the objective of this study was to determine the effect of orally-administered bLF on the morphological and functional development of the neonatal piglet intestine. Due to the positive effects of bLF on cell proliferation in vitro, we hypothesized that oral bLF would increase intestinal cell proliferation in vivo. We further hypothesized that increased proliferation would translate into improved villus morphology and digestive enzyme activity.

**Materials and Methods**

*Animal Protocol and Dietary Treatments*

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Illinois. The animal study was conducted as described by Comstock and colleagues (Comstock et al 2014). Briefly, colostrum-deprived piglets were randomized into one of three treatment groups varying in bLF content. Sow-milk replacer formula was made by Animix (Juneau, WI) with a Lf-enriched bovine whey (DVM; Veghel, The Netherlands). The three diets were control formula (0.4 g bLF/L (130 mg bLF/(kg/BW·d); CON, n=34), or formula with added bLF at either 1g bLF/L (367 mg bLF/(kg/BW·d); LF1; n=29) or 3.6 g bLF/L (1300 mg bLF/(kg/BW·d); LF3, n=29). The powdered diets were reconstituted as 20% solids (200 g/L) prior to feeding. All three diets were isocaloric and isonitrogenous and composition of diets has been
previously published (Comstock et al 2014). The bLF preparation was isolated from bovine milk and was 97% pure LF (DMV). The two LF concentrations were selected to reflect the mean dose of LF consumed by the breastfed human infant (LF1) or 5-times the dose on a mg/kg/BW·d basis (LF3). This dose assumed that human infants (~5.5 kg BW) consume about 1L human milk/d with an average LF concentration of 1.85 g/L (Ronayne et al 2000). Formula was offered 22 times daily at a rate of 360 mL/(kg/BW·d) for 7 or 14 days postpartum.

For the first 36h postpartum, piglets were oro-gastrically administered sow serum to provide IgG for passive immunity as previously described (Comstock et al 2014). Because piglets were colostrum-deprived, there was some mortality during the study which has been previously described and provided in a supplemental figure by Comstock et al 2014. Piglets were individually housed throughout the duration of the study in environmentally controlled rooms (25°C) capable of maintaining six piglets per cage separated by Plexiglas partitions. Radiant heaters were attached to the tops of the cages to maintain an ambient temperature of 30°C.

Sample Collection

Tissues were collected on d7 or d14 postpartum. Piglets were injected i.p. with 10mM 5-ethynyl-2'-deoxyuridine (EdU) at 1mL/kg BW 2h prior to euthanasia to assess intestinal proliferation. Pigs were euthanized and intestinal samples collected as previously described (13). The intestine was divided into segments: duodenum (Duo, first 10%), jejunum (Jej, middle 75%), and ileum (Ile, final 15%) to be measured and weighed. Sections (1-2cm) of Duo, Jej and Ile were taken and fixed in 10% formalin or
Optimal Cutting Temperature (OCT) freezing medium or immediately snap-frozen in liquid nitrogen. Fecal samples and mucosa from the Duo, Jej and Ile were collected and immediately snap-frozen.

**Fecal bLF analysis**

bLF in fecal samples was analyzed by semi-quantitative Western blot. After homogenization in PBS with protease inhibitor and extraction with 8M urea, 2mM PMSF, and 2mM DTT, samples were vacuum dried overnight. Samples were separated using reducing NuPAGE® Novex® SDS-PAGE 4-12% Bis-Tris gradient gel (Life Technologies, Grand Island, NY). Briefly, 195µg of sample was loaded into each well, and each gel included molecular weight markers and an LF standard (Sigma, St. Louis, MO). bLF was detected with a polyclonal goat anti-bLF antibody (Bethyl Labs, Montgomery, TX), followed by a secondary antibody (anti-goat conjugate IR800, Rockland, Gilbertsville, PA). Western blots were imaged using an Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE). Li-Cor Image Studio software was used to analyze the results. The units, percent of standard per µg of feces, were calculated by taking the sum of the intensity of bLF fragments in the sample divided by the intensity of the bLF standard multiplied by 100 and then divided by the quantity (µg) of sample loaded.

**Intestinal Histomorphology**

Villus and crypt histomorphology were assessed as previously described (Hester et al 2012). Briefly, formalin-fixed, paraffin-embedded sections (6µm) were stained with
haematoxylin and eosin by the Diagnostic Laboratory at the University of Illinois College of Veterinary Medicine (Urbana, IL). Digital images (20x) were obtained using the Nanozoomer Digital Pathology System (Hamamatsu, Bridgewater, NJ; University of Illinois Institute for Genomic Biology Microscopy and Imaging Facility). Images were evaluated using Axio Vision 4.8 (Zeiss, Germany). Quantitative measurements were made from 10-15 well-oriented crypt-villus systems in the Duo and Jej for villus height (μm), villus area (mm$^2$), crypt depth (μm) and crypt area (mm$^2$). An example image is shown in Figure 3.1.

Intestinal Proliferation

Formalin-fixed, paraffin-embedded Jej samples were sectioned (6μm) onto slides. Incorporation of EdU was detected ex vivo with a Click-iT EdU Cell Proliferation Kit (Life Technologies, Carlsbad, CA) using Alexa Fluorophore 555 for EdU labeling and 4',6-diamidino-2-phenylindole (DAPI) to label all nuclei allowing results to be standardized for total nuclearity. For proliferation, 6-8 images per animal were taken using the confocal microscope at 20x magnification, using z-stacks and 2x2 tiling at 512 x 512 in the x and y direction. EdU-incorporation was quantified using AxioVision fluorescence quantification software (Carl Zeiss, Thornwood, NY). Tiff image files for the DAPI and EdU channels were overlaid to create a DAPI+EdU image (Figure 3.2A). The crypt region was outlined and, based on color intensity within that area for blue (DAPI) or red (EdU), pixel area was measured and converted into μm$^2$ (at 0.49μm/pixel) (Figure 3.2B). The percent proliferation is expressed as the ratio of red area (area of proliferation) to blue area (total cellularity) multiplied by 100. After initial analysis of 4
animals per group, no differences in proliferation were observed between LF1 and LF3, thus remaining LF1 piglets were not analyzed.

**Mucosal Enzyme Activity and Protein and DNA Content**

**Lactase activity:** Mucosal homogenates were prepared as previously described (Hartke et al 2005). Briefly, 0.2 grams frozen mucosa from Duo, Jej and Ile were homogenized in 2 ml of homogenization buffer (0.9% saline solution, 223 μmol/L iodoacetic acid, 1 μmol/L phenylmethylsulfonylfluoride). Lactase activity was measured as previously described (Dudley et al 1994). Briefly, mucosal homogenates were incubated in 0.6M lactose buffer (dissolved in 0.0625M Maleate Buffer) for 60 minutes at 37 °C. The reaction was stopped by the addition of 2.0% zinc sulfate and 1.8% barium hydroxide, and the amount of glucose released was detected using a glucose oxidase reagent (Thermo Scientific, Middletown, VA). Lactase activity is expressed as μmol glucose released per min per mg of protein.

**Dipeptidylpeptidase IV (DPP IV) activity:** Peptidase activity was measured according to the method of Petersen and colleagues (Petersen et al 2002). Briefly, 0.2 grams frozen mucosa from Duo, Jej and Ile were homogenized into 2 ml of 1.0% Triton X-100 (Fisher Scientific, Pittsburgh, PA). DPP IV activity in the homogenate was quantified using a chromogenic substrate solution of 10mM α-L-glutamic acid 4-nitroanilide (Bachem Bioscience, Inc., King of Prussia, PA) in 50 mM Tris-HCl (pH=8.0). Using a spectrophotometer, homogenate enzyme activity was measured kinetically over six min at an absorbance of 405 nm. Enzyme activity was calculated using the Beer and Lambert Law by multiplying the slope of the absorbance values over
the six minutes by the dilution factor (range of 1:5-1:20) and a constant (4.34). Peptidase enzyme activity is expressed as U per mg of protein.

**Mucosal protein and DNA content:** A Bradford protein assay (Bio-Rad, Hercules, CA) was performed to measure mucosal protein content in the homogenates for lactase, peptidase and DNA content assays. To quantify double stranded DNA, mucosal homogenates were prepared as for the lactase assay. Homogenates were diluted in TE [Tris-EDTA, pH 7.5] buffer and incubated with PicoGreen (Life Technologies, Carlsbad, CA) for 5 min. PicoGreen fluoresces when it binds to double-stranded DNA. The fluorescence was quantified using a fluorometer (Molecular Devices, Sunnyvale, CA). Results are reported as absolute values as well as μg double-stranded DNA normalized by mg protein concentration using the Bradford results.

**Crypt cell mRNA expression**

Since no differences in proliferation were observed between LF1 and LF3, only the Ctrl and LF3 treatment groups were used for crypt cell mRNA analyses.

**Laser Capture Microdissection (LCM):** OCT-embedded Jej segments were cut in serial sections (8µm thick) using a cryostat, mounted onto PEN membrane slides (Arcturus, Life Technologies, Carlsbad, CA), and stored at −80°C until used for LCM. Frozen sections were fixed and stained using the HistoGene LCM frozen section staining kit (Arcturus) according to the manufacturer’s instructions. Staining was immediately followed by LCM performed on a Veritas LCM and Laser Cutting System (Molecular Devices, Sunnyvale, CA). Cells were collected from the crypt region of each slide onto separate CapSure HS LCM caps (Arcturus) as shown in Figure 3.3. Following LCM,
crypt regions were immediately processed using a PicoPure RNA isolation kit (Arcturus) according to the manufacturer’s instructions.

**Gene Expression by Quantitative Real Time Polymerase Chain Reaction (RT-qPCR):** Briefly, using Superscript VILO Master Mix (Invitrogen, Life Technologies), cDNA from crypt cell RNA was obtained following manufacturer’s recommended protocol. TaqMan PreAmp Master Mix (Applied Biosystems, Life Technologies) was used to amplify low abundance cDNA prior to qPCR. Crypt cell expression of cyclin D2 (CCND2; Ss03382534_s1), lactoferrin receptor/Intelectin-2 (ITLN2; Ss03374218_m1) and β-catenin (CTNNB1; Ss03394811_g1) were assessed using porcine specific TaqMan gene expression assays (Applied Biosystems, Life Technologies). 60S ribosomal protein L19 (RPL19; Ss03375624_g1) was used as an endogenous control. Amplification efficiency was equal among the targets. The relative standard curve method was used for quantitation. Standard curves consisted of dilutions of pooled whole tissue Jej cDNA. Normalized values for each target were calculated by dividing the target quantity mean by the RPL-19 quantity mean. A fold-difference was calculated for each measurement by dividing the normalized target values by the normalized calibrator sample. Tissue from the d7, CON animals was used as the calibrator in each instance.

**Statistical Analysis**

Statistical analyses were performed using SAS Version 9.2 (Cary, NC). BW was compared among groups by repeated-measures analysis of variance (ANOVA) using PROC MIXED. Sow had a significant effect on proliferation. Therefore, all data were analyzed by 2-way ANOVA using PROC MIXED with fixed effects of diet (CON vs.
LF1 vs. LF3) and day (d7 vs d14) and random effects of sow. In addition, fixed effect of intestinal region (Duo vs. Jej vs. Ile) was included for intestinal mass, lactase activity, DPP IV assay, PicoGreen DNA assay, and villus and crypt morphology. Post hoc testing was done using Least Squares Means with a Tukey adjustment to obtain an adjusted P value. Model assumptions of normality were tested before running the Proc Mixed model. If data were not normally distributed, they were either: 1) transformed to be normally distributed or 2) analyzed by non-parametric tests. Specifically, qPCR data for β-catenin expression was log-transformed to obtain a Shapiro Wilk P < 0.05. Fecal bLF was analyzed using a nonparametric Wilcoxon Two-sample test in SAS. Differences were considered significant at P<0.05 and trends at 0.05<P<0.10. Data are expressed as mean ± SEM.

Results

Formula intake and BW gain

Dietary bLF did not affect BW or daily weight gain (kg/d). Total formula intake was 5.15 ± 0.25 L (d0-d7) and 14.2 ± 0.54 L (d0-d14). Piglet BW on d7 and d14 were 2.49 ± 0.07 kg and 4.19 ± 0.11 kg, respectively. By d7, animals had gained an average of 0.94 ± 0.04 kg, and by d14, animals had gained an average of 2.62 ± 0.07 kg.

Dietary bLF intake and Fecal bLF excretion

The diets contained 0.33, 1.07 and 3.6 g bLF/L diet for CON, LF1, and LF3, respectively. Therefore, g bLF consumed per kg BW on d7 was 0.12, 0.39, and 1.31 for CON, LF1 and LF3, respectively. On d14, g bLF consumed per kg BW was 0.12, 0.43
and 1.32 for CON, LF1 and LF3, respectively. Fecal bLF was greater in LF1 and LF3 animals compared with CON (P<0.0001) and greater in LF3 animals compared with LF1 (P=0.0095) (Figure 3.4). No significant effect of day or interaction was observed.

*Intestinal Weight*

Intestinal mass (g) was normalized by intestinal length (cm) and by BW (kg) for statistical analysis. D14 animals had greater intestinal weight for length than d7 animals (P<0.0001). Effects of intestinal region were observed, wherein Ile weight per cm intestine was greater than Duo (P<.0001) or Jej (P=0.0022). No interactions between day and region or effect of treatment were observed. The mean intestinal weight per intestinal length for d7 animals was 0.17 ± 0.01g/cm, 0.20 ± 0.01g/cm and 0.22 ± 0.01g/cm for Duo, Jej and Ile sections, respectively. The mean intestinal weight per length for d14 animals was 0.26 ± 0.01g/cm, 0.26 ± 0.01g/cm and 0.30 ± 0.01g/cm for Duo, Jej and Ile sections, respectively.

When intestinal weight was normalized by BW, d14 animals had greater intestinal weight (g/kg) than d7 animals (P=0.0005). In addition, effects of intestinal region were observed, with differences between Duo and Jej, Jej and Ile, and Duo and Ile (P<.0001). The mean intestinal weight per kg BW for d7 animals was 3.91 ± 0.10, 34.72 ± 0.82, 7.59 ± 0.36 for Duo, Jej and Ile sections, respectively. The mean intestinal weight per kg BW for d14 animals was 4.90 ± 0.20, 37.06 ± 1.15, 8.23 ± 0.21 for Duo, Jej and Ile sections, respectively. Lastly, there was an overall effect of diet (P=0.0175) between CON and LF3 animals (P=0.013), with LF3 piglets having heavier intestinal weight per kg BW than CON animals. No interactions were observed.
**Histomorphology**

**Villi:** Villus length (P=0.0002) and area (P<0.0001) were greater at d14 than d7 (Table 3.1). There was also a significant regional effect, where Duo villi were shorter (P=0.0002) and had less area (P<0.0001) than Jej villi. Diet had no significant effect on villus length or area and no significant interactions were observed.

**Crypts:** Dietary bLF increased crypt depth (Figure 3.5) and crypt area (Figure 3.6) in the Duo and Jej. bLF-fed animals had deeper (LF3, P=0.0005; LF1, P=0.0019) and larger (LF3, P=0.0085; LF1, P=0.0351) crypts compared with CON, while LF1 and LF3 did not differ in depth (P=0.59) or area (P=0.56). D14 animals had significantly larger crypts than d7 animals in the Duo and Jej (Figure 3.5); however, no significant day effect was observed with crypt depth. Crypts in the Duo were deeper (P<0.0001) and larger (P<0.0001) than crypts in the Jej. No interactions were observed.

**Proliferation**

Proliferation was increased by dietary bLF (Figure 3.7). Animals consuming 1g/L (LF1, P=0.0018) or 3.6g/L bLF (LF3, P=0.0009) had increased Jej proliferation relative to CON animals. There was no difference in proliferation between animals consuming LF1 or LF3 (P=0.65) or between d7 and d14 animals (P=0.52).
Intestinal Enzyme Activity

**Lactase:** A significant day effect was observed where d7 animals had greater lactase activity (μmol glucose/min/g protein) than d14 animals (Table 3.2). Lactase activity in the Jej was greater than in the Duo (P<0.0001) and Ile (P<0.0001).

**DPP IV activity:** Neither diet nor day affected DPP IV activity (U/mg protein). However, DPP IV activity differed by intestinal region, with a distal to proximal gradient, wherein activity was significantly greater in the Ile, than Jej and Duo and Jej than Duo (Table 3.2).

Intestinal Mucosal Protein and DNA content

Mucosal DNA content, a marker of cellularity, was not affected by bLF consumption (P=0.57). Mucosal protein content was not affected by bLF consumption, day or region. Protein content was 66.89 ± 2.75, 59.83 ± 1.91, 63.96 ± 2.26 mg protein/g mucosa for Duo, Jej, and Ile samples, respectively. When DNA content was expressed relative to protein content (μg DNA/mg protein), there was a significant interaction between day and region. On d14, Duo samples contained more double-stranded DNA than Jej (P=0.0124) or Ile (P=0.0393) samples. Additionally, the concentration of double-stranded DNA was significantly greater in d7 Ile than d14 Ile samples. The average DNA content normalized by protein at d7 was 2.55 ± 0.20, 2.35 ± 0.11, 2.77 ± 0.13μg DNA/mg protein for Duo, Jej and Ile, respectively. The average DNA content normalized by protein at d14 was 2.48 ± 0.16, 1.87 ± 0.12, 1.79 ± 0.15μg DNA/mg protein for Duo, Jej and Ile sections, respectively.
Intestinal Crypt Cell mRNA Expression

β-catenin expression was not affected by animal age, but was affected by dietary intake. The crypt cells of LF3 animals expressed more β-catenin than the crypt cells of CON animals (Figure 3.8). Cyclin D2 and intelectin expression were not significantly different between CON and LF3 fed animals. The average Cyclin D2 expression for d7 animals was 1.0 ± 0.33 and 2.78 ± 1.18 fold difference for CON and LF3, respectively. The average Cyclin D2 expression for d14 animals was 0.76 ± 0.14 and 1.29 ± 0.65 fold difference for CON and LF3, respectively. The average intelectin expression for d7 animals was 1 ± 0.26 and 1.06 ± 0.31 fold difference for CON and LF3, respectively. The average intelectin expression for d14 animals was 1.13 ± 0.17 and 1.16 ± 0.19 fold difference for CON and LF3, respectively.

Discussion

Diet is a major factor regulating the growth and maturation of the neonatal small intestine (Cera et al 1988; Berseth et al 1983). As intestinal proliferation is a primary driver of intestinal growth and development, the goal of the current study was to determine whether dietary bLF would be bioactive in the neonatal piglet intestine. The piglet was selected as it is a well-accepted preclinical model for the human infant in terms of intestinal growth and function (Puiman et al 2008). Further, we selected dietary doses of bLF that represented those that a breastfed human infant could be exposed to (LF1) and a supraphysiological dose (LF3).

Previous research has shown that both hLF and bLF stimulate intestinal cell proliferation when added to culture medium in vitro (Nguyen et al 2014; Jiang et al 2012;
Nichols et al 1987). Moreover, mice suckling dams transgenic for hLF for 10d had greater intestinal mass, intestinal length and ratio of maltase-to-lactase activity in the duodenum compared to pups nursing non-transgenic dams (Zhang et al 2001). However, the mean concentration of hLF in the transgenic milk was 12 g/L, which is markedly higher than that found in mature human milk (2 g/L) (Rai et al 2014; Ronayne et al 2000). Additionally, weaned pigs fed 1 g LF/kg diet for 15 days had greater mean daily gain and villus height (Wang et al 2006). Weaning is associated with increased risk for diarrhea and impaired intestinal structure and function in pigs (Wijtten et al 2011).

These findings suggest that dietary LF could be beneficial for pigs during weaning. One aim of this study was to determine the impact of dietary LF on intestinal epithelial cell proliferation, and crypt and villous integrity, in vivo, during the early weaning period. The key findings of the current study were that piglets fed formula containing bLF for the first 14 days of life had increased jejunal intestinal crypt proliferation and crypt depth and area. Additionally, jejunal crypt cells isolated by LCM had increased β-catenin expression. The conserved Wnt/β-catenin pathway regulates stem cell pluripotency and cell fate decisions during development (Crosnier et al 2006). Although, ~80% of newly synthesized intestinal cells differentiate into enterocytes or absorptive cells that line the intestinal villi (Van der Flier et al 2009), no significant effect of dietary bLF on villus morphology or digestive enzyme activity was observed. As will be discussed below, this may be due to the length of the study being insufficient to observe migration and maturation of newly synthesized cells. This study and those described above suggest that dietary LF could be beneficial to pigs during weaning by
increasing intestinal epithelial cell proliferation and improving or protecting intestinal crypt and villous structure during this time of insult to the intestine.

LF is resistant to proteolytic degradation in early life; in particular, holo-LF, or the iron-bound form, is more resistant to degradation than apo-LF (Spik et al 1982). This also allows for some LF to pass through the GI tract and substantial quantities of LF have been found in the stool of breastfed infants (Davidson et al 1987). Immunologically and structurally intact bLF was detected in the piglet feces, suggesting that some bLF resisted digestion and could potentially interact with LF receptors (LFR) present in the piglet intestine.

We postulate that bLF stimulated proliferation by direct interaction with the LFR for the following reasons: The LFR has been localized to the crypt region of the small intestine (Cera et al 1988, Suzuki et al 2005) and LF has been shown to stimulate proliferation \textit{ex vivo} in isolated rat crypt cells (Nichols et al 1987) and \textit{in vitro} using cells of crypt origin (Jiang et al 2011). Lönnerdal and colleagues cloned the pig LFR (Liao et al 2007), which is similar to the human LFR (Suzuki et al 2001), and 100% homologous to intelectin-1. Intejectin-1 is present in lipid rafts and has been proposed to help stabilize the brush border membrane, preventing loss of digestive enzymes to the gut lumen and protecting the glycolipid domains from pathogens (Wrackmeyer et al 2006). The expression of intejectin-2 was assessed as this intejectin has been fully sequenced in the pig and shows 91% sequence homology with intejectin-1 (Pemberton et al 2004). The bovine intejectin receptor has also been sequenced (Blease et al 1993), but its homology to either the human or pig receptors has not been reported. Importantly, previous studies showing that bovine LF, but not bovine transferrin, inhibited the binding of pig milk LF
to LFR on brush border membrane vesicles isolated from piglet intestine (Gislason et al 1993), suggesting that bLF would interact with intestinal LFR in the piglet in vivo.

We recently showed that dietary bLF increased intelectin-2 mRNA expression in natural killer cells in piglets (Liu et al 2013). However, there was no effect of bLF on intestinal intelectin-2 mRNA expression in the current study. We did not measure receptor protein density or binding, thus cannot rule out effects of dietary bLF on intestinal LFR abundance. Liao and coworkers reported that LFR protein abundance increased significantly from 1 wk to 3 wks of age in the piglet duodenum, whereas no change with age was observed for the jejunum. Interestingly, at 1 wk of age, most of the LFR was localized intracellularly, rather than on the brush border membrane (Liao et al 2007). This may be due to the fact that both apo- and holo-LF are taken up by Caco-2 cells via clathrin-mediated endocytosis (Jiang et al 2011). Uptake of LF-intelectin complexes via an endosome were detected immunochemically as punctate signals within the cells recently by Akiyama et al (Akiyama et al 2013). In addition, LF has been shown to translocate into the nucleus and bind to specific DNA binding sites thereby regulating gene transcription and influencing cell proliferation and differentiation (Liao et al 2012). Taken together, these previous observations suggests a mechanism by which ingested LF effects intestinal proliferation by first being internalized at the intestinal epithelium, followed by nuclear translocation to transcriptionally regulate intestinal proliferation (Akiyama et al 2013; Liao et al 2012; Jiang et al 2011).

Typically, in bovine and human milk, only 10-20% of LF is holo-LF, whereas most is in the unsaturated state (apo-LF) (Lönnerdal et al 2011). In the current study, the bLF was incorporated into formulas containing 120 mg iron/kg diet (Comstock et al
Since bLF can bind additional iron in vitro (Lönnerdal et al. 2011), it is possible that some bLF could bind the formula iron within the formula or piglet intestine, forming a high proportion of holo-LF. The two forms of LF appear to induce cell proliferation through different signaling pathways. For example, treating Caco-2 cells with either apo- or holo-LF activated the PI3K/Akt signaling pathway, while only apo-LF increased ERK1 signaling (Jiang et al. 2012).

The MAP kinase (MAPK) pathway that includes signaling through ERK1 and ERK2 is most commonly associated with LF’s effect on intestinal proliferation (Jiang et al. 2012; Lönnerdal et al. 2011). However, MAPKs can also cross-talk with other signaling pathways. One such pathway is the Wnt/β-catenin pathway, which is also known to regulate cell proliferation (Krejci et al. 2012). In addition, it has been suggested that the ERK pathway acts independently of the PI3K/Akt pathway to activate Wnt/β-catenin signaling (Krejci et al. 2012). The Wnt/β-catenin signaling pathway is a highly conserved mammalian pathway known as the primary driving force behind proliferation of intestinal epithelial cells (Van der Flier et al. 2009). Specifically, β-catenin is the central player regulated by Wnt, and activation of the Wnt pathway leads to larger crypts through expression of β-catenin (Crosnier et al. 2006). We hypothesized that bLF may affect the Wnt canonical signaling pathway through increased transcriptional β-catenin expression, and our results demonstrate this is likely one mechanism by which bLF increased crypt proliferation as well as crypt size. However, future studies in which nuclear translocation of β-catenin is directly measured is needed to verify this pathway.

Triggering of the MAPK pathway could affect expression of downstream molecules, such as cyclin D, that regulate cell cycle progression to the S-phase (Jiang et
al 2012). However, we failed to detect changes in cyclin expression of crypt cells. Up-regulated cyclin D1 transcription by LF has been observed in vitro, and these effects on cyclin D1 were blocked in the presence of a PI3K or ERK inhibitor, confirming the downstream effects of LF (Jiang et al 2012). It is possible that changes in cyclin proteins are more difficult to detect in vivo as cells are at various stages of the cell cycle, whereas in vitro cells would be mostly synchronized after the addition of LF to the media.

Surprisingly, dietary bLF did not affect villus height or hallmarks of intestinal development such as disaccharidase and peptidase activity, as previously demonstrated in response to dietary LF (Wang et al 2006; Zhang et al 2001). The lack of response in these outcomes could be due to the short duration of the study. Newly synthesized cells typically displace older, functionally differentiated cells as they migrate up the villus. In the piglet, it takes a minimum of 1-2 wks for intestinal epithelium turnover following birth (Moon et al 1971). It has been reported to take up to 3 wks for complete cell turnover (Skyrzypek et al 2005; Smith et al 1978), leading to increased absorptive ability and functional maturity in the young gut by 3 wks postnatal age. Continuing the study to d21 may have enabled detection of LF effects on villus height and lactase and DPP IV activities.

In summary, this study supports in vitro studies that show LF stimulates proliferation and is the first to show that milk-borne bLF stimulates crypt cell proliferation in neonatal pigs. The use of the piglet model in this research allows for these findings to be applicable to humans. Like humans, the pig is a monogastric omnivore with highly comparable gastrointestinal anatomy (Meurens et al 2012). The American Academy of Pediatrics recommends infants be breastfed exclusively during the first 6 months of life,
however approximately 20% of infants in the United States never receive breast milk (CDC, 2013). The inclusion of bLF in infant formula could provide the formula fed infant with a diet closer to that of human milk and may contribute to intestinal development during the neonatal period.
Figure 3.1 Representative image of a crypt-villus system used for histomorphology measurements taken in AxioVision 4.8 (Zeiss, Germany). Quantitative measurements were made in the Duo and Jej for villus height (μm), villus area (μm²), crypt depth (μm) and crypt area (μm²).
Figure 3.2 Images taken on the confocal microscope and saved as tiff image files for the DAPI and EdU channels were overlaid to create a DAPI+EdU image using AxioVision fluorescence quantification software (Figure 3.2A). The crypt region was outlined and, based on color intensity within that area for DAPI or EdU, pixel area was measured and converted into μm² (Figure 3.2B).
Figure 3.3 Laser capture microdissection (LCM) was used to capture crypts from OCT-embedded Jej sections. Sections were cut and mounted onto PEN membrane slides, frozen sections were fixed and stained prior to LCM (Figure 3.3A). Cells were collected from crypt region of each slide onto a CapSure HS LCM cap (Figure 3.3B). Figure 3.3C shows an image of an HS LCM cap with isolated crypt cells.
Figure 3.4 Fecal bLF excretion in piglets fed formula containing 0.4 (CON), 1.0 (LF1) or 3.6 (LF3) g bLF/L for 14 d. Results are expressed as a percent of bLF standard normalized by µg dry weight of feces. There was no effect of day, therefore values were pooled by diet. Values are means ± SEM, n= 10-13 per diet. bLF, bovine lactoferrin
Figure 3.5 Crypt depth (μm) of duodenum (Figure 3.5A) and jejunum (Figure 3.5B) of piglets fed formula containing 0.4 (CON), 1.0 (LF1) or 3.6 (LF3) g bLF/L. There was no effect of day, so values were pooled by diet. Values are means ± SEM, n= 10-13 per diet by day. bLF, bovine lactoferrin
Figure 3.6 Crypt area (mm²) of duodenum (Figure 3.6A) and jejunum (Figure 3.6B) of piglets fed formula containing 0.4 (CON), 1.0 (LF1) or 3.6 (LF3) g bLF/L for 7 or 14 d. Values are means ± SEM, n= 10-13 per diet by day. bLF, bovine lactoferrin
Figure 3.7 Intestinal proliferation in the jejunum of piglets fed 0.4 (CON), 1.0 (LF1) or 3.6 (LF3) g bLF/L. (Figure 3.7A) Representative images of intestinal sections illustrating fluorescent detection of EdU+ proliferating cells and DAPI+ nuclei. The crypt region was outlined and, based on color intensity within that area for blue (DAPI+) or red (EdU), pixel area was measured and converted into μm² (at 0.49μm/pixel). (Figure 3.7B) The percent proliferation is expressed as the ratio of the area in red (area of proliferation) to the area in blue (total nuclei) multiplied by 100%. Because there was no effect of day, values were pooled by diet. Values are means ± SEM, n= 7-10 for CON and LF3 and n= 3-4 for LF1. bLF, bovine lactoferrin; DAPI, 4’,6-diamidino-2-phenylindole; EdU, 5-ethynyl-2’-deoxyuridine
Figure 3.8 Taqman porcine specific CTNNB1 (β-catenin) mRNA expression was measured in crypt cells isolated from the jejunum of piglets fed 0.4 (CON) or 3.6 (LF3) g bLF/L. Crypt cells were isolated by laser capture microdissection. RPL-19 was used as an endogenous control to normalize the expression of β-catenin. Results are expressed as fold difference relative to control d7 animals. Because there was no effect of day, values were pooled by diet. Values are means ± SEM, n= 5-6 for CON and LF3
Table 3.1. Villus length and area in the duodenum and jejunum of d7 and d14 piglets

<table>
<thead>
<tr>
<th></th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>P-value by Region</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Villus Length (µm)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d7*</td>
<td>514 ± 18.7</td>
<td>673 ± 29.2</td>
<td>P&lt;.0001</td>
</tr>
<tr>
<td>d14</td>
<td>632 ± 30.2</td>
<td>811 ± 50.0</td>
<td>P&lt;.0001</td>
</tr>
<tr>
<td><strong>Villus Area (mm²)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d7*</td>
<td>58.0 ± 2.60</td>
<td>70.0 ± 3.37</td>
<td>P=0.0037</td>
</tr>
<tr>
<td>d14</td>
<td>81.5 ± 5.28</td>
<td>98.0 ± 7.39</td>
<td>P=0.0037</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n= 10-13 per region by day.

Villus length and crypt depth differed by region at both d7 and d14. *In addition, there was an effect of day, independent of region for both measures (P <0.05)
Table 3.2 Lactase and dipeptidyl peptidase IV activity in the duodenum, jejunum and ileum of d7 and d14 piglets.

<table>
<thead>
<tr>
<th></th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>Ileum</th>
<th>P-value by Region</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lactase</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(μmol glucose/min/g protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d7*</td>
<td>185±23.2a</td>
<td>331±28.7b</td>
<td>162±27.1a</td>
<td>P&lt;.0001</td>
</tr>
<tr>
<td>d14</td>
<td>151±14.1a</td>
<td>320±31.7b</td>
<td>85.7±13.6a</td>
<td>P&lt;.0001</td>
</tr>
<tr>
<td><strong>Dipeptidyl peptidase IV</strong></td>
<td>1.52±0.08a</td>
<td>2.32±0.10b</td>
<td>2.96±0.15c</td>
<td>P&lt;.0001</td>
</tr>
<tr>
<td>(U/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SEM, n= 10-13 per region by day.

Means in a row without a common letter differ by region. *In addition, for lactase there was an effect of day, independent of region for both measures (P <0.05)
CHAPTER 4

DIETARY bLf AND B. INFANTIS SUPPLEMENTATION ALTERED S. AUREUS LOAD AND TISSUE CYTOKINE EXPRESSION IN A SYSTEMIC S. AUREUS INFECTION IN THE NEONATAL PIGLET

Abstract

In a clinical trial, premature infants fed formula with bLf had decreased blood-borne staphylococcal infections, but the mechanism was not known. Additionally, Lf promotes the growth of B. infantis, a predominant species in the breast-fed infant intestine. Herein, the effect of bLf alone or in combination with B. infantis on the course of S. aureus infection was assessed. Colostrum-deprived pigs had umbilical catheters placed within 12h of birth and were fed formula with 4g/L bLf (LF) or whey protein (CON); half of the piglets in each group were further randomized to receive B. infantis ($10^9$ CFU/day). On d7, piglets were infected intravenously with S. aureus ($10^5$ CFU/kg BW, S54F9) and were euthanized on d12.

Piglets had elevated (P<0.05) rectal temperature beginning at 36h post-infection. LF piglets had elevated rectal temperatures and improved weight gain on d10, 72 h post-infection. LF piglets had decreased staphylococcal load at the kidney (P<0.05) and LF tended to decrease load at the lung (P<0.10) compared to CON, with no effect of B. infantis. When assessing the incidence of S. aureus infection, LF tended to decrease incidence at the kidney (P<0.10), and incidence was lowest in the combined group (LF+B. infantis) at the lung (P<0.05). Thus, bLf decreased translocation of S. aureus from the blood to the tissues, which could potentially reduce fatal organ dysfunction caused by blood-born S. aureus infections.
Using RT-qPCR, immune cell gene expression was measured at the lung, kidney and spleen tissues. At the kidney, LF had an immunoregulatory role where the provision of bLf ameliorated increased Toll-like receptor 2 (TLR2) expression observed in *B. infantis* alone animals (P<0.05). bLf also decreased kidney IL-10 expression, with no effect on IFNγ. At the lung, LF had an inflammatory role by increasing IFNγ mRNA expression (P<0.05), with no effect on TLR2 or IL-10 mRNA expression. Contrary to our hypothesis, *B. infantis* had an inflammatory role at the spleen leading to increased IFNγ expression of the immune cells of the spleen. The potential mechanisms by which bLf and *B. infantis* regulate the immune response to *S. aureus* infection will be discussed in Chapter 5 of this dissertation.

**Introduction**

*S. aureus* is the most common and fatal bacterial infectious disease worldwide in the neonatal population (Park et al 2013). Antibiotic-resistant strains of *S. aureus* have emerged and have limited treatment options for life-threatening *S. aureus* infections in infants (Maraqa et al 2011) that lead to organ dysfunction, such as endocarditis, pneumonia, osteomyelitis and septic shock (Graves et al 2010). A recent clinical trial and subsequent meta-analysis showed that lactoferrin (Lf) reduced septicemia to *Staphylococcus* species in premature infants when supplemented in infant formula (Manzoni et al 2009), however, the mechanism of action was not investigated in that study.

Experiments conducted in rodents support a beneficial effect of LF against *S. aureus* and suggest potential underlying mechanisms of action. When added to the diet,
Lf reduced systemic *S. aureus* load in *S. aureus* challenged mice (Bhimani et al. 1999). Several studies have suggested improved clearance in the presence of Lf is due in part to a Th1 response. For example, Lf-transgenic mice demonstrated an enhanced Th1 response to *S. aureus* infection and these mice showed greater IFN-γ response and increased ability to clear the bacterial infection (Guillen et al. 2002). Work in our laboratory showed that dietary bLf modified the immune system response such that Th1 responses were favored. Specifically, peripheral blood mononuclear cells (PBMC) isolated from piglets fed formula with 3.7g/L bLf for 14 days secreted more IFN-γ ex vivo than PBMC isolated from piglets fed a control formula containing 0.4g/L bLf (Comstock 2014).

Herein, we are investigating the combination of bLf and a probiotic bacterial strain *B. longum subp. infantis*, which is the predominant bifidobacterial strain in breast-fed infant gut microbiota. Previous studies have described interactions between bLf and bifidobacteria. For example, bLf supplemented into infant formula created a favorable environment for the growth of bifidobacteria (Kawaguchi et al. 1989) and *in vitro* studies have shown that bLf binds to *B. infantis* and promotes its growth (Rahman et al. 2008). Thus, we hypothesized that the combination of *B. infantis* and Lf would be more efficacious than either ingredient alone for promoting the Th1 response and improving bacterial clearance from tissues.
Materials and Methods

Animal Protocol

All animal care and experimental procedures were in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Illinois. The study design is shown in Figure 4.1 Pregnant sows at the University of Illinois Swine Research Center were monitored for farrowing beginning on d110 of gestation and female piglets (n=49) were removed prior to ingestion of colostrum. Sow serum was administered to the piglets via oral gavage to provide passive immunity at a volume of 5 ml/kg BW at birth, 12, 24 and 36 hours postpartum. The study was conducted in three separate replicates from May to November 2013. Piglets were individually housed in cages in environmentally-controlled rooms (25°C). Plastic heating pads in each enclosure were used to maintain an ambient temperature of 30°C. Piglets were fed non-medicated, sow-milk replacer (Milk Specialties Global Animal Nutrition, Carpentersville, IL) with 4g/L bLf (LF; Bioferrin 200; Glanbia, Kilkenny, Ireland) or whey protein (CON; Provon 192; Glanbia) 20-times daily at a rate of 360 ml/kg BW/d. Half of the piglets in each group were further randomized to receive B. infantis (ATCC 15697; 10⁹ CFU/day); Whey + B. infantis (B. infantis) and LF + B. infantis (COMB). The piglets were monitored each morning for formula intake, weight gain and stool consistency using the following scale: 1=firm; 2=stiff-flowing; 3=easy-flowing/loose; 4=watery/diarrhea; 5=severe diarrhea (Correa-Matos et al 2003). On d12 postpartum, 120h post-infection, piglets were euthanized by an intravenous injection of sodium
pentobarbital (72 mg/kg BW, Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI) via the umbilical catheter.

**B. infantis Preparation and Storage**

*B. infantis* ATCC 15697 was grown from a frozen stock in deMan, Rogosa, Sharpe (MRS) broth (Difco) medium supplemented with cysteine (0.05%). *B. infantis* was grown anaerobically in a Coy anaerobic chamber as previously described (Francl et al 2012). Following the initial inoculum, cells were passed (1%) into fresh MRS + cysteine to be grown to an optical density (OD) of 0.6 in an anaerobic chamber in a nephelo flask. The final transfer was grown to 0.6 in order to obtain mid-exponential phase growth of the bacterial cells. A 10 ml aliquot was collected by centrifugation (4,000 rpm, 4°C, at 10 minutes). Finally, the cell pellet was prepared for frozen stock in a 1:1 25% glycerol solution and cell pellet suspension in sterile PBS. Each batch of *B. infantis* was validated for viability from frozen stock. *B. infantis* was grown on Modified Reinforced Clostridial Agar (RCM) anaerobically and the bacterial colonies counted. On average, each stock contained 5 x 10^8 colony forming units (CFU) per ml. Piglets received 2 ml of a 5 x 10^8 CFU/ml from frozen stock three times per day. The total dosage per day was approximately 10^9 CFU. Prior to administration, *B. infantis* was washed in PBS to remove glycerol and the bacterial pellet was resuspended in a final volume of 10ml PBS for administration.
Umbilical Catheterization

The surgical procedure was conducted as described in the Appendix and shown in Figure 4.1

S. aureus Infection

On d7, animals were infected with *S. aureus* strain S54F9 \((10^5)\) at 1ml per kg body weight suspended in 0.9% sterile isotonic saline. Rectal temperature and activity were assessed every 12h post infection up to 120h (d5) post-infection. A subjective activity score was developed based on Correa-Matos et al (2003), where each piglet was visually assessed for physical activity and categorized as: active, weak, or lethargic. Furthermore, piglets were evaluated according to their response to a variety of stimuli such as feeding, handling during weighing and measuring of rectal temperature. Activate piglets responded to all three events, weak piglets responded to only two events, and lethargic piglets were unresponsive during the entire observation period.

*S. aureus Load*

Immediately after euthanasia, blood was collected by cardiac puncture into heparin-laced vials. 5 grams of whole organ cross sections for the kidney (left, cortex and medulla), lung (left, caudal lobe), heart (apex, left and right ventricles) and spleen were collected (Smith and Schenk, 1998). Tissues were homogenized in sterile PBS (20ml) using the stomacher at a 1:5 dilution and 200 µl hand-plated at 1:5, 50 µl spiral plated at 1:5, and 50 µl plated using a spiral plater (Neu-Tec Group Inc., Farmingdale, NY) at 1:10 in cold PBS on Mannitol salt agar (BD, Franklin Lakes, NJ) a selective and differential
media for *S. aureus*. All tissues were plated in triplicates at each dilution, and the plates were incubated for 48h at 37 °C before being counted. Colony counts were averaged for each set of triplicates and corrected for the dilution factor used. Final results for *S. aureus* load are expressed as colony forming units per gram tissue (CFU/g). A limit of detection was set at 10 *S. aureus* colonies per plate; if the average of the triplicates was less than 10 colonies the animal was considered to be negative for *S. aureus* infection.

*Tissue Cytokine Analysis*

**RNA Extraction:** Frozen kidney, lung and spleen samples (100 mg) were homogenized with 1 mL of TRIzol reagent (Invitrogen Life Technologies, Grand Island, NY). Samples were incubated at room temperature for 5 min, then transferred to a microcentrifuge tube and centrifuged at 12,000xg at 4°C for 15 min. The aqueous phase (400 µL) was transferred to a new tube and 500 µL of isopropanol was added. Samples were incubated at room temperature for 10 min and centrifuged at 12,000xg at 4°C for 10 min. After removal of the supernatant, the RNA pellet was washed with 1 mL of 75% ethanol and centrifuged at 7,500xg for 5 min. The ethanol was removed and the pellet was allowed to dry on ice (5-10 min). The pellet was dissolved in 20 µL of nuclease-free water (Invitrogen Life Technologies) and the RNA was quantified using a NanoDrop 1000 instrument (Thermo Scientific, Wilmington, DE). Samples were diluted to achieve an RNA concentration of no more than 500 ng/µL and submitted to the Keck Center for Comparative and Functional Genomics (University of Illinois, Urbana, IL) for analysis of RNA quality via a 2100 Bioanalyzer instrument (Agilent Technologies, Inc., Santa Clara, CA).
Measurement of Gene Expression by Real time Quantitative Reverse Transcription Polymerase Chain Reaction: RNA samples with an integrity number (RIN) > 6 were transformed into cDNA utilizing a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed using TaqMan gene expression assays (Table 4.1). The expression of RPL-19 gene was used as an endogenous control. The relative standard curve method was used for quantitation. Standard curves consisted of dilutions of cDNA created from spleen mRNA pooled from all of the treatment groups. Normalized values for each target were calculated by dividing the target quantity mean by the RPL-19 quantity mean. A fold-difference was calculated for each measurement by dividing the normalized target values by the normalized calibrator sample. Animals fed whey (CON) were used as the calibrator in each instance.

Statistical Analysis

Statistical analyses were performed using SAS Version 9.2 (Cary, NC). Body weight, rectal temperatures and serum cytokines were tested between groups by repeated-measures analysis of variance (ANOVA) using PROC MIXED. Other data were analyzed by 2-way ANOVA using PROC GLM procedure. Due to female piglets all weighing approximately 1.5 kg at birth and randomized from 5-6 sows per replicate it was not necessary to account for random effects in the model. The statistical model for S. aureus incidence and normalized target values for tissue gene expression used fixed effects for treatment (LF vs. Whey), Probiotic (Received or did not receive B. infantis), and interaction between treatment and probiotic. Staphylococcal load was analyzed using a
nonparametric Wilcoxon Rank Sum on CFU/g for LF vs. CON and *B. infantis* administration, where the one-sided Z-statistic was used due to a sample size greater than 10. In the event of a significant main effect a post hoc Least Significant Difference test was used. Statistical significance was defined as $P<0.05$ and trends reported as $P<0.10$. Data are presented as mean ± SEM.

**Results**

*Formula intake and BW gain*

Daily formula intake averaged 793 ± 30 ml/d for the first wk of life (d1-d7) and 1092 ± 27 ml/d following infection in the second wk of life (d7-12 postpartum). Piglets received 4g/L of supplemental protein as whey (CON) or bLF (LF). Mean supplemental dietary protein intake was 4 g/day at d7 and 4.8 g/day at d12.

Dietary bLf improved weight gain following infection (Figure 4.2). Piglet body weight for all groups prior to infection on d7 was similar among the groups: CON (2.6 ± 0.12 kg), *B. infantis* (2.8 ± 0.12 kg), COMB (2.8 ± 0.08kg) and LF (2.8 ± 0.09 kg). However, following infection, a significant treatment effect was observed where LF-fed animals (3.4 ± 0.08 kg) had greater body weight than CON (3.0 ± 0.13 kg) on d10, with no effect of *B. infantis*. LF-fed animals continued to have improved weight gain for the remainder of the study on d 11 and 12.

*Rectal Temperature*

There was a significant overall effect where LF-fed animals had greater rectal temperatures then CON ($P=0.0056$) with no interaction between LF and hour (Figure
There was an overall significant effect of day (P<0.0001) and all animals experienced elevated rectal temperatures by 36 hours post-infection compared with baseline, 12 and 24 hours post-infection, and continued to have elevated rectal temperatures until euthanasia at 120 hours post-infection. A normal rectal temperature ranges for a piglet from 100°F (37.8°C) to 103°F (39.4°C) (Merck Veterinary Manual; reported in °F) however, there are no previous reports in the literature on colostrum-deprived piglets at one wk infected with *S. aureus* therefore each piglets change in rectal temperature was relative to the baseline in our studies. At baseline, animals had rectal temperatures of 39.2 ± 0.2, 39.1 ± 0.2, 39.0 ± 0.1, and 39.1 ± 0.1°C for CON, *B. infantis*, LF and COMB, respectively. By 36 hours post-infection, animals had rectal temperatures of 39.3 ± 0.1, 39.5 ± 0.2, 40.0 ± 0.2 and 40.0 ± 0.2°C for CON, *B. infantis*, LF and COMB, respectively.

*S. aureus Load*

**Severity:** To assess disease severity, absolute values for *S. aureus* load were analyzed. The data is represented as colony forming units (CFU) per gram of tissue. *S. aureus* was not detected in the blood. LF significantly reduced staphylococcal load at the kidney (P=0.0174), and tended to decrease load at the lung (P=0.0652) and heart (P=0.0639), with no effect at the spleen (**Figure 4.4**). There were no effects of *B. infantis* administration on staphylococcal load at the tissue. LF-fed animals had a load of 1,791 ± 1,138, 3,630 ± 1,488, and 1,465 ± 800 CFU/g at the kidney, lung and heart, respectively, whereas CON animals had a *S. aureus* load of 13,060 ± 10,304, 14,607 ± 7,746, 2,667 ±
2,134 CFU/g at the kidney, lung and heart, respectively. Overall, LF decreased *S. aureus* load by 7.3, 4 and 1.8 fold at the kidney, lung and heart, respectively.

**Incidence:** In addition to staphylococcal load counts, incidence of infection was determined where 10 colonies were considered positive for *S. aureus* infection. Overall, COMB decreased incidence of lung infection by 38% relative to CON animals, and LF tended to decrease kidney infection by 26% relative to CON animals.

LF combined with *B. infantis* significantly decreased incidence of infection at the lung (P=0.02) with no effect of LF treatment or *B. infantis* alone (Table 4.2). At the lung, the incidence of infection was 100, 100, 77, and 62% for CON, *B. infantis*, LF, and COMB treatments, respectively.

LF alone tended to decrease the incidence of infection at the kidney (P=0.0684), with no effect at the heart or spleen from LF alone, in combination with *B. infantis*, or *B. infantis* alone (Table 4.2). At the kidney, the incidence of infection was 67, 55, 39, and 31% for CON, *B. infantis*, LF, and COMB treatments, respectively. On average, LF-fed animals had a 35% incidence of infection at the kidney, whereas CON animals had a 61% incidence.

**Immune cell gene expression at the tissue**

**Lung:** LF-fed animals had significantly increased IFNγ expression at the lung relative to CON (P=0.0366), with no effect of *B. infantis* alone or COMB (Figure 4.5). The average IFNγ expression was 1.0 ± 0.28, 0.8 ± 0.25, 2.0 ± 0.54 and 1.5 ± 0.40 fold difference for CON, *B. infantis*, LF and COMB, respectively. No significant differences were observed for TLR2 or IL-10 expression for LF, *B. infantis* or COMB. The average
TLR2 expression was 1.0 ± 0.15, 0.9 ± 0.17, 0.9 ± 0.12 and 1.2 ± 0.30 fold difference for CON, *B. infantis*, LF and COMB, respectively. The average IL-10 expression was 1.0 ± 0.20, 1.0 ± 0.26, 1.6 ± 0.45 and 1.4 ± 0.61 fold difference for CON, *B. infantis*, LF and COMB, respectively.

**Kidney:** *B. infantis* significantly increased immune cell kidney TLR2 mRNA expression (P=0.0072) which was ameliorated by LF with a significant interaction between LF and *B. infantis* (P=0.0425) (**Figure 4.6**). The average TLR2 expression was 1.0 ± 0.16, 2.5 ± 0.63, 1.1 ± 0.11 and 1.3 ± 0.16 fold difference for CON, *B. infantis*, LF and COMB, respectively. LF significantly decreased IL-10 expression at the kidney (P=0.0262) with a *B. infantis* trend (P=0.0934) and no interaction was observed between LF and *B. infantis* (**Figure 4.7**). The average IL-10 expression was 1.0 ± 0.3, 2.3 ± 1.0, 0.4 ± 0.09 and 0.7 ± 0.23 fold difference for CON, *B. infantis*, LF and COMB, respectively. No significant differences were observed for IFNγ expression for any treatment groups. The average IFNγ expression was 1.0 ± 0.95, 1.2 ± 1.1, 0.6 ± 0.5 and 1.2 ± 1.3 fold difference for CON, *B. infantis*, LF and COMB, respectively.

**Spleen:** *B. infantis* significantly increased IFNγ mRNA expression (P=0.0145), with no effect of LF or the interaction between LF and *B. infantis* (**Figure 4.8**). The average IFNγ expression was 2.5 ± 2.1 (*B. infantis*), 1.1 ± 0.29 (LF) and 2.7 ± 0.76 (COMB) fold higher than CON (1.0 ± 0.16). No significant differences were observed for TLR2 or IL-10 mRNA expression for any treatment group. The average TLR2 expression was 1.0 ± 0.11, 1.2 ± 0.34, 1.2 ± 0.14 and 1.4 ± 0.18 fold difference for CON, *B. infantis*, LF and COMB, respectively. The average IL-10 expression was 1.0 ± 0.07, 1.0 ± 0.22,
1.0 ± 0.12 and 1.1 ± 0.19 fold difference for CON, *B. infantis*, LF and COMB, respectively.

**Discussion**

Premature infants are especially vulnerable to systemic *S. aureus* infections due to the use of central venous catheters leading to catheter-borne blood stream infections, administration of systemic antibiotics which decrease diverse microbial colonization and fecal bifidobacteria, and delayed introduction of human milk (Gewolb et al 1999). Antibiotic-resistant strains of *S. aureus* have emerged and have limited treatment options for life-threatening *S. aureus* infections in infants (Maraqa et al 2011), such as endocarditis, pneumonia, osteomyelitis and septic shock (Graves et al 2010).

The piglet is considered the best model for the human infant in terms of physiology, immune response and anatomy (Odle et al 2014; Meurens et al 2012). Piglets are capable of reproducing the gradual pathophysiologic changes and mimic clinical characteristics of neonatal sepsis (Kato et al 2004). *S. aureus* is a dominant cause of widespread septicemia in pigs and humans (Jensen et al 2010) and some strains have been shown to cross from pigs to pig farmers, complicating hospital care (Morcillo et al 2012). Taken together, the piglet was chosen as the best model to study blood-borne life threatening *S. aureus* infections in the neonate.

Herein, two clinical outcomes (body weight and temperature) that would be monitored in a neonate were evaluated. Lf prevented weight loss caused by *S. aureus* infection. Several studies have shown that Lf improves weight gain. In a study where formula-fed term infants were supplemented with 850mg/L bLf, there was a trend toward
increased weight gain during the first 6 months of age (King et al 2007). These findings were consistent with a previous study in which enhanced weight gain was observed in healthy term infants fed iron-bound bLf supplemented based on an iron concentration of 1.8 mg Fe/L (Hernell and Lönnerdal, 2002). Lf supplementation at 1.0 g/kg BW improved average daily gain in weanling pigs, a time of high stress that often leads to increased outbreaks of diarrhea, depressed feed intake and growth (Wang et al 2007). The improved growth performance with Lf supplementation has been in part attributed to its role on regulating the innate immune system and providing protection against microbial infection. Lf reduced diarrhea as effectively as an antibiotic (Wang et al 2007). This effect was consistent with another study that reported decreased diarrhea with either dietary Lf or antibiotics (Shan et al 2007). In addition to effectively reducing diarrhea, Lf reduced potentially pathogenic bacteria and increased bifidogenic bacteria, whereas the antibiotic reduced both pathogenic and beneficial bacteria (Wang et al 2007).

Lf’s documented role in stimulating the innate and adaptive immune systems suggests that animals supplemented with Lf would be better able to mount the necessary inflammatory responses to clear bacteria than CON piglets. As a part of the innate immune system, fever is used as a form of host defense to improve bacterial clearance through phagocytosis. Herein, an overall treatment effect was observed where LF-fed animals had increased rectal temperatures. Importantly, LF decreased Staphylococcal load at the kidney and tended to decrease load at the lung. This supports previous research where Lf supplementation reduced Staphylococcal load at the kidney in S. aureus challenged mice (Bhimani et al 1999).
In addition to absolute *S. aureus* load counts, the incidence of infection was determined where 10 colonies per plate, or 250 CFU/g, was used as the threshold for infection and tissues exceeding this level were considered positive for *S. aureus* infection. LF in combination with *B. infantis* significantly decreased incidence of infection in the lung, and LF alone tended to decrease the incidence of infection in the kidney. The guidelines as defined by the CDC National Healthcare Safety Network (CDC-NHSN) for patients less than 1 year of age states 15 colony-forming units per dish must be cultured from an intravascular cannula to meet the criteria for an arterial or venous infection (Grothe et al 2010; Horan et al 2008). In our studies, tissues that had less than 15 colony-forming units per MSA plate averaged only 2-5 colonies per plate.

Lf-transgenic mice have an increased ability to clear *S. aureus* following a challenge (Guillen et al 2002). Lf increased IFNγ mRNA expression in lung following infection, suggesting an inflammatory Th1 response that would improve bacterial clearance. In the current study, LF also tended to decrease Staphylococcal load at the lung, however the incidence of *S. aureus* infection was lowest in the COMB group and IFNγ mRNA expression was increased in LF alone but not COMB. Despite the incidence being lowest in COMB at the lung, LF alone still decreased incidence by 20%, where 80% of animals were positive for infection, relative to CON and *B. infantis* where 100% of animals were positive for infection. Thus, bacterial incidence may be a more sensitive measure than tissue cytokine expression to observe the benefit of COMB in decreasing incidence.

In the kidney, LF ameliorated the increased TLR2 expression caused by the probiotic and decreased IL-10 expression, suggesting LF has a regulatory role at the
immune cells of the kidney to decrease *S. aureus* load. *In vitro* work has shown that *S. aureus* down-regulates the inflammatory T-cell response by trigging IL-10 production by monocytes via TLR2 activation (Chau et al 2009). Increased IL-10 production is a survival mechanism by which *S. aureus* is able to better colonize the host by suppressing the immune response (Chau et al 2009). Therefore, Lf’s role on TLR2 in this instance was protective. In addition to this, Lf suppressed IL-10 production, thus, promoting a more inflammatory response at the kidney. A high level of IL-10 is known to decrease generation of the Th1 response (Fiorentino et al 1991). It has been shown that in addition to promoting a Th1 response through increased IFNγ production, Lf also increased the IL-12: 10 ratio in lipopolysaccharide (LPS) stimulated splenocytes to promote the Th1 response (Hwang et al 2007). The presence of IL-12, a cytokine that promotes the Th1 response in addition to IFNγ, leads to significantly decreased production of IL-10 (Hwang et al 2007).

Contrary to our hypothesis, *B. infantis* actually increased spleen IFNγ mRNA expression however this had no impact on the load severity or *S. aureus* incidence. Lf did not have a Th1 effect at the immune cells of the spleen. Lf may have differential activity at different organs which has been demonstrated by the differences observed between the lung and kidney *S. aureus* load and tissue cytokine expression in these studies. Several studies have shown that LF actually inhibited the Th1 response. In one study, bLf and human Lf (hLf) inhibited the Th1 response and promoted the Th2 response in cell lines (Zimecki et al 1996). In another study, human milk derived Lf suppressed IFNγ production and enhanced IL-10 production in human anti-CD3/CD28 activated CD4+ T cells after 24 hours (Li et al 2006). Others have suggested that LF plays a role in the
Th1/Th2 balance depending on the stimulus and location and timing. For instance, spleen cells from mice orally supplemented with Lf for 6 wks and ex vivo stimulated with Concanavalin A for 48 hours increased Th2 cytokine IL-4 (Sfeir et al 2004). In contrast, spleen cells isolated from piglets fed formula with 3.7g/L bLf and stimulated ex vivo with Phytohaemagglutinin or LPS for 72 hours secreted more IFN-γ (Comstock 2014).

Overall, in the presence or absence of B. infantis, LF decreased S. aureus translocation from the blood, potentially by regulating host immune response to improve bacterial clearance that will be further discussed in Chapter 5 of this dissertation.
Figure 4.1 Study design. Newborn colostrum-deprived piglets (n=49) were randomized to: CON (4g/L Whey), B. infantis (4g/L Whey + $10^9$ B. infantis), LF (4g/L bLf) and COMB (4g/L bLf + $10^9$ B. infantis). Piglets received an oral gavage of serum at 0, 12, 36 and 48 hours post-partum. Lf or Whey protein at 4 g/L was added to sow milk replacer (SMR) diet. $10^9$ B. infantis was administered via oral gavage. Within 12 hours of birth, piglets received two umbilical catheters, one for sample collection and one for infection. On day 7 post-partum, all piglets were infected with S. aureus strain S54F9. Piglets were euthanized on day 12 post-partum.
**Table 4.1** Quantitative real-time PCR primers for TaqMan Gene Expression Assays used for tissue PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Custom Taqman assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNG (IFNγ)</td>
<td>Ss03391053_g1</td>
</tr>
<tr>
<td>IL-10</td>
<td>Ss03382372_u1</td>
</tr>
<tr>
<td>TLR2</td>
<td>Ss03381278_u1</td>
</tr>
</tbody>
</table>
Figure 4.2 Piglet weight gain over time d0-d12 post-partum. There was a significant difference between LF-fed and Whey-fed animals beginning at day 10 post-partum. There was no effect of *B. infantis*, therefore values were pooled for LF (n=26) which included LF and COMB groups; and Whey (n=23) which included CON and *B. infantis* groups. Values are means ± SEM. bLF, bovine lactoferrin
Figure 4.3 Piglet rectal temperatures pre-infection and over the course of infection beginning on day 7 (time 0). There was a significant difference of time and LF. By 36 hours post-infection, animals had elevated rectal temperatures relative to baseline, 12 and 24 hours post-infection. There was an overall effect of treatment, where LF-fed animals had elevated rectal temperatures. There was no effect of *B. infantis*, therefore values were pooled for LF (n=26) which included LF and COMB groups; and Whey (n=23) which included CON and *B. infantis* groups. Values are means ± SEM. bLF, bovine lactoferrin
Figure 4.4 *S. aureus* load expressed as colony forming units (CFU) per gram of tissue at the kidney, lung and heart at euthanasia (day 12 post-partum; or 120 hours post infection). No differences were detected at the spleen. CFU was determined based on the plate count method where a tissue dilution was plated in triplicates on mannitol salt agar, a selective and differential media for *S. aureus* and the number of cells was counted on the plate (one colony = one viable live cell). LF-fed animals had significantly decreased CFU/g at the kidney, and tended to have decreased CFU/g at the lung and heart. There was no effect of *B. infantis*, therefore values were pooled for LF (n=26) which included LF and COMB groups; and Whey (n=23) which included CON and *B. infantis* groups. Values are means ± SEM. bLF, bovine lactoferrin
Table 4.2 Percent incidence of *S. aureus* infection, where less than 10 *S. aureus* colonies per plate was considered negative for infection. Percentage incidence at the kidney, lung, spleen and heart and treatments represented down the column.

<table>
<thead>
<tr>
<th></th>
<th>Kidney</th>
<th>Lung</th>
<th>Spleen</th>
<th>Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Percent Incidence (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>66.7</td>
<td>100\textsuperscript{a}</td>
<td>58</td>
<td>77</td>
</tr>
<tr>
<td><em>B. infantis</em></td>
<td>54.5</td>
<td>100\textsuperscript{a}</td>
<td>64</td>
<td>60</td>
</tr>
<tr>
<td>LF</td>
<td>38.5\textsuperscript{+}</td>
<td>76.9\textsuperscript{a}</td>
<td>69</td>
<td>64</td>
</tr>
<tr>
<td>COMB</td>
<td>30.8\textsuperscript{+}</td>
<td>61.5\textsuperscript{b}</td>
<td>42</td>
<td>33</td>
</tr>
</tbody>
</table>

Values are mean percentages, n=11-13 per group. Means in a column without a common letter differ by treatment, P <0.05 (ANOVA); \(+\) trend 0.05 < P < 0.10.
Figure 4.5 INFγ mRNA expression at the lung at 120 hours post-infection or day 12 post-partum using RT-qPCR. Data expressed as fold difference. Normalized values for INFγ were calculated by dividing the target quantity mean by the RPL19 quantity mean. A fold-difference was calculated for each measurement by dividing the normalized target values by the average normalized target value for CON animals. All samples that were statistically compared to each other were run on the same plate. LF-fed animals had significantly elevated INFγ mRNA expression at the lung, with no effect of B. infantis. Values are means ± SEM, n=10-12 per group. bLF, bovine lactoferrin; COMB, combined
Figure 4.6 TLR2 mRNA expression at the kidney at 120 hours post-infection or day 12 post-partum using RT-qPCR. Data expressed as fold difference. Normalized values for TLR2 were calculated by dividing the target quantity mean by the RPL19 quantity mean. A fold-difference was calculated for each measurement by dividing the normalized target values by the average normalized target value for CON animals. All samples that were statistically compared to each other were run on the same plate. There was a significant interaction effect, where *B. infantis* animals had significantly elevated TLR2 mRNA expression at the immune cells in the kidney, which was ameliorated with the addition of Lf as shown in the COMB group. Values are means ± SEM, n=8-11 per group. bLF, bovine lactoferrin; COMB, combined.
Figure 4.7 IL-10 mRNA expression at the kidney at 120 hours post-infection or day 12 post-partum using RT-qPCR. Data expressed as fold difference. Normalized values for IL-10 were calculated by dividing the target quantity mean by the RPL19 quantity mean. A fold-difference was calculated for each measurement by dividing the normalized target values by the average normalized target value for CON animals. All samples that were statistically compared to each other were run on the same plate. LF-fed animals had significantly decreased IL-10 mRNA expression at the immune cells in the kidney, with no effect of *B. infantis*. Values are means ± SEM, n=8-11 per group. bLF, bovine lactoferrin; COMB, combined
Figure 4.8 IFNγ mRNA expression at the immune cells in the spleen at 120 hours post-infection or day 12 post-partum using RT-qPCR. Data expressed as fold difference. Normalized values for IFNγ were calculated by dividing the target quantity mean by the RPL19 quantity mean. A fold-difference was calculated for each measurement by dividing the normalized target values by the average normalized target value for CON animals. All samples that were statistically compared to each other were run on the same plate. *B. infantis* animals had significantly increased IFNγ mRNA expression at the immune cells in the spleen, with no effect of Lf. Values are means ± SEM, n=9-11 per group except for *B. infantis* where n=3. bLF, bovine lactoferrin; COMB, combined
CHAPTER 5

DIETARY BLF AND B. INFANTIS SUPPLEMENTATION ALTERED SYSTEMIC IMMUNE RESPONSE IN AN S. AUREUS INFECTION IN THE NEONATAL PIGLET

Abstract:

Infants are susceptible to infection at birth (Wynn and Levy, 2010). Treatment of life threatening S. aureus infections in the neonatal population is further complicated by the emergence of antibiotic resistant strains, disproportionally affecting the morbidity and mortality of infants less than 3 months old in the neonatal intensive care unit (Zervou et al 2014; Maraqa et al 2011).

Premature infants fed formula with bLf had decreased blood-borne staphylococcal infections (Manzoni et al 2009). Additionally, Lf promotes the growth of B. infantis (REF). Herein, the effect of bLf alone or in combination with B. infantis on the course of S. aureus infection was assessed. Colostrum-deprived piglets had umbilical catheters placed at birth and were fed formula with 4g/L bLf (LF) or whey protein (CON); half of the piglets in each group were further randomized to receive B. infantis (10⁹ CFU/day). On d7, piglets were infected intravenously with S. aureus (10⁵ CFU/kg BW, S54F9) and euthanized on d12.

Lf piglets had decreased serum IL-10, increased lymphocyte percent and tended to have decreased segmented neutrophils in whole blood prior to infection. LF also decreased nucleated red blood cells in whole blood, and decreased the percent change for monocytes and B cells of the PBMC population from 72 to 120hr post-infection. Whereas, B. infantis increased serum IL-10 following infection, with a peak IL-10
concentration at 96 and 108h post-infection. *B. infantis* also decreased the memory T cell population at 120h post-infection as well as the percent change from 72 to 120h post-infection. Importantly, the combination of LF and *B. infantis* increased Foxp3 expression in a CD4+ enriched population as assessed by RT-qPCR, with no effect from LF or *B. infantis* alone, suggesting the combination provides the optimal mode by which *B. infantis* is able to alter CD4+ T cell phenotypes towards a regulatory response.

**Introduction**

Lactoferrin (Lf) is abundant in mammalian milk where it is an iron-binding glycoprotein, suggesting one way by which Lf is thought to exert its antimicrobial effects by preventing microbes from accessing adequate iron. Lf is also present in neutrophil granules, and the release of Lf at sites of injury have been reported to inhibit infiltration of inflammatory neutrophils while attracting monocytes, suggesting Lf plays an important role in regulating inflammation (Bournazou et al 2009; de la Rosa et al 2008). In addition to having effects on the innate immune system, reports in the literature suggest that Lf is important in generating a Th1 adaptive immune response in mice (Bhimani et al 1999; Guillen et al 2002) and piglets (Comstock et al 2014), leading to an increased inflammatory response during infection and improving bacterial clearance.

Additionally, Lf promotes the growth of beneficial commensal bacteria bifidobacteria spp., especially *B. infantis* (Rahman et al 2008). *B. infantis* is a predominant bifidobacteria species in human milk and in the breast-fed infant intestine (Grönlund et al 2007). *B. infantis* supplemented mice in a systemic LPS model showed reduced NF-kB activation *in vivo* and increased Foxp3 T regulatory (T reg) expressing
cells (O’Mahony et al 2008). Oral *B. infantis* increased IL-10 secretion and induced Foxp3 regulatory CD4+ T cell expression in human PBMC (Konieczna et al 2012). Taken together, *B. infantis* may play a regulatory role in balancing inflammatory response due to microbial insult. Thus, we hypothesized that dietary Lf would promote a Th1 response, whereas *B. infantis* would promote a T reg response. Additionally, The combination of Lf and *B. infantis* would further improve the T reg response due to Lf’s role in promoting *B. infantis* growth.

**Materials and Methods**

*Animal Protocol*

The catheterizations and animal protocol were conducted as described in the Appendix and Chapter 4.

*Sample Collection*

Blood samples were collected into non-heparinized vacuum tubes via the sampling umbilical catheter prior to infection and every 12h post-infection until 120h post-infection for cytokine analyses. Heparinized blood was collected at 72 and 120h post infection for PBMC isolation. A fresh blood smear was performed prior to infection and at 120h post-infection for a complete blood count and differential analysis by the hematology department at the University of Illinois College of Veterinary Medicine (Urbana, IL). On d12, 120h post-infection, piglets were euthanized by an intravenous injection of sodium pentobarbital (72 mg/kg BW, Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI). A sample collection timeline is shown in Figure 5.1.
**Serum Cytokines**

Serum was obtained by centrifugation of blood samples collected prior to and every 12h post-infection and was analyzed using porcine-specific ELISA kits for IFNγ, IL-6 and IL-10 (R&D Systems, Minneapolis, MN). For samples with concentrations below the detection limit of the assay (the lowest point on the standard curve), a value was established by using detection limit was determined for each ELISA plate using the standard curve and one half the detection limit (approximately 10-12 pg/ml), as used in the literature for a sandwich ELISA for values below the detection limit (Chung et al 2010; Van Emon et al 2008).

**Flow Cytometry to Identify PBMC subpopulations**

PBMC were obtained by Ficoll-Hypaque centrifugation of heparinized blood shown in Figure 5.2. Cells were resuspended in flow staining buffer (PBS, 1% BSA, 0.1% sodium azide). The immune cell phenotypes were assessed by BD™ LSR II flow cytometry unit (BD Biosciences). The relative number of subpopulations was determined using FlowJo 7.0 software (FlowJo, Ashland, OR) as shown in Figure 5.3.

T lymphocytes: were identified by mouse anti-pig CD3-biotin (Southern Biotech) and visualized with streptavidin-phycoerythrin (PE) Cy7 (Southern Biotech). To further differentiate CD4+ and CD8+ T cells, cells were stained with mouse anti-pig CD4-fluorescein isothiocyanate (FITC) (Southern Biotech) and mouse anti-pig CD8-PE (Southern Biotech). Results were expressed as a percent of CD3 events for single positive CD4 and CD8 and double positive T cells (Memory T cell; CD4+CD8+).
Macrophages: were identified by CD172a-biotin (AbD Serotec), CD163-PeCy5, and CD14-FITC (BD Biosciences). Results were expressed as a percent of CD172a events for single positive CD14 or double positive for CD14 and CD163.

B Cells: were identified by mouse anti-pig CD21-PE (Southern Biotech) and mouse anti-pig MHCII-FITC (AbD Serotec). Results were expressed as a percent of total gated PBMC events double positive for CD21 and MHCII.

**CD4+ T cell enrichment**

An enriched CD4+ T cell population was isolated from PBMC. Non-target cells were labeled with an antibody cocktail and magnetically labeled with MACS microbeads. The Porcine Specific Antibody cocktail included: B cell (CD21), Granulocyte/MØ/neutrophil (CD172a), cytotoxic T cell (CD8), monocyte (CD14), and gamma delta T cell (PGBL22A; recognizes TCR delta chain). Non-target cells were retained on the MACS column, and target cells (CD3+CD4+) were allowed to pass through to be collected as the enriched unlabeled fraction. Purity was assessed using flow cytometry as shown in Figure 5.4.

**Gene Expression by Quantitative Real Time Polymerase Chain Reaction (RT-qPCR):** RNA was isolated from the enriched CD3+CD4+ population using an RNA Eukaryotic Mini Kit (Qiagen, Valencia, CA), and cDNA transcribed using Superscript VILO Master Mix (Invitrogen, Life Technologies, Grand Island, NY). qPCR was performed using TaqMan gene expression assays (Life Technologies) for Foxp3 (Treg), T-bet/TBX21 (Th1), GATA3 (Th2), and IL-10 (Table 5.1). A pre-amplification step was necessary for all markers except GATA3, therefore GATA3 was not pre-amplified prior
to qPCR. Following RT, cDNA was pre-amplified using TaqMan PreAmp Master Mix (Applied Biosystems, Life Technologies) for Foxp3, T-bet and IL-10.

**Statistical Analysis**

Statistical analyses were performed using SAS Version 9.2 (Cary, NC). Data was analyzed by 2-way ANOVA using PROC GLM procedure for CBC/differential, flow cytometry absolute percentage values, RT-qPCR for CD4 T cell expression. Due to female piglets all weighing approximately 1.5 kg at birth and randomized from 5-6 sows per replicate it was not necessary to account for random effects in the model. Data were analyzed by 2-way ANOVA using PROC MIXED repeat measures, for serum cytokines and immune cells. The statistical model in both instances used fixed effects for treatment (LF vs. Whey), Probiotic (*B. infantis*), and interaction between treatment and probiotic, with an additional fixed effect of time for repeated measures analysis. In the event of a significant main effect a post hoc Least Significant Difference test was used. Statistical significance was defined as P<0.05 and trends reported as 0.05 < P < 0.10. Data are presented as mean ± SEM.

**Results**

*CBC/differential*

Fresh blood samples were collected for complete blood counts (CBC), including red blood cell count (RBC), hemoglobin (Hgb), hematocrit (Hct), mean corpuscular volume (MCV), white blood cell count (WBC), platelet count (Plt), Nucleated RBC (NRBC) count and leukocyte differential counts including % neutrophils (Neu), % lymphocytes
(Lym), % monocytes (Mon), % eosinophils (Eosin) and % basophils (Baso) performed at the University of Illinois Clinical Pathology Laboratory.

Analysis was performed prior to infection, d7 postpartum, and at euthanasia, d12 postpartum. Prior to infection, LF-fed animals had significantly increased Lym % (P=0.0196) (Figure 5.5), and tended to have decreased segmented or mature Neu % (P=0.0782) (Figure 5.6). On average, the Lym % was 43 ± 3.7 and 31 ± 3.0% for LF and CON, respectively, and Neu % 53 ± 3.8 and 62 ± 3.4% for LF and CON, respectively. Following infection, LF-fed animals had significantly decreased NRBC (P=0.0472), a key marker of inflammation (Figure 5.7). On average, NRBC counts were 3.5 ± 0.9 and 7.3 ± 1.7 for LF and CON, respectively. No other differences were observed in the other parameters measured prior to infection or at euthanasia. Additionally, B. infantis alone or in combination with LF had no effect. CBC/differential values for pre-infection and 120 hours post-infection are presented in Table 5.2 and Table 5.3, respectively.

Serum Cytokine Analyses

IFNγ Serum IFNγ was measured pre-infection and at 24, 36, and 72h post-infection. All animals were below the detection limit for the measured time points.

IL-6 Serum IL-6 was measured pre-infection and at 72 h post-infection, a time point determined during the pilot study. Only 3 animals (2 LF-fed, and 1 CON) had detectable IL-6 at 72h post-infection. All animals were below the detection limit pre-infection.

IL-10 Serum IL-10 was measured pre-infection and every 12h post-infection. On average, approximately 17 animals had detectable serum IL-10 per time point, with the
majority of animals, 29 out of 49, having detectable IL-10 at 24h post-infection. However, there was an overall significant effect of time where animals produced the highest concentration of IL-10 at 96 and 108h post-infection (P= 0.0328) (Figure 5.8).

LF-fed animals had decreased IL-10 prior to infection with a concentration of 10 ± 0.9 and 32 ± 8.5pg/ml for LF and CON, respectively (P=0.0077) (Figure 5.9). IL-10 was within the detection limit of the assay for 20%, 5 out of 26, LF-fed animals, and for 60%, 13 out 22, CON-fed animals. No effect of *B. infantis* or interaction with LF was observed prior to infection. There was an overall significant effect of *B. infantis* during the course of infection, where *B. infantis* fed animals had increased serum IL-10 (P=0.0255) (Figure 5.8). Peak IL-10 concentration was 86 ± 47 and 74 ± 33pg/ml for *B. infantis* fed 96 and 108h post-infection, respectively, whereas animals that did not receive *B. infantis* had a serum concentration of 29 ± 6.6 and 18 ± 3.0 for 96 and 108h post-infection, respectively. Overall, *B. infantis* increased IL-10 concentration in the serum, with a 3- and 4.2-fold increase at 96 and 108h post-infection, respectively, the time points at which serum IL-10 concentration was greatest.

*Identification of Immune Cell Populations by Flow Cytometry*

B cell subpopulation was measured at 72 and 120h post-infection, and the absolute values at each of these time points did not significantly differ for any of the treatment groups. On average, the absolute percentage of B cells that were double positive for MHCII^+^CD21^+^ at 72h was 4.2 ± 0.8, 6.1 ± 1.3, 6.0 ± 1.1 and 5.3 ± 1.0% for CON, *B. infantis*, LF and COMB, respectively (Figure 5.10). At 120h, the absolute B cell
percentages were 9.9 ± 2.9, 11.1 ± 2.6, 8.2 ± 1.8 and 6.6 ± 2.0% for CON, B. infantis, LF and COMB, respectively (Figure 5.10).

Animals experienced a significant increase in the percentage of B cells from 72 to 120h post-infection, with an interaction between treatment LF and day (P=0.0370) when analyzed using repeated measures ANOVA. The percent change at 120 hours was 240 ± 166, 177 ± 57, 151 ± 77 and 112 ± 55% for CON, B. infantis, LF and COMB, respectively (Figure 5.10).

T cell subpopulations were measured at 72 and 120h post-infection. Single positive (CD4+ or CD8+) did not differ significantly between treatment groups, however B. infantis significantly decreased double positive (CD4+CD8+) memory T cells at 120h post-infection (P=0.0343) and B. infantis significant decreased the percent change in memory T cells at 120h from 72h (P=0.0271) (Figure 5.11). There was no day effect when analyzed using repeat measures ANOVA, however there was a B. infantis and day interaction (P=0.0132) consistent with the analysis that demonstrates B. infantis decreased memory T cells at 120h.

On average, the percentage of double positive T cells as a percent of CD3+ at 72 hours was 9.6 ± 1.2, 9.5 ± 0.9, 9.2 ± 1.1 and 10 ± 1.4% for CON, B. infantis, LF and COMB, respectively. At 120h, the percentage of double positive T cells as a percent of CD3+ at 72h was 11 ± 1.2, 8.3 ± 0.6, 11 ± 1.2 and 9.0 ± 0.8% for CON, B. infantis, LF and COMB, respectively. The percent decrease at 120h was 119 ± 45, 92 ± 26, 124 ± 52 and 91 ± 28% for CON, B. infantis, LF and COMB, respectively (Figure 5.11).

On average, the absolute percentage of CD4+ as a percent of CD3+ at 72h was 71 ± 4.0, 70 ± 3.7, 74 ± 1.7 and 68 ± 2.0% for CON, B. infantis, LF and COMB, respectively.
At 120h, the absolute percentage of CD4$^+$ as a percent of CD3$^+$ was 70 ± 2.9, 70 ± 3.8, 71 ± 2.8 and 66 ± 3.7% for CON, *B. infantis*, LF and COMB, respectively. The absolute percentage of CD8$^+$ as a percent of CD3$^+$ at 72h h was 4.7 ± 0.6, 5.2 ± 0.7, 5.6 ± 1.1 and 4.1 ± 0.5% for CON, *B. infantis*, LF and COMB, respectively. At 120h, the absolute percentage of CD8$^+$ as a percent of CD3$^+$ was 6.0 ± 0.9, 5.2 ± 0.6, 5.3 ± 0.7 and 6.3 ± 0.8% for CON, *B. infantis*, LF and COMB, respectively.

Monocyte subpopulation was measured at 72 and 120h post-infection, and the absolute values did not significantly differ among the treatment groups. On average, the percentage of CD14$^+$CD163$^+$ monocytes as a percent of CD172$^+$ at 72 hours was 18 ± 5.2, 20 ± 4.2, 23 ± 4.4 and 22 ± 4.6% for CON, *B. infantis*, LF and COMB, respectively. At 120h, the percentage of CD14$^+$CD163$^+$ monocytes as a percent of CD172$^+$ was 25 ± 7.5, 23 ± 5.2, 19 ± 4.6 and 19 ± 5.1% for CON, *B. infantis*, LF and COMB, respectively.

There was no day effect when analyzed using repeat measures ANOVA, however there was a trend for a LF by day interaction (P=0.1007), consistent with the finding that LF animals tended to have a lower percentage of change at 120h. The percent change of monocytes from 72 to 120h post-infection tended to decrease in LF-fed animals (P=0.0717). The percent change at 120h was 192 ± 197, 201 ± 202, 102 ± 77 and 104 ± 94 % for CON, *B. infantis*, LF and COMB, respectively (Figure 5.12).

NK cell subpopulation was measured at 72 and 120h post-infection, and the absolute values did not differ significantly among the treatment groups. On average, the percentage of CD3$^-$CD8$^+$ NK cells at 72h was 2.1 ± 1.0, 3.5 ± 1.1, 3.4 ± 1.1 and 1.4 ± 0.4% for CON, *B. infantis*, LF and COMB, respectively. At 120h, the percentage of CD3$^-$
CD8+ NK cells was 6.4 ± 2.3, 5.0 ± 1.5, 3.9 ± 1.2 and 7.8 ± 4.5 % for CON, *B. infantis*, LF and COMB, respectively.

Animals experienced a significant increase in the percentage of NK cells from 72 to 120h post-infection (P=0.0401), with no significant effect of treatment or for any interaction when analyzed using repeated measures ANOVA. Similarly, no effect on percent change in NK cells were observed for any treatment group. The percent change at 120h compared to 72h was 420 ± 104, 936 ± 603, 329 ± 172 and 490 ± 314 % for CON, *B. infantis*, LF and COMB, respectively.

*mRNA expression in CD4+ T cell enriched populations*

There was a significant effect of the COMB treatment (P=0.0064), where animals fed LF or *B. infantis* alone had decreased Foxp3 expression, and the combination of LF and *B. infantis* restored Foxp3 expression to CON expression (Figure 5.13). The average expression relative to CON animals was 0.14 ± 0.03, 0.19 ± 0.07 and 1.0 ± 0.37 fold difference for *B. infantis*, LF and COMB, respectively.

No significant differences were observed for GATA3 or IL-10 expression between the treatment groups. T-bet expression was below the level of detection and was therefore not measured. On average, relative to CON animals, GATA3 expression was 1.3 ± 0.22, 1.2 ± 0.28 and 1.2 ± 0.14 fold difference for *B. infantis*, LF and COMB, respectively. IL-10 expression relative to CON animals was 0.07 ± 0.05, 0.05 ± 0.01 and 0.2 ± 0.07 fold difference for *B. infantis*, LF and COMB, respectively.
Discussion

The nature of the immune response is critical to the outcome of disease during an infection. While inflammatory responses are necessary to clear infection, a prolonged inflammatory state can lead to damage that is detrimental. For instance, in a mouse septic arthritis model, a Th1 inflammatory response improved bacterial clearance; however mice that had a prolonged Th1 response developed chronic arthritis. In another study, it was shown that during the early stages of a systemic \textit{S. aureus} infection IFN\textgreek{g} receptor knockout mice had a higher mortality than their wild-type littermates. However, wild-type mice had a higher mortality than IFN\textgreek{g} receptor knockout mice in the later phase of infection (Zhao et al 1995). This suggests that IFN\textgreek{g} may have an protective effect against \textit{S. aureus} in early infection, but a detrimental effect later in the course of the infection.

Under some circumstances, Lf has been shown to stimulate the production of a Th1 response via IFN\textgreek{g}, while decreasing the anti-inflammatory IL-10 response. As described in Chapter 4, Lf-fed animals had decreased \textit{S. aureus} load at the lung and kidney by different immune cell mechanisms, where at the lung Lf increased IFN\textgreek{g} expression and at the kidney Lf decreased IL-10 expression. We have chosen to supplement \textit{B. infantis} in this model to potentially provide protection in the later phase of infection per the hypothesized role of probiotic in this model. An over production of inflammatory cytokines could lead to increased mortality and comorbidities associated with the inflammation. \textit{B. infantis} is known to down-regulate inflammatory responses, and \textit{B. infantis}-supplemented mice in a systemic LPS model show reduced NF-k\textgreek{b} activation \textit{in vivo} and increased T regulatory expressing cells locally and systemically, thus, providing one potential mechanism of protection (O’Mahony et al 2008). In addition, Lf has been
shown to promote *B. infantis* *in vitro*, suggesting another potential mechanism by which these dietary components could interact in the model (Rahman et al 2008).

A variety of studies have shown LF has immunomodulatory activities affecting the function of many immune cell types including lymphocytes, mø, and dendritic cells. It has been reported that oral Lf has a stimulatory effect on T and B cell lymphocyte proliferation in the spleen of immune-compromised mice (Artym et al, 2003). Shan et al showed oral administration of Lf enhanced porcine peripheral blood and spleen lymphocyte proliferation (Shan et al 2007). This is consistent with the finding in our studies that Lf-fed animals had increased lymphocyte percent in whole blood prior to infection as measured by the CBC/differential analysis on whole blood. The changes observed in Lf-fed animals prior to infection could provide an explanation for the decreased incidence of *S. aureus* infection in those animals due to the ability of these animals to mount an immune response more rapidly. Lf-fed animals also had decreased serum IL-10 prior to infection, also suggesting they were able to mount a quicker response with bacterial insult. In addition to this, Lf animals may have a more controlled adaptive inflammatory response. Prior to infection, Lf animals tended to have decreased neutrophils in the blood and following infection Lf significantly decreased nucleated red blood cells (NRBCs), or immature RBCs that are used as a marker of inflammatory injury.

*B. infantis* had an overall effect of increased serum IL-10, which is consistent with what has been observed in mice and humans supplemented with *B. infantis* (Konieczna et al 2012; O’Mahony et al 2008). High concentrations of IL-10 have consistently been shown to be a strong indicator of septic shock and predictor of mortality during infection
due to its broadly immunosuppressive function (Heper et al 2006). We had hypothesized that *B. infantis* would promote anti-inflammatory cytokine IL-10, however we also hypothesized the addition of Lf would increase IFNγ in the serum. In our studies, no IFNγ was detected in the serum. One possible explanation for this could be that IFNγ is an intracellular cytokine and its presence may be too low in the serum to detect. A possible future direction would be to intracellularly stain T lymphocytes for IFNγ to see if there are any differences with Lf feeding.

However, we did not detect T-bet expression in CD4+ T cells isolated from the blood, a Th1 differentiation marker that leads to production of IFNγ by Th1 cells. Therefore, the Th2 bias in our animals may have been too great to detect differences in Th1 differentiation and IFNγ by these T cells at the time point we chose, at 5 days post-infection or 12 days post-partum. It is well known that neonates are Th2-skewed at birth which gradually diminishes. This Th2 skew is thought to take place to suppress IFNγ and is due to an intrinsic bias in the neonate to develop an IL-4 expression and secretion Th2 phenotype in neonatal CD4+ T cells (Hebel et al 2014). We measured Th2 differentiation by GATA3 and all animals expressed GATA3 abundantly with no differences with treatment. We did however observe increased tissue IFNγ mRNA, therefore in order to rule out a Th1 response as having been low or not occurring in our animals, we would need to isolate CD4+ T cells at the tissues and measure T-bet expression at the tissue where active infection was occurring and IFNγ expression was detected.

With increased serum IL-10 concentration, in addition to previous literature reporting that *B. infantis* increased Foxp3 expression (Konieczna et al 2012), we had hypothesized *B. infantis* would increase Foxp3 expression in CD4+ T cells, with the greatest increase
in the COMB group where we expected Lf to promote *B. infantis* growth. However, we observed a decrease in Foxp3 expression relative to CON in LF or *B. infantis* alone, with a restoration of Foxp3 expression in the COMB group. It did not come as a surprise that Tregs, regulatory T cells, were decreased due to dietary Lf. Previous *in vitro* work has shown both bLf and recombinant human Lf (rhLf) reduced Treg lymphocytes in a PBMC culture (Gonzalo de la Rosa et al 2008). Consistent with the IL-10 serum cytokine data, *B. infantis* animals also had decreased memory T cells with a significant decrease at 120h, and a decrease from 72 to 120h post-infection. This decrease in immunological memory can be explained by the immunosuppression due to elevated IL-10. Due to the Foxp3 expression not being greater in *B. infantis* animals, the elevated IL-10 may have been due monocytes and not T lymphocytes.

Macrophages and B cells are valuable as antigen presenting cells which is necessary for the initiation of the innate immune response leading to an antigen-specific adaptive immune response. Macrophages are also highly phagocytic cells and therefore play an essential role in controlling of infection in addition to secreting cytokines essential to initiation of specific CD4+ T cell responses having an inflammatory or anti-inflammatory role depending on timing and stimulus. Research has shown macrophages contain Lf receptors and that Lf serves as a chemoattractant and can increase the phagocytic activity of macrophages at the site of infection (Soehnleine et al 2009; Gahr et al 1991). As was observed in our model for the bacterial load analysis in Chapter 4, there was infection at the tissue and the blood was cleared of infection. We examined immune cell populations at the blood and expected circulating monocytes to be recruited to the tissue, where they are able to clear infection through phagocytosis. This is consistent with our finding that
Lf animals tended to have a decreased percentage of monocytes from 72 to 120h post-infection as we would expect the monocytes to migrate from the blood and into the tissues.

B cells are also critical for presenting foreign antigens to T cells in addition to developing a specific antibody response to the pathogen. All animals experienced a significant increase in the percentage of B cells from 72 to 120h post-infection as we would expect with the development of a humoral response to *S. aureus*. However, LF-fed animals had significantly less of an increase in B cells relative to CON. The literature supports the existence of Lf receptor on B cells, and the strong ability of Lf to promote the differentiation of immature B-cells into antigen-presenting cells to promote the immune response (Siqueiros-Cendón et al 2014; Actor et al 2009). The result observed in our studies is possibly due to Lf-fed animals having already cleared infection prior to euthanasia.

Overall, *B. infantis* increased IL-10 that led to a suppressed immune response independent of Foxp3 expression. While Lf did not increase serum IFNγ or T-bet expression as we had expected, dietary Lf restored Foxp3 expression in COMB animals and altered the immune status prior to infection by increasing circulating lymphocytes. Lf also reduced NRBCs and monocytes at the blood following infection. It is possible Lf fed animals had decreased incidence of infection and therefore elicited less of a response then CON. It's careful to note the timeline of collection in these studies (Figure 5.1) as we would expect a different result with a longer study or if samples were collected at a different time.
Figure 5.1 Sample Collection Timeline for CBC/differential, ELISA and flow cytometry analysis. Blood was collected for serum every 12 hours, whole blood was submitted for a CBC/differential prior to infection and at 120 hours post-infection, and lastly PBMC were isolated for flow cytometry at 72 and 120 hours post-infection. In addition to flow cytometry, PBMC isolated at 120 hours were used for CD4$^+$ T cell enrichment. The collection timeline was chosen to measure the innate immune response and early adaptive immune responses beginning at 72 hours post-infection.
Figure 5.2 PBMC isolated from neonatal piglets by Ficoll-Hypaque centrifugation of heparinized blood. The sample is predominated by a mixed lymphoid population (98% lymphocytes) and less than <2% of nucleated cells represent neutrophils and monocytes.
Figure 5.3 Flow cytometry gating procedure. The whole PBMC population was selected on the side-scatter vs forward scatter plot (A). For T cells, stained CD3⁺ cells (PE-Cy5) were selected after comparing the stained and unstained populations (A). Single stained cells for CD4⁺ (FITC) and CD8⁺ (PE) were used to determine gating (A). The same principles applied for other subsets (B cell, Monocyte, NK cell). An example of B cell (B) and Monocyte (C) gating is shown. For NK cell gating (not shown) the CD3⁻ population would be selected and NK cells would be those cells positive for CD8.
Figure 5.4 Flow cytometry following CD4+ T cell enrichment to determine purity of enriched fraction. An enriched CD4+ T cell population was isolated from piglet PBMC infected with *S. aureus*. Non-target cells were labeled with an antibody cocktail and magnetically labeled with MACS microbeads. Non-target cells were retained on the MACS column and target cells were allowed to pass through to be collected. On average, 85% of the enriched (pass through) population contained CD3+CD4+ T cells.
Table 5.1 Quantitative real-time PCR primers for TaqMan Gene Expression Assays used for CD4⁺ T cell PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Custom Taqman assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBX21</td>
<td>Ss03373719_s1</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Ss03376695_u1</td>
</tr>
<tr>
<td>GATA3</td>
<td>Ss03388351_m1</td>
</tr>
<tr>
<td>IL-10</td>
<td>Ss03382372_u1</td>
</tr>
</tbody>
</table>
Figure 5.5 Percentage of lymphocytes in whole blood prior to *S. aureus* infection in 7-day old piglets. The results were obtained from a CBC/differential analysis performed at the University of Illinois Clinical Pathology Laboratory. LF-fed animals had significantly increased percent of lymphocytes in whole blood prior to infection. There was no effect of *B. infantis*, therefore values were pooled for LF (n=26) which included LF and COMB groups; and Whey (n=23) which included CON and *B. infantis* groups. Values are means ± SEM. bLF, bovine lactoferrin
Figure 5.6 Percentage of mature neutrophils in whole blood prior to infection on day 7 post-partum. The results were obtained from a CBC/differential analysis performed at the University of Illinois Clinical Pathology Laboratory. LF-fed animals tended to have decreased percent of mature neutrophil in whole blood prior to infection. There was no effect of *B. infantis*, therefore values were pooled for LF (n=26) which included LF and COMB groups; and Whey (n=23) which included CON and *B. infantis* groups. Values are means ± SEM. bLF, bovine lactoferrin
Figure 5.7 Nucleated red blood cells (NRBC) in whole blood 120 hours post-infection.

The results were obtained from a CBC/differential analysis performed at the University of Illinois Clinical Pathology Laboratory. LF-fed animals tended to have decreased NRBC in whole blood 120 hours post-infection. There was no effect of *B. infantis*, therefore values were pooled for LF (n=26) which included LF and COMB groups; and Whey (n=23) which included CON and *B. infantis* groups. Values are means ± SEM.

bLF, bovine lactoferrin
<table>
<thead>
<tr>
<th>Pre-Infection (unit)</th>
<th>CON</th>
<th>B. infantis</th>
<th>LF</th>
<th>COMB</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (/μL)</td>
<td>3.81 ± 0.39</td>
<td>3.75 ± 0.25</td>
<td>4.19 ± 0.20</td>
<td>3.85 ± 0.18</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>7.54 ± 0.72</td>
<td>7.51 ± 0.53</td>
<td>8.23 ± 0.40</td>
<td>7.69 ± 0.40</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>25.4 ± 2.16</td>
<td>24.7 ± 1.82</td>
<td>26.5 ± 1.25</td>
<td>25.2 ± 1.33</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>69.6 ± 2.26</td>
<td>66.8 ± 1.30</td>
<td>67.2 ± 2.21</td>
<td>68.9 ± 1.44</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>19.9 ± 0.28</td>
<td>20.0 ± 0.26</td>
<td>19.7 ± 0.41</td>
<td>20.0 ± 0.34</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>29.6 ± 0.68</td>
<td>30.6 ± 0.49</td>
<td>31.2 ± 1.04</td>
<td>30.6 ± 0.81</td>
</tr>
<tr>
<td>NRBC (/200 WBC)</td>
<td>2.00 ± 0.87</td>
<td>3.20 ± 0.93</td>
<td>2.50 ± 1.05</td>
<td>3.00 ± 1.06</td>
</tr>
<tr>
<td>WBC Count (/μL)</td>
<td>8.92 ± 2.10</td>
<td>11.1 ± 1.03</td>
<td>10.9 ± 1.27</td>
<td>9.66 ± 1.91</td>
</tr>
<tr>
<td>Seg (%)</td>
<td>66.9 ± 4.37</td>
<td>59.4 ± 4.95</td>
<td>54.2 ± 5.08</td>
<td>51.9 ± 5.86</td>
</tr>
<tr>
<td>Band (%)</td>
<td>0.86 ± 0.86</td>
<td>7.00 ± 5.16</td>
<td>0.60 ± 0.50</td>
<td>1.10 ± 0.71</td>
</tr>
<tr>
<td>Lymph (%)</td>
<td>30.1 ± 4.43</td>
<td>31.5 ± 4.13</td>
<td>42.6 ± 5.36</td>
<td>43.2 ± 5.50</td>
</tr>
<tr>
<td>Mono (%)</td>
<td>1.86 ± 0.91</td>
<td>1.70 ± 0.45</td>
<td>2.20 ± 0.55</td>
<td>2.60 ± 0.52</td>
</tr>
<tr>
<td>Baso (%)</td>
<td>0.14 ± 0.14</td>
<td>0.10 ± 0.10</td>
<td>0.10 ± 0.10</td>
<td>0.20 ± 0.20</td>
</tr>
<tr>
<td>Eosin (%)</td>
<td>0.14 ± 0.14</td>
<td>0.30 ± 0.30</td>
<td>0.30 ± 0.15</td>
<td>1.00 ± 0.42</td>
</tr>
</tbody>
</table>
Table 5.3 CBC/differential analysis per treatment group 120 hours post-infection

<table>
<thead>
<tr>
<th>Post Infection (unit)</th>
<th>CON</th>
<th>B. infantis</th>
<th>LF</th>
<th>COMB</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (/µL)</td>
<td>3.55 ± 0.21</td>
<td>3.34 ± 0.20</td>
<td>3.37 ± 0.16</td>
<td>3.48 ± 0.08</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>6.62 ± 0.33</td>
<td>6.23 ± 0.30</td>
<td>6.32 ± 0.23</td>
<td>6.54 ± 0.16</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>22.4 ± 1.02</td>
<td>21.2 ± 1.09</td>
<td>21.6 ± 0.80</td>
<td>22.7 ± 0.47</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>66.5 ± 1.71</td>
<td>65.0 ± 1.54</td>
<td>64.6 ± 1.36</td>
<td>66.2 ± 1.06</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>18.8 ± 0.41</td>
<td>18.8 ± 0.34</td>
<td>19.0 ± 0.59</td>
<td>18.8 ± 0.29</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>29.9 ± 0.59</td>
<td>29.5 ± 0.22</td>
<td>29.3 ± 0.47</td>
<td>28.7 ± 0.29</td>
</tr>
<tr>
<td>NRBC (/200 WBC)</td>
<td>5.36 ± 2.09</td>
<td>9.18 ± 2.61</td>
<td>3.92 ± 1.46</td>
<td>3.15 ± 1.11</td>
</tr>
<tr>
<td>WBC Count (/µL)</td>
<td>9.96 ± 2.93</td>
<td>11.4 ± 2.23</td>
<td>10.8 ± 2.00</td>
<td>10.8 ± 1.58</td>
</tr>
<tr>
<td>Seg (%)</td>
<td>57.1 ± 5.79</td>
<td>60.7 ± 4.74</td>
<td>57.3 ± 3.19</td>
<td>60.5 ± 4.06</td>
</tr>
<tr>
<td>Band (%)</td>
<td>1.36 ± 0.53</td>
<td>3.00 ± 1.85</td>
<td>0.92 ± 0.33</td>
<td>1.00 ± 0.47</td>
</tr>
<tr>
<td>Lymph (%)</td>
<td>37.2 ± 6.06</td>
<td>31.6 ± 3.07</td>
<td>36.4 ± 3.14</td>
<td>34.5 ± 3.64</td>
</tr>
<tr>
<td>Mono (%)</td>
<td>3.45 ± 0.74</td>
<td>2.91 ± 0.77</td>
<td>4.23 ± 0.97</td>
<td>2.97 ± 0.78</td>
</tr>
<tr>
<td>Baso (%)</td>
<td>0.36 ± 0.28</td>
<td>0.09 ± 0.09</td>
<td>0.62 ± 0.38</td>
<td>0.22 ± 0.12</td>
</tr>
<tr>
<td>Eosin (%)</td>
<td>0.55 ± 0.16</td>
<td>0.73 ± 0.30</td>
<td>0.54 ± 0.14</td>
<td>0.78 ± 0.23</td>
</tr>
</tbody>
</table>
Figure 5.8 Serum IL-10 concentrations measured pre-infection and every 12 hours post-infection and a repeat analysis measures was performed. Blood collected at the catheter was used for serum to measure IL-10 using a porcine specific ELISA kit (R&D Systems, Minneapolis, MN). There was an overall significant effect of time where animals produced the highest concentration of IL-10 at 96 and 108h post-infection. There was also an effect of treatment, where animals who received *B. infantis* had elevated IL-10. There was no effect of Lf, therefore values were pooled for *B. infantis* (n=26) which included *B. infantis* and COMB groups; and No *B. infantis* or No Probiotic (n=23) which included CON and LF groups. Values are means ± SEM.
Figure 5.9 Serum IL-10 concentrations measured pre-infection were analyzed by cross-sectional analysis. Blood collected at the catheter was used to obtain serum to measure IL-10 using a porcine specific ELISA kit (R&D Systems, Minneapolis, MN). There was an effect of treatment, where animals who received LF had decreased IL-10 prior to infection. There was no effect of B. infantis. Values are means ± SEM, n=11-13 per group. bLF, bovine lactoferrin; COMB, combined
Figure 5.10 B cell population was measured using flow cytometry at 72 and 120 hours post-infection from isolated PBMC and identified as double positive for CD21 and MHCII as a percent of PBMC (CD21+MHCII+). The relative number of subpopulations was determined using FlowJo 7.0 software (FlowJo, Ashland, OR). The absolute values at each of these time points did not significantly differ for any of the treatment groups, however all animals experienced a significant increase in the percentage of B cells from 72 to 120 hours post-infection. Furthermore, LF-fed animals, including LF and COMB, had less of a percent change compared with Whey, including B. infantis and CON.

Values are means ± SEM, n=8-9 per group. bLF, bovine lactoferrin; COMB, combined
Figure 5.11 Memory T cell population was measured using flow cytometry at 72 and 120 hours post-infection from isolated PBMC and identified as double positive for CD4 and CD8 as a percentage of CD3 positive (CD3⁺CD4⁺CD8⁺). The relative number of subpopulations was determined using FlowJo 7.0 software (FlowJo, Ashland, OR). *B. infantis* significantly decreased memory T cells at 120 hours post-infection. There was no significant change over time from 72 to 120 hours post-infection. Additionally, *B. infantis*, including *B. infantis* and COMB animals, had significantly decreased percent change in memory T cells compared with animals that did not receive *B. infantis*, including LF and CON. Values are means ± SEM, n=8-9 per group. bLF, bovine lactoferrin; COMB, combined
Figure 5.12 Monocyte population was measured using flow cytometry at 72 and 120 hours post-infection from isolated PBMC and identified as double positive for CD14 and CD163 as a percentage of CD172 positive (CD172⁺CD14⁺CD163⁺). The relative number of subpopulations was determined using FlowJo 7.0 software (FlowJo, Ashland, OR). The absolute values at each of these time points did not significantly differ for any of the treatment groups, and there was no significant change over time. However, LF-fed animals, including LF and COMB, tended to have less of a percent change compared with Whey, including B. infantis and CON. Values are means ± SEM, n=7-9 per group. bLF, bovine lactoferrin; COMB, combined
Figure 5.13 Foxp3 CD4+ T cell expression at 120 hours post-infection from a CD4+ T cell enriched population obtained using MACS depletion. RT-qPCR method was used to measure cDNA expression and data expressed as fold difference. Normalized values for Foxp3 were calculated by dividing the target quantity mean by the RPL19 quantity mean. A fold-difference was calculated for each measurement by dividing the normalized target values by the average normalized target value for CON animals. All samples that were statistically compared to each other were run on the same plate. COMB animals had significantly increased Foxp3 relative to B. infantis and LF-fed animals, however Foxp3 expression did not differ between CON and COMB. Values are means ± SEM, n=5-8 per group. bLF, bovine lactoferrin; COMB, combined
CHAPTER 6
CONCLUSIONS AND FUTURE DIRECTIONS

The overall focus of this dissertation was the impact of bovine Lf (bLf) supplementation on gastrointestinal development and how bLf and B. infantis combined mediate the immune response during a systemic S. aureus infection to promote bacterial clearance. Three aims were proposed and the conclusions from each aim are as follows.

At the intestine, LF increased proliferation, beta-catenin mRNA expression, and crypt size. The implications of improved intestinal maturation suggest a more mature immune response that begins at the intestine and effects systemic immunity. The direct connection of local immunity to systemic immunity due to improved intestinal maturation is a key future direction to further explore. In our research, we have looked at the trophic effects of Lf, and effects of oral Lf on a systemic immune response.

When fed prior to and during a systemic S. aureus infection, Lf decreased S. aureus load at the kidney while tending to decrease S. aureus load at the lung. Lf-fed animals had altered immune status prior to infection as assessed by CBC/differential and serum IL-10 analysis. Lf also increased inflammatory responses specifically at the lung as evidenced by increased IFNγ mRNA expression, and had an immunomodulatory role at the kidney as evidenced by altered TLR2 and IL-10 expression. Lastly, Lf affected immune cell populations as assessed by flow cytometry during the adaptive immune response beginning at 72h post infection with a decreased percent change for monocytes and B cells in the PBMC population.

Lf alone decreased S. aureus incidence and severity (tissue load), while B. infantis induced anti-inflammatory and T regulatory responses. B. infantis decreased inflammatory immune responses by increasing serum cytokine IL-10 and kidney IL-10
expression. In the presence of Lf, *B. infantis* was beneficial, especially in the lung where *S. aureus* translocation incidence was decreased. In the absence of Lf, *B. infantis* may have been harmful due to suppressed immune response which led to decreased memory T cell formation. Overall, the combination of Lf and *B. infantis* in formula may improve the neonatal immune response, while decreasing potentially harmful over-inflammatory responses to infection.

In light of these results, there are several additional future directions to further our understanding. We have gained a great depth of knowledge and the *S. aureus* model is very valuable for investigating a key clinical problem that impacts infants, and also to understand how dietary Lf educates the immune system both at the intestine, and through direct interaction outside the intestine. It is well known that Lf is able to sequester iron in biological fluids and destabilize the membranes of microorganisms. This is one mechanism by which Lf is believed to be bacteriostatic. Therefore, one future direction would be to conduct additional experiments to delineate how Lf and *S. aureus* interact with each other. Lastly, due to combined effects, we are interested in investigating the question of how *B. infantis* and Lf interact and alter the microbiota composition. The microbiota in turn impacts the immune response and this would help to take us a step further in connecting the local environment of the intestine with the local and systemic immune systems.
References


Corthesy B, Gaskins HR, Mercenier A. Cross-talk between probiotic bacteria and the host immune system. *ASN J. Nutr* 2007; 137: 781S-790S.


Mohamed MA, Cunningham-Rundles S, Dean CR, Hammad TA, Nesin M. Levels of pro-inflammatory cytokines produced from cord blood in-vitro are pathogen dependent and increased in comparison to adult controls. *Cytokine* 2007; 39: 171-177.


Palmqvist N, Foster T, Tarkowski A, Josefsson E. Protein A is a virulence factor in *Staphylococcus aureus* arthritis and septic death. *Microb Pathog* 2002; 33: 239-249.


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Vogel HJ. Lactoferrin, a bird’s eye view. Biochem Cell Biol 2012; 90: 233-244.


APPENDIX

ESTABLISHING A SYSTEMIC S. AUREUS INFECTION MODEL IN THE NEONATAL PIGLET

Abstract:

*Staphylococcus aureus* (*S. aureus*) is a primary cause of death due to sepsis in neonatal intensive care units. Fatal sepsis is characterized by systemic inflammatory response syndrome (SIRS), leading to systemic inflammation and organ dysfunction. Herein, a clinically-relevant piglet model of SIRS secondary to *S. aureus* infection was developed. The *S. aureus* strain: S54F9 used in this study had been used in a model of *S. aureus* septicemia in 8-wk-old pigs (Leifsson et al 2010; Nielsen et al 2009).

The goal herein was to establish an efficacious dose for systemic *S. aureus* infection in piglets. Colostrum-deprived pigs had umbilical catheters placed within 12h of birth and were fed sow’s milk replacer formula. On d7, piglets were either non-infected (n=2) or injected IV with 1ml/kg BW *S. aureus* (strain S54F9) at $10^3$ (low; n=3) or $10^5$ (high; n=3) CFU/ml. Blood was repeatedly sampled from the catheter to follow the course of cytokine response and piglets were euthanized 10-14d post-infection.

Most infected animals developed a transient fever (>39.4°C) post-infection. Infected animals had elevated WBC count and percentage of neutrophils, characteristic of SIRS. *S. aureus* was detected in the spleen (n=5), kidney (n=3), lung (n=3) and heart (n=2) of infected animals and high-dose pigs developed rear limb septic arthritis confirmed by culture. Serum cytokine responses were variable; however, IL-10 was highest in the first 24 h post-infection, whereas IL-6 peaked between 72-96 h post-infection. In conclusion, we have established a piglet model of systemic *S. aureus* infection that can be used to investigate therapeutic nutritional interventions.
Introduction

The goal of the preliminary study was to establish the piglet *S. aureus* model. The first step was to determine an efficacious dose of *S. aureus* to administer to the piglets and to define the time course of response to infection. S54F9 was initially isolated from a pig pulmonary abscess and had been previously used to study systemic *S. aureus* infection in weaning pigs (Leifsson et al 2010; Nielsen et al 2009). S54F9 has been generously provided by the microbiologist, Dr. Bent Aalbæk, from the veterinary department at the University of Copenhagen and was cultured as recommended.

Materials and Methods

*S. aureus* growth and preparation for infection

Briefly, the bacteria were provided via BBL Culture Swab, and the swab was enumerated on mannitol salt agar (MSA), a selective and differential medium for staphylococci (Figure A.1). *S aureus* forms yellow colonies on MSA. One colony was picked and propagated in 10ml LB broth for overnight growth at 37 °C for 24 hours. The *S. aureus* was frozen in a 1:1 12.5% glycerol buffer for long term storage.

Prior to experimentation, we established the relationship between the turbidity of our culture (optical density) using a spectrophotometer and our approximate cell concentration (colony forming units/ ml) as shown in Figure A.1. To determine how many cells give a specific OD, we grew our culture for different amounts of time, measured our OD, and plated them on MSA to obtain a standard curve. We grew our
cells to mid-log for the day of experiment (OD range 0.4-0.6), where bacteria are not competing for nutrients, are metabolically active and experience minimal stress.

On the day of infection, *S. aureus* were grown to an OD of 0.5, which corresponded to a concentration of \( \sim 4 \times 10^8 \) based on our standard curve. The cell suspension was centrifuged and the bacterial pellet washed with sterile isotonic saline twice prior to a 3-fold and 5-fold dilution in isotonic saline to obtain our target high *S. aureus* \( (10^5) \) and low *S. aureus* \( (10^3) \) doses. The dose was administered at 1ml/kg body weight. Non-infected animals were administered 0.9% sterile isotonic saline at 1ml per kg body weight. Control animals were used to ensure animals were reacting to active infection and not the environmental conditions of our animal facility or due to the fact that they were colostrum deprived.

*Animal Protocol*

All animal care and experimental procedures were in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Illinois. The study design is shown in **Figure A.2**. One sow was monitored for farrowing and piglets (n=8) removed prior to ingestion of colostrum. Sow serum was administered to provide passive immunity via oral gavage. Serum was administered at a volume of 5ml/kg body weight at birth, 12, 24 and 36 hours postpartum. The suckling newborn piglet absorbs all intact immunoglobulins by macromolecular absorption in the first 24 hours (Butler et al 2009). Piglets were individually housed in cages in environmentall-controlled rooms (25°C). Plastic heating pads in each enclosure will be used to maintain
an ambient temperature of 30°C. Piglets were fed non-medicated, sow-milk replacer (Milk Specialties Global Animal Nutrition, Carpentersville, IL) 20-times daily at a rate of 360 ml/kg BW/d. The piglets were monitored each morning for formula intake, weight gain and stool consistency using the following scale: 1=firm; 2=stiff-flowing; 3=easy-flowing/loose; 4=watery/diarrhea; 5=severe diarrhea (Correa-Matos et al 2003).

Umbilical Catheterization

Piglets underwent a surgical procedure within 12 h of birth to place two umbilical catheters; one for administering the *S. aureus* and the other for blood sampling using established methods (Donovan et al 1997). Briefly, piglets were lightly sedated with 2% isoflurane (IsoFlo, Abbott Laboratories, North Chicago, IL). The abdomen was washed and an iodine disinfectant applied to the umbilicus and surrounding region. To numb the umbilicus, lidocaine (Henry Schein, Melville, NY) was injected subcutaneously into multiple sites surrounding the umbilical stump. One catheter (3.5 french polyvinyl chloride catheter; Tyco Healthcare Group, Mansfield MA) was inserted 22 cm into the dorsal aorta to a position near the heart. A second catheter for blood sampling was inserted and advanced 20 cm. Catheters were sutured to the umbilical stump and secured to the body with suture and elastic tape. Piglets were fitted into a jacket to allow free movement within the cages. Catheter patency was maintained by flushing twice daily with heparinized saline (10 IU heparin/ml in 0.9% NaCl). Piglets received one systemic dose of Enrofloxacin (2.5 mg/kg; Bayer, Shawnee Mission, KS). *S. aureus* forms biofilms in catheters and these biofilms present a complication in the treatment of *S. aureus* in the hospital environment making this a clinically feasible model for
transmission (Thurslow et al 2011). The prolonged maintenance of the catheters in pre-term neonates in the intensive care further enhances contamination and translocation of *S. aureus* from skin to blood stream.

**Sample Collection**

Blood samples were collected into non-heparinized vacuum tubes via the sampling umbilical catheter. Piglets were euthanized by day 14 post-partum (Figure A.2) by an intravenous injection of sodium pentobarbital (72 mg/kg BW, Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI).

**S. aureus Load**

Immediately after euthanasia, blood, kidney, lung, heart and spleen were collected to determine *S. aureus* load. Tissues were be homogenized in sterile PBS using the stomacher at a 1:10- 1:5 dilution and serial diluted in cold PBS to be plated on Mannitol salt agar using a spiral plater (Neu-Tec Group Inc., Farmingdale, NY) at neat, 1:2, 1:5, 1:10, and 1:100 dilutions. Plates were incubated for 48 hours at 37 °C and colony forming units were counted and results expressed as cfu/ml. Plates were quantifiable at a minimum 30-40 colonies/plate and considered positive at a minimum of 7-10 colonies/plate for the purposes of establishing our model.

**Complete Blood Count and Differential**

Blood was collected into EDTA-coated tubes and inverted immediately after collection to distribute anticoagulant. Blood tubes were submitted to the hematology
department at the University of Illinois, College of Veterinary Medicine for a CBC and
differential blood count. In future studies, we plan to do a fresh blood smear to be
submitted for CBC because several of our samples coagulated and we were not able to
analyze them.

Serum Cytokines

IL-6 and IL-10 were measured by ELISA using commercially available porcine
specific ELISA kits (R&D Systems, Minneapolis, MN). These cytokines were selected as
they were shown to be significantly elevated in patients with severe sepsis, and sustained
elevation distinguished between survivors and non-survivors (Gogos et al 2000). IL-6
was chosen over TNFα based on studies which have shown infants have a more robust
IL-6 response (Angelone et al 2006).

Results

A febrile response (Table A.1) was observed in three high *S. aureus* and two low
*S. aureus* piglets, however no pattern was observed based on *S. aureus* dose. The
hematological results (Table A.2) indicated characteristics of a bacterial infection, where
high neutrophil percent and an elevated white blood cell count were observed in the
blood.

Elevated IL-6 (Figure A.3) was present later in infection- at 72 and 96 h post-
infection-in all animals, except for one low dose *S. aureus* animal that had no elevated
IL-6 during any time point of infection. Elevated IL-10 (Figure A.4) was present in
early infection within the first 24 hours post-infection, except for one low dose *S. aureus*
animal that had no elevated IL-10. All three high *S. aureus* animals had approximately the same amount of IL-10 at 24 hours, whereas low dose animals exhibited no consistent pattern.

The *S. aureus* load results (*Table A.3*) indicate the presence of *S. aureus* at the spleen, kidney, lung and heart. The dose is too low to detect *S. aureus* in the blood. High dose pigs tended to have a higher *S. aureus* load at the lung, heart and spleen.

Lastly, we observed gross pathological findings of *S. aureus* infection. All high *S. aureus* animals had a swollen joint characteristic of osteomyelitis. Animals had visible abscess and lung lesions, with a higher incidence in high *S. aureus* animals, as previously observed with strain S54F9 (Leifsson et al 2010; Nielsen et al 2009). Taken together, we chose to use the higher dose in our piglet studies. Despite having a small number of subjects, we expect a more consistent response in the high dose *S. aureus* group. Based on preliminary experiments, high dose *S. aureus* animals had a more common pathology for strain S54F9, and all animals in this group responded to the dose with elevated serum cytokines whereas in the low *S. aureus* group, we had animals that were non-responders.
Figure A.1 *S. aureus* growth and preparation for infection. BBL Culture Swab was enumerated on mannitol salt agar, a selective and differential medium where *S. aureus* forms yellow colonies (A). Several colonies were picked and grown for stock. Prior to experimentation, the relationship between turbidity of culture (optical density or OD) and viability (colony forming units/ml or CFU/ml) was determined (B).
Newborn, Colostrum-deprived

Control or CON (n=2) “non-infected”

$S.\ aureus\ 4 \times 10^3$ at 1ml/kg BW (n=3)

$S.\ aureus\ 4 \times 10^5$ at 1ml/kg BW (n=3)

Infect All Pigs IV

D14: collected tissue for $S.\ aureus$ load, blood for analysis, and observed gross pathology.

Collect serum: baseline, and every 12 hours post-infection for serum cytokines. Measure rectal temperature every 12 hours

Umbilical Catheterization

Day: 0 0.5 1 2 3 4 5 6 7 8 9 10 11 12 13 14

**Figure A.2** Study design. Newborn colostrum-deprived piglets (n=8) were randomized to: CON “non-infected;” low dose $S.\ aureus\ (10^3);\ or\ high\ dose\ S.\ aureus\ (10^5)\). Piglets received an oral gavage of serum at 0, 12, 36 and 48 hours post-partum and were all fed sow milk replacer (SMR) diet. Within 12 hours of birth, piglets received two umbilical catheters, one for sample collection and one for infection. On day 7 post-partum, all piglets were infected with $S.\ aureus$ strain S54F9. Piglets were euthanized by day 14 post-partum.
Table A.1 Rectal temperature pre-infection, peak febrile response and hour for *S. aureus* animals infected with low ($10^3$) or high ($10^5$) dose. A febrile response was observed in three high *S. aureus* and two low *S. aureus* piglets.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Start Temp (°C)</th>
<th>Peak Fever (°C)</th>
<th>Time (hr)</th>
<th>Difference (°C)</th>
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</thead>
<tbody>
<tr>
<td>High</td>
<td>38.5</td>
<td>40.2</td>
<td>24</td>
<td>1.7</td>
</tr>
<tr>
<td>High</td>
<td>39.4</td>
<td>41.2</td>
<td>72</td>
<td>1.8</td>
</tr>
<tr>
<td>High</td>
<td>38.7</td>
<td>39.8</td>
<td>84</td>
<td>1.1</td>
</tr>
<tr>
<td>Low</td>
<td>40.4</td>
<td>42.1</td>
<td>36</td>
<td>1.7</td>
</tr>
<tr>
<td>Low</td>
<td>38.5</td>
<td>41.3</td>
<td>48</td>
<td>2.8</td>
</tr>
<tr>
<td>Low</td>
<td>39.2</td>
<td>39.8</td>
<td>12</td>
<td>0.6</td>
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</table>
Table A.2 Hematological Results for high (n=2) \textit{S. aureus} pigs and CON (n=1). Fresh blood was collected at necropsy to be submitted to University of Illinois Clinical Pathology Laboratory. Characteristics of a bacterial infection, where high neutrophil percent (segment and band) and an elevated WBC count were observed.

<table>
<thead>
<tr>
<th>ID</th>
<th>High Dose</th>
<th>High Dose</th>
<th>CON</th>
<th>Ref. Range</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Units</td>
<td>Units</td>
<td>Units</td>
<td></td>
</tr>
<tr>
<td>WBC Count</td>
<td>/ul</td>
<td>21.1 x 10$^3$</td>
<td>18.9 x 10$^3$</td>
<td>6.75 x 10$^3$</td>
</tr>
<tr>
<td>Seg %</td>
<td>%</td>
<td>80</td>
<td>64</td>
<td>38</td>
</tr>
<tr>
<td>Band %</td>
<td>%</td>
<td>5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Lymph %</td>
<td>%</td>
<td>9</td>
<td>22</td>
<td>53</td>
</tr>
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</table>
Figure A.3 Serum IL-6 was measured pre-infection and every 12 hours post-infection.

Blood collected at the catheter was used for serum to measure IL-6 using a porcine specific ELISA kit (R&D Systems, Minneapolis, MN). Elevated IL-6 was present later in infection at 72 and 96 hours post-infection in all except, except for one low dose *S. aureus* animal that had no elevated IL-6 during any time point of infection.
**Figure A.4** Serum IL-10 was measured pre-infection and every 12 hours post-infection. Blood collected at the catheter was used for serum to measure IL-10 using a porcine specific ELISA kit (R&D Systems, Minneapolis, MN). Elevated IL-10 was present in early infection within the first 24 hours, except for one low dose *S. aureus* animal that had no elevated IL-10 during any time point of infection. All three high *S. aureus* animals had approximately the same amount of IL-10 at 24 hours, whereas low dose animals exhibited no pattern.
Table A.3 *S. aureus* load data expressed as CFU/g. Results indicate the presence of *S. aureus* at the spleen, kidney, lung and heart. The dose is too low to detect *S. aureus* in the blood. High dose pigs tended to have a higher *S. aureus* load at the lung, heart and spleen.

<table>
<thead>
<tr>
<th><em>S. aureus</em> Dose</th>
<th>Time Post Infection/Postnatal Age</th>
<th>Spleen</th>
<th>Kidney</th>
<th>Lung</th>
<th>Heart</th>
</tr>
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<tbody>
<tr>
<td>High</td>
<td>100hr- Day 11</td>
<td>1.2x10</td>
<td>3.3x10</td>
<td>2.0x10</td>
<td>Pos* too dense</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
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<td>168hr- Day 14</td>
<td>8.0x10</td>
<td>7.3x10</td>
<td>3.6x10</td>
<td>9.0x10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>High</td>
<td>168hr- Day 14</td>
<td>8.3x10</td>
<td>1.7x10</td>
<td>3.3x10</td>
<td>3.3x10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Low</td>
<td>110hr- Day 11</td>
<td>7.0x10</td>
<td>2.8x10</td>
<td>2.0x10</td>
<td>2.0x10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Low</td>
<td>168hr- Day 14</td>
<td>4.8x10</td>
<td>3.2x10</td>
<td>1.66x10</td>
<td>2.0x10</td>
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<td>2</td>
<td>2</td>
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<tr>
<td>Low</td>
<td>80hr- Day 10</td>
<td>0</td>
<td>0</td>
<td>3.3x10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td></td>
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<tr>
<td>CON</td>
<td>168hr- Day 14</td>
<td>6.7x10</td>
<td>0</td>
<td>3.3x10</td>
<td>3.3x10</td>
</tr>
<tr>
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