Defining the Role of Lactoferrin at the Host-Microbe Interface

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LIST OF ABBREVIATIONS

AMP	Antimicrobial Peptide
CD	Cluster of Differentiation
CFU	Colony Forming Units
CP	Calprotectin
Cps	Capsular Serotype
EOD	Early Onset Disease
GAS	Group A *Streptococcus*
GBS	Group B *Streptococcus*
HMO	Human-Oligosaccharide
IAP	Intrapartum Antibiotic Prophylaxis
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
LB	Luria-Bertani Broth
Lf	Lactoferrin
LOD	Late Onset Disease
LPS	Lipopolysaccharide
MIC	Minimum Inhibitory Concentration
MLST	Multi-Locus Sequence Typing
MOI	Multiplicity of Infection
NET	Neutrophil Extracellular Traps
npx	NADH Peroxidase
OD	Optical Density
PBS	Phosphate Buffered Saline
PM	Placental Macrophages
PMA	Phorbol 12-myristate-13-acetate
PPROM	Preterm Premature Rupture of Membrane
ROS	Reactive Oxygen Species
SEM	Scanning Electron Microscopy
SNP	Single Nucleotide Polymorphism
STs	Sequence Types
TGF	Tumor Growth Factor
THB	Todd-Hewitt Broth
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor

INTRODUCTION

Group B *Streptococcus* is the leading infectious agent of preterm birth and adverse pregnancy outcomes

Group B *Streptococcus* (GBS), or *Streptococcus agalactiae*, is one of the leading causes of infection-related adverse pregnancy and neonatal outcomes, which include stillbirth, chorioamnionitis, preterm birth, and neonatal sepsis and meningitis (1,2). The pathogen can infect by vertical transmission, either through ascending infection from the colonized mother, or exposure during vaginal birth (3). It is estimated around 18% of pregnant women are colonized with GBS, making colonization the leading risk factor for disease. Generally, GBS is a commensal bacterium, but some strains can transition into an invasive pathogen, through mechanisms not well understood. The most current comprehensive systematic review and meta-analysis estimated the grouped incidence of neonatal morbidity and mortality to be 0.47 per 1000 worldwide, including ones attributed to GBS-associated preterm birth, stillbirth, and neonatal GBS infection (4). For standard care, the CDC recommends routine screening for GBS at 36 weeks of pregnancy and administration of intrapartum antibiotic prophylaxis (IAP) to mothers who test positive (1). Despite recommended precautions and intervention, IAP appears to have little effect on the rate of late-onset disease in newborns or preterm birth and stillbirths, which occurs after a week of life (1,5-6). Currently, it is estimated that these GBS-related adverse outcomes impact 97,000 to 4 million pregnancies annually. Unsurprisingly, the overuse of IAP contributes to antibiotic resistance in GBS. One study in the United States where screening and IAP are standard of care, showed 32% and 15% of GBS isolates were resistant to erythromycin and clindamycin (7), respectively. A similar study in South Africa, where IAP is not commonly used, provides a stark contrast, with only 4% and 2% of GBS isolates reported to be resistant to erythromycin and clindamycin, respectively (8). Given this ineffective intervention against GBS and observed increases in antibiotic resistance, novel strategies must be developed to combat GBS-related adverse pregnancy outcomes.

Ascending Infection of GBS from the vaginal mucosa leads to disease

GBS colonizes up to 30% of pregnant women, and while most remain asymptomatic, some strains can cause disease through ascending infection from the vaginal mucosa into the uterus. This process involves trafficking of bacteria from the vagina to other reproductive tissues, ultimately invading placental membranes (chorion and amnion), the amniotic cavity, and the fetus (9). GBS employs virulence factors to promote colonization, adhesion and invasion of host cells, and modulation of the inflammatory response (10). One such virulence factor is the formation of biofilms, which is critical for persistence and chronic infection (10). These sessile communities protect the bacterium from immune assault and the hostile vaginal environment, allowing for successful establishment on the vaginal epithelium (11).

A fine balance of inflammation is critical for a healthy pregnancy during infection

The developing fetus is semi-allogenic to the mother so immune tolerance must be maintained for a successful pregnancy (12). Placental macrophages (PMs), or Hofbauer cells, are fetal-derived leukocytes that reside within the connective tissue core of the placental villous tree (13). PMs, representing around 20-30% of human gestational tissue leukocytes, play a key role in placental invasion, angiogenesis, tissue remodeling, and development (13,14). These phagocytes typically display a tolerogenic M2 or M2-like phenotype, constitutively express anti-inflammatory cytokines such as IL-10 and TGF-β1 (15), and suppressors of cytokine signaling (16), which is crucial in supporting an optimal environment for fetal development (17). A highly inflammatory environment and macrophage polarization to classical pro-inflammatory M1 state are associated with abnormal pregnancies, including spontaneous abortions, preterm labor, and preeclampsia (1).

Upon contact with the GBS, PMs will phagocytose the pathogen and initiate a proinflammatory immune response (18). The release of pro-inflammatory cytokines and chemokines by the phagocytes and other resident immune cells leads to recruitment of neutrophils. Neutrophils aid in the clearance of

GBS by a repertoire of defenses including reactive oxygen species (ROS), antimicrobial peptides (AMP), and other enzymes (19).

Lactoferrin is expressed in response to GBS infection

One AMP produced by neutrophils is lactoferrin, a glycoprotein with two iron binding domains, each of which chelates a single iron ion with high affinity (20), starving invading bacteria of this crucial metal (21). In addition to chelating iron, two regions of the protein exhibit potent antimicrobial activity against a range of bacteria, fungi, and viruses (22). Previous studies in our lab have demonstrated that lactoferrin expression is upregulated in response to GBS infection in a mouse model (23). In addition to its antimicrobial properties, immune-modulatory properties have been described (24).

Rationale for study

Despite antibiotic treatment and vaccine attempts, GBS remain a leading cause of perinatal infections. With the rise of antibiotic resistant strains of GBS, novel therapies independent of traditional chemical antibiotics are warranted. Furthermore, there remains a gap in knowledge in the host-microbe interactions during pregnancy. My central hypothesis is that lactoferrin possesses antimicrobial activity against GBS while interacting with placental macrophages to modulate the immune response to maintain an immune-tolerant environment for the developing fetus.

Chapter I outlines the current knowledge of the lactoferrin against *Streptococci species* and other bacterial pathogen in the literature. In Chapter II, I assess the antimicrobial and anti-biofilm property of lactoferrin against a clinical strain and establish a mechanism. In Chapter III, I further my studies from Chapter II and conducted my studies against a genetically and diverse panel of clinical GBS strains. On the immune-modulatory side of lactoferrin, I describe how placental macrophages response to treatment with lactoferrin in Chapter IV. In Chapter V, I investigate if lactoferrin possesses antimicrobial and antibiofilm property against other bacterial pathogens threatening public health. Finally, I examined the antibiotic enhancing activity of lactoferrin against GBS and other bacterial pathogens in Chapter VI. Together, my studies will establish the role of protective role of lactoferrin

during GBS infection and propose lactoferrin as a viable therapeutic candidate against multiple bacterial pathogens.

A version of the following section (*Chapter I, Lactoferrin is a critical mediator of both host immune response and antimicrobial activity in response to Streptococcal infections infection*) was originally published in *American Chemical Society Infectious Disease*. (Apr 2020).

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CHAPTER I

Lactoferrin is a critical mediator of both host immune response and antimicrobial activity in response to streptococcal infections

Introduction

A variety of biological processes is conserved across all kingdoms of life and requires nutrient metals as cofactors. Invading bacterial pathogens require nutrient iron as a cofactor for cellular processes including DNA replication, respiration, peroxide reduction, electron transport, cell division, and stress response. In response, the host exploits this need for nutrient metals such as iron by deploying a variety of factors to bind iron with high affinity, effectively starving the invading pathogen in a process termed, “nutritional immunity” (25). Nutritional immunity in the vertebrate host includes numerous antimicrobial peptides such as calprotectin, calgranulin C, ferritin, hemoglobin, transferrin, and lactoferrin. Lactoferrin is highly abundant in host tissues infected with bacterial pathogens such as *streptococcal spp*. Interestingly, several of these nutritional immunity proteins also have immunoregulatory properties. This chapter will focus on the intersection of lactoferrin’s involvement in antimicrobial activity and immune regulation and the pathogenesis of *Streptococcus*.

Streptococcus and human health

The genus of *Streptococcus,* which includes a group of gram-positive cocci bacteria, is one of the leading causal agents of human infectious diseases (26). The genus is separated into 49 species and eight subspecies, 35 of which are attributed as the source of invasive infections in humans. Four of these Streptococcal species, which include *S. pyogenes* (Group A *Streptococcus*, or GAS), *S. agalactiae* (Group B *Streptococcus*, or GBS), *S. mutans*, and *S. pneumoniae*, are common causes of bacterial infections in humans. Collective, these species of bacteria create an immense public health burden (27).

Invasive GAS infections include a number of clinical manifestations locally in the skin, joints, soft tissue, or the lower respiratory tracts, or globally as bacteremia without a focus of infection (28). GAS

is one of the top human-infection related cause of death, with 1.8 million new cases reported per year with an estimate of 517,00 deaths annual (29,30). GAS infections can result in fatality in one of two ways: necrotizing fasciitis and sepsis associated with pregnancy or the puerperium. The former is a rare rapidly spreading infection of the soft tissue that results in the necrosis of fascia, skin, and subcutaneous fat (31), while the latter results from infection of tissues made vulnerable by pregnancy and birth, such as, breast tissue, genital tract, and surgical wound (32). Additionally, all invasive GAS infections can be complicated by streptococcal toxic shock syndrome (STSS), a condition characterized by hypotension and often accompanied by fever or rash with rapid progression to shock and multi-organ failure (33). Of those infected, only 10% of patients develop STSS, but early intervention is important as over 25% of these patients will result in death within 24 hours of presentation (34). GAS is also responsible for non-lethal disease conditions ranging from mild infections, such as impetigo and pharyngitis, to invasive diseases (35). Serious immune sequelae may be triggered after repeated GAS infections, including rheumatic heart disease and acute glomerulonephritis (36). Overall, the skin appears to be the most frequent portal of entry as marked by a third of patients have evidence of skin wounds or lesions (37,38). In particular, injecting drug users at vulnerable to acquiring GAS (39).

S. agalactiae, or Group B *Streptococcus* (GBS), is one of the leading infection-related causes of preterm birth, chorioamnionitis, neonatal sepsis, funisitis, bacteremia, mastitis, and invasive soft tissue infections in diabetics (1,18,40). Newborns, neonates, pregnant mothers, elderlies, and the immunocompromised are susceptible to GBS infection (2). GBS colonizes 20-30% of healthy adults, in the urogenital tract of females and lower gastrointestinal tract of both males and females (41). The human gastrointestinal tract serves as the major reservoir of GBS, where it exists as commensal flora (3). Between 20-30% of pregnant women are estimated to be colonized with GBS, making colonization the leading risk factor for disease (42). In one longitudinal study in South Africa, Kwatra *et al.* found that up to 50% of women in their study population was transiently colonized with GBS at some point during their pregnancies (43).

GBS is a large health burden, especially to mothers and neonates in developing countries. An estimate of 21.7 million pregnant mothers were colonized with GBS in 2015. With GBS infection, there is a higher risk of maternal and neonatal death and development (5). The most recent report of global vaginal GBS colonization estimates a prevalence of 18%, after adjusting for sample collection and methodology, ranging from the highest prevalence in the Caribbean (35%), to the lowest regional prevalence in Southern and Eastern Asia (11–13%) (42). An estimated four million newborns deaths are reported each year within the first 4 weeks of life globally, and one in four of these deaths stems from severe infection including pneumonia or sepsis. 99% of neonatal deaths occur in low- and middle-income countries (44). In developed countries, GBS and *Escherichia coli* are collectively responsible for approximately 70% of early-onset neonatal sepsis of both preterm and term infants (45). Neonatal colonization occurs in approximately 40–75% of births from GBS colonized mothers; 1–2% of these cases resulted in invasive disease (46–49). The most recent comprehensive systematic review and meta-analysis estimated the grouped incidence of neonatal morbidity and mortality to be 0.47 per 1000 worldwide, including ones attributed to GBS-associated stillbirth, preterm birth, and neonatal GBS infection (4).

S. mutans is the primary etiologic agent of human dental caries (50), and occasionally infective endocarditis (51). The bacterium can form biofilms on the surface of teeth, referred to as dental plaque biofilm (52). In fact, *S. mutans* is the prime initiator of plaque and a potent producer of acid, which results in exacerbation of tooth decay and oral disease. *S. mutans* has evolved to exist as part of the normal member of oral dental biofilms, unlike other pathogens that produce virulence factors that disrupt the host (53). As a result, *S. mutans* can easily enter the bloodstream during dental surgery, resulting in colonization of injured heart valves and induction of endocarditis (54). As dental caries are the most common and costly oral disease worldwide, *S. mutans* is the etiological root underlying this burden (55).

S. pneumoniae causes pneumonia, meningitis, otitis media, and septicemia (56). The pneumococcus is a commensal member in the pharynx and upper respiratory tract of healthy

individuals but may cause localized infections, such as otitis media, particularly in children. Invasive pneumococcal infection may become established at a variety of sites including the meninges and lung parenchyma. *S. pneumoniae* has been widely linked to respiratory infections in immunocompromised populations (57–59). Additionally, it is the bacterial major cause of community acquired pneumonia (CAP) and is an important cause of bacteremia, particularly in infants and older adults (2).

The populations most susceptible to invasive pneumococcal disease are children younger than two years old, adults above the age of 65, and those with certain underlying conditions, such as HIV infection, multiple myeloma and chronic kidney, liver or pulmonary disease (60). An estimate of about four million illnesses within the United States and about 450,000 hospitalizations per year are attributed to this bacterium (61). Furthermore, studies indicate that 10% of patients with invasive pneumococcal diseases die of their illnesses (62).

S. pneumoniae is spread through airborne droplets, and as part of the commensal microbiota of the upper respiratory tract, *S. pneumoniae* invades its host by colonizing the nasopharynx asymptomatically (57,63). After colonization, if the bacterium is not cleared by the immune system, the bacterium is spread *via* horizontal dissemination into the lower airways and other tissues and organs and becomes pathogenic (63).

Lactoferrin and the innate immune system

The innate immune system plays a critical role in defense against streptococcal infections. Briefly, the epithelial cells and resident antigen-presenting cells (macrophages and dendritic cells) interact with the pathogen, resulting in the production of proinflammatory cytokines. In a study with humanized mice, intravenous or intraperitoneal infection of GBS resulted in the upregulation of the cytokines TNF-α, IL-1β, IL-6, IFN-γ, IL-8, and IL-10 (64). IL-1β, IL-8 (or CXCL-8), and CXCL1 are crucial in facilitating the chemotaxis of neutrophils to the site of infection (65). Depletion of bone marrow neutrophils in a murine model resulted in increased bacterial burden of many bacterial species,

including GAS, illustrating the critical role neutrophils play in combatting bacterial infections such as those caused by Streptococcal species (66).

Neutrophils are phagocytic granular innate immune cells that are the first line of defense against invading bacterial pathogens. Neutrophils can both directly and indirectly kill bacteria. Reactive oxygen species (ROS) produced by NADPH oxidase activity is one of the potent antimicrobial strategies asserted by neutrophils (67). The reactive oxygen radicals, including H_2O_2, O^-_2, and $HO^\bullet$, concentrate in the phagosome, where it assaults the invading bacterial pathogens (68). Furthermore, neutrophils express myeloperoxidase (MPO) at high levels during oxidative burst, generating more reactive oxygen radicals (69). Finally, Nitric oxide radicals ($NO^\cdot$) are produced by the inducible nitric oxide synthase 2 (iNOS or NOS2) (70) to impair bacterial enzymes and growth (71).

In addition to phagocytosing bacteria, neutrophils degranulate and release a variety of proteins to aid in bacterial clearance. Neutrophils contain three types of granules with respect to antimicrobial killing. The primary granules, or azurophilic granules, contain the most toxic mediators, which include elastase, cathepsin, myeloperoxidase, and defensin (19). Meanwhile, the secondary granules, also known as specific granules, possess lactoferrin, lysosome, NADPH oxidase, and other antimicrobial peptides and enzymes (72). Finally, neutrophils excrete tertiary granules, which hold gelatinase, cathepsin, and collagenase. Neutrophils also release antimicrobial elements independent of granules by excreting DNA neutrophil extracellular traps (NETs). NETs are DNA structures decorated with antimicrobial agents, which compose of nuclear chromatin, histones, and other antimicrobial proteins that serve to immobilize and kill or inhibit the growth of invading microbes (73). Together, granule release and NET excretion generate antimicrobial defense arm against infection of many *Streptococcus* species.

One such antimicrobial defense against Streptococcal infections is lactoferrin, which is expressed within secondary granules and decorated on NETs. Recent work indicates that *S. agalactiae* induces the excretion of NETs that are enriched with lactoferrin (23). Lactoferrin is an antimicrobial

globular glycoprotein that is expressed by macrophages, eosinophils, and neutrophils in response to infection (74). Outside of immune cells, lactoferrin is expressed in a variety of tissues and fluids including breast milk, colostrum, tear fluid, saliva, and mucous (75).

Structure- function relationships of lactoferrin

Lactoferrin is an iron-binding glycoprotein with a size of 80 kDa. The protein consists of a single polypeptide chain containing 692 amino acids, which is folded into two symmetrical lobes (N and C) with the two regions connected with a hinge region (76) **(Figure 1)**. Each lobe can bind one Fe^{3+} (ferric) iron together with one CO_3^{2-} iron within the cleft of the lobe that contains a nonheme iron-binding site (76). While lactoferrin predominately binds iron, it can also bind Cu^{2+}, Mn^{2+}, and Zn^{2+} at lower affinity (77). Two regions of lactoferrin have been studied in the iron-independent activity of the glycoprotein. This first region contains the first 47 residues starting at the N-terminus and is referred to as lactoferricin (24). The residues from this region come together to form a β-α-β unit at the surface of human lactoferrin, isolated from the iron-binding regions. The exposed region includes nine amino acid side chains projecting from its surface. This region is located in a highly charged N-terminal tail (residues 1 to 5; GRRRR) and an amphipathic region near the C terminus of helix A (residues 28 to 31; RKVR) that are in close proximity in the folded human lactoferrin protein (76,78). This highly basic region has been implicated in direct bacterial killing (79). Furthermore, this region of the N-terminus has been shown to bind to the DNA sequences upstream of various genes and act to regulate transcriptional activation (80,81). Another major binding site is at residues 269-285 in the N-lobe of human; this region is termed lactoferrampin (24). These two peptides are generated by pepsin cleaving human lactoferrin (82). Overall, the glycoprotein is very basic, which allows for interaction with negatively charged molecules in solution and on cell surfaces (83).

The lactoferrin gene possesses polymorphisms in human population with varying effects on microbial clearance. One such single-nucleotide polymorphism (SNP) occurs in a lysine (K)/arginine (R) polymorphism at the 29th position of the N- terminal region. For lactoferrin with a lysine at the 29th

position (hLf-K) and lactoferrin with an arginine at the 29th position (hLf-R), the K and R alleles had frequencies of 24% and 76%, respectively, among 17 healthy human subjects and 72% and 28%, respectively, among 9 individuals with localized juvenile periodontitis (84). In a study from Velliyagounder *et al*., hLf-K was found to significantly improve antimicrobial activity against *S. mutans* and *S. mitis* compared to hLf-R (84). However, no difference was observed in antimicrobial activity against Gram-negative bacteria. The study also performed genetic analysis and revealed that the K allele (hLf-K) was more frequent in their sample of patients with localized juvenile periodontitis, which is an aggressive and rapid form of periodontitis that disproportionally affect African American adolescents (85). Interestingly, the group found that the K allele (hLf- K) was more prevalent in the African American population, which is correlated with higher incidence of the disease. The authors hypothesized that the more bactericidal allele (hLf-K) kills more Gram-positive bacteria, which changes the microflora and allows for the Gram-negative bacteria *Aggregatibacter actinomycetemcomitans* to colonize (84). Importantly, *A. actinomycetemcomitans* colonization is linked to localized juvenile periodontitis (85).

In a different study, Barber *et al.* used various genetic tools to investigate the evolution of the lactoferrin across the human ancestral timeline. The group revealed that the lactoferrin gene acquired iron-independent antimicrobial capability through positive selection across the evolutionary history of primates (86). They also pinpointed signatures of natural selection, specifically pressure from microbial pathogens, acting on lactoferrin in human populations, suggesting that lactoferrin genetic diversity has impacted the evolutionary success of both ancient primates and humans. For instance, the authors call attention to bacterial receptors that target transferrin, a related gene, for iron acquisition. This repeated targeting of transferrin may provide advantage for antimicrobial activity to arise in the n-lobe of lactoferrin. Furthermore, the authors also describe a polymorphism in the lactoferrin gene that also correlated to health disparities. Like the SNP at position 29 described previously, the polymorphism at the 47th amino acid is also more prevalent amongst the African-related population. The African populations possess the Arg variant of lactoferrin at the 47th position. Interestingly, these populations

are disproportionally affected by preterm birth and dental caries, both with *Streptococcus*-infection etiology, highlighting the importance of the peptide in antimicrobial defense and human health (87,88).

Immunoregulatory functions of lactoferrin

While lactoferrin is largely recognized as an antimicrobial peptide, the glycoprotein also possesses immunoregulatory properties, both dependent and independent of iron chelation. Polymorphonuclear neutrophils are recruit to the site of infection in an inflammatory event. Once the PMN reach the site, the cells will release ROS to aid in the clearance of the bacterial pathogen. However, reactive oxygen radicals are not only detrimental to bacterial cells, but also will non-discriminately damage the surrounding host tissue. One consequence of cell damage by ROS assault is the release of ferric and ferrous iron. The free iron may then participate in the Haber-Weiss reaction to generate new free radicals, further exacerbating the damage. Lactoferrin plays a crucial role in preventing further damage by chelating iron released by the wounded tissue, resulting in the alleviation of oxidative stress at the site of inflammation (89).

Independent of iron chelation, lactoferrin can interact with receptors of immune cells to dampen a pro-inflammatory response (90). The glycosaminoglycans of membrane proteoglycans on cell surfaces account for 80% of binding by lactoferrin at a low affinity (10^{-5}-10^{-6} M) (91). One consequence of this interaction is the downregulation of pro-inflammatory cytokines by the immune cells. Lactoferrin has been shown to bind to bacterial lipopolysaccharide (LPS), the ligand for TLR4, thus mitigating TLR-4 mediated pro-inflammatory cytokine production by macrophages (92). Another study revealed that lactoferrin can bind to soluble CD14 (sCD14), which normally complexes with LPS to induce production of IL-8, resulting in the inhibition of the cytokine production by epithelial cells (93). As lactoferrin is known to bind DNA, internalization of the peptide into monocytic cells can inhibit the NF-kB transcription factor binding to the TNF-α promoter and downregulate LPS-induced cytokine production (92). The modulation of cytokine production by lactoferrin ultimately modifies the balance of Th1 and Th2 responses (94).

Aside from altering the cytokine profile of an immune response, binding of lactoferrin to some cell-surface molecules leads to changes in immune cell activation, recruitment, and function. As previously discussed, lactoferrin binds sCD14, preventing the induction of IL-8. This interaction also prevents the expression of E-selectin and intercellular adhesion molecule 1 (ICAM-1), which disrupts recruitment of leukocytes to the site of infection (95,96). Furthermore, the same study demonstrated that lactoferrin can compete with IL-8 for proteoglycans and their further presentation to leukocytes. Lactoferrin has also been shown to hinder hydrogen peroxide production mediated by LPS binding to L-selectin of PMNs (96). Taken together, lactoferrin can target many key processes of the innate immune response and assert its immunoregulatory effects.

Lactoferrin and nutritional immunity

With two binding pockets to bind iron ions at high affinity, lactoferrin is a prime scavenger for free-flowing iron. Iron is involved in a variety of critical biological processes, including DNA synthesis, formation of heme, enzyme co-factor, electron transport system, ATP synthesis, and nitrate reduction in the nitrogen cycle. In bacteria, iron is an essential micronutrient for biofilm formation, cell development, and cell growth (97,98). As a result, there is an arms-race to acquire the imperative nutrients between the human host and the pathogen. To mitigate bacterial invasion, the human immune system produces molecules that bind free-flowing transitional metals in a strategy coined “nutritional immunity” (25). Among these iron-chelating proteins include lactoferrin and transferrin (99). Lactoferrin, however, can retain iron at a much lower pH compared to transferrin, allowing for a more potent iron-sequestration activity (100). The importance of nutritional immunity is highlighted by the mechanism in which bacteria have evolved to combat this selective pressure. While only about 10^{-9}–10^{-18} M iron is available within the host, most microbes need around 10^{-8} M iron for normal cellular functions (101). Members of the *Streptococcus* genus have gained iron acquisition mechanisms including siderophores and import channels to overcome the host pressure (102).

Antimicrobial activity of lactoferrin

Lactoferrin has been implicated in defense against a wide range of invasive organisms by a variety of mechanisms. Many studies have shown that lactoferrin is able to aid in the control of bacteria, viruses, fungi, and parasites (100). Among bacterial pathogens, lactoferrin has been shown to exhibit potent antimicrobial activity against *Streptococcus*, *Shigella, Salmonella*, *Staphylococcus*, and *Enterobacter* genera (103). Supplementation with lactoferrin (and other oral enzymes and proteins) in toothpaste reduced oral bacterial growth and viability and biofilm formation, a result that was attributed to perturbations in the integrity of bacterial membranes, specifically in *S. mutans* (104). Both gram-negative and gram-positive bacteria have molecules that are targeted by lactoferrin, lipopolysaccharides and lipoteichoic and teichoic acids, respectively, resulting in the depolymerization of the cell membrane (105). This ultimately leads to the disruption and permeabilization of the bacterial membrane, resulting in cell death. Furthermore, the N-terminus of lactoferrin has a serine protease-like activity, which impairs the expression of cell-surface virulence factors (106). In one study with *E. coli*, lactoferrin disrupted the proteins of the type III secretion system, which is employed by many gram-negative pathogens to export effector proteins into the host cell for successful invasion (107).

While lactoferrin can defend against bacterial pathogens by direct killing, it is also able to protect by inhibiting bacterial resistance structures known as “biofilms”. Biofilms are communities of microorganisms that form highly complex three-dimensional structures that adhere to surfaces and protect against nutrient deprivation and immune assault (108). The biofilm-altering role of the antimicrobial peptide lactoferrin has been studied intensively in *Streptococcus* species, due to the fact that lactoferrin is abundant in mucosal surfaces such as those found in mammalian milk and the oral cavity, which are natural reservoirs for *Streptococcus* (109,110). Allison and colleagues reported that lactoferrin significantly inhibits *S. mutans* biofilm at all dilutions, including those within the physiological range found in milk (3 mg/mL), a result that was not observed with other milk components such as casein and lactose (109). Other *in vitro* studies using lactoferrin alone, or in combination with lactoperoxidase and/or lysozyme, determined that these antimicrobial molecules inhibit oral

streptococcal species, such as *S. mutans,* biofilm formation by preventing bacterial adhesion (111–118). One plausible explanation for the mechanism by which lactoferrin inhibits Streptococcal biofilm formation is by iron sequestration. Indeed, this is supported by results reported by Berlutti, *et al.*, who demonstrated that bovine apo-lactoferrin enhances *S. mutans* aggregation, but holo-lactoferrin (iron-loaded) represses bacterial aggregation. Additionally, the authors discovered that bovine lactoferrin had the inclination to inhibit adhesion of *S. mutans* to abiotic surfaces independent of its capacity to bind iron, demonstrating its iron-independent biofilm-repressing activity (119). Furthermore, a recent study by Angulo-Zamudio, *et al.* demonstrated that lactoferrin has the capacity to disaggregate pneumococcal biofilms and inhibit the acquisition of antibiotic resistance via DNAse activity (120). Together, these results underscore the importance of lactoferrin in modulating streptococcal aggregation and biofilm formation/maintenance.

In addition to being bactericidal and disrupting biofilms, lactoferrin can engage the innate immune system to aid in bacterial clearance. One study demonstrated that lactoferrin was able to substitute for antibodies to activate the classical pathway of complement and induce opsonization of unencapsulated *S. agalactiae* efficiently (121). Complement is a family of proteins of the innate immune system that activate in a cascade with the result of pore formation by the membrane attack complex on bacterial membranes or opsonization by phagocytes (122). In contrast, other studies have shown that lactoferrin inhibits the complement pathway through preventing the formation of the C3 convertase (123). As lactoferrin has been shown to have immunomodulatory effects, it is possible that lactoferrin inhibits some but not all the complement pathways. One study suggests that lactoferrin impedes the classical pathways but activate the alternative pathways during *Staphylococcus aureus* infection (124). More studies will need to be conducted to firmly establish the interaction between lactoferrin and the complement system.

Utility as a biomarker

With lactoferrin being an peptide of the immune system, it has been associated in many infectious diseases. As a result, changes in expression levels may allow for lactoferrin to be used as a sensitive biomarker for the diagnosis of many human diseases. In the studies of oral health, increased levels of lactoferrin in the saliva is positively correlated with chronic periodontitis (125). In a different study, salivary lactoferrin levels were elevated in periodontal disease in patients who are either HIV positive or negative, suggesting that lactoferrin is a suitable biomarker for periodontal disease for individuals who are immunocompromised (126). A recent clinical study has established that lactoferrin can be used as an effective biological recognition element to selectively detect *S. sanguinus*, one of the most prevalent bacterial species in the onset of periodontal diseases (127). In the realm of maternal and neonatal health, lactoferrin was shown to be elevated in the amniotic fluid of patients with chorioamnionitis (128). Additionally, elevated plasma lactoferrin levels are indicative of newborn preterm infants with sepsis (129). Taken together, lactoferrin may serve as a powerful biomarker for a variety of human diseases.

Potential use as an antimicrobial strategy

Lactoferrin, along with its peptide derivatives (lactoferricin, lactoferrampin, and LF1-11), have been studied as a strategy to defend against a range of infectious diseases (130). *In vivo* studies using lactoferrin alone or in combination with lactoperoxidase and/or lysozyme have revealed that lactoferrin has the capacity to inhibit colonization of many oral streptococcal species, such as *S. mutans,* in dental caries/lesions by repressing biofilm formation and preventing bacterial adhesion for up to one-month post-treatment (115). Lactoferrin supplementation in toothpaste reduced oral *S. mutans* burden, an important intervention that could be utilized to defend against caries or periodontal disease progression associated with *Streptococcus* (104).

Currently, lactoferrin and its peptide derivatives have shown clinical efficacy against urinary tract infections and restoration of the normal gut flora in inflammatory bowel disease patients (131). Other

studies in murine models have shown reduced infection of methicillin-resistant *S. aureus* strain 2141 (MRSA)(132), *S. aureus* 25923 (133), *Klebsiella pneumoniae* ATCC 43816 (132), and various multidrug-resistant *Acinetobacter baumannii* (134) after an intravenous injection of Lf1-11. There has also been success using lactoferrin in combination with antibiotics to target antibiotic resistance bacteria responsibly for mammary gland infections (135). In addition, lactoferrin showed enhanced antimicrobial activity in combination with current treatment against antibiotic resistant *Helicobacter pylori* infections (136). Finally, lactoferrin has shown promise as a candidate to combat or prevent bacterial vaginosis (137,138), neonatal sepsis (139), urinary track information by uropathogenic *E. coli* (140), and necrotizing enterocolitis (141). Transferrin, another iron chelating protein with overlapping functions as lactoferrin, when used in combination meropenem and ciprofloxacin, improved the efficacy of the antibiotics against *A. baumannii* and *K. pneumoniae* (142,143). Furthermore, combination therapy with transferrin reduced the frequency of antibiotic resistance in the same studies. With transferrin and lactoferrin possessing overlapping physiological functions, it is likely that the same effects will be observed when these antibiotics are used in combination with lactoferrin. As lactoferrin has shown to protect against other bacterial infections such as GBS (23), there is merit in studying the use of lactoferrin and its derivatives as a chemotherapeutic strategy against bacterial infections.

Conclusions

Lactoferrin is produced by epithelial cells and innate immune cells such as granulocytes and participates in nutritional immunity by binding two molecules of iron (73,75) **(Figure 2)**. Lactoferrin has potent antimicrobial effects against a wide range of microbial infections, including those caused by *Streptococcus* spp (23). However, the antimicrobial peptide also possesses immunoregulatory properties, making it a very versatile protein with many functions. Lactoferrin interacts with cell surface membranes via proteoglycan receptors as well as pathogen-associated molecular patterns such as LPS to inhibit TLR4 and soluble CD14 receptors to ultimately suppress proinflammatory signaling and cytokine production (92,93). Lactoferrin can bind to host DNA and repress NFκB- dependent induction

of TNF-α, thereby exerting anti-inflammatory activities (92). Lactoferrin can also inhibit hydrogen peroxide production by innate immune cells (93,94). Streptococcal cells induce NET formation in which these immune cells excrete DNA decorated with antimicrobial molecules such as lactoferrin (23). Lactoferrin, subsequently inhibits bacterial growth and biofilm formation. Lactoferrin also binds to the surface of streptococcal cells to promote complement-binding and opsonization (121). Furthermore, many studies have revealed lactoferrin could be a promising candidate as a biomarker for diagnosis or as a potential treatment to combat microbial infections. As *Streptococcus* associated disease is a rising global health burden, the investigation of the intersection between lactoferrin immune defense and *Streptococcus* biology will provide crucial scientific knowledge to address the rising concern.

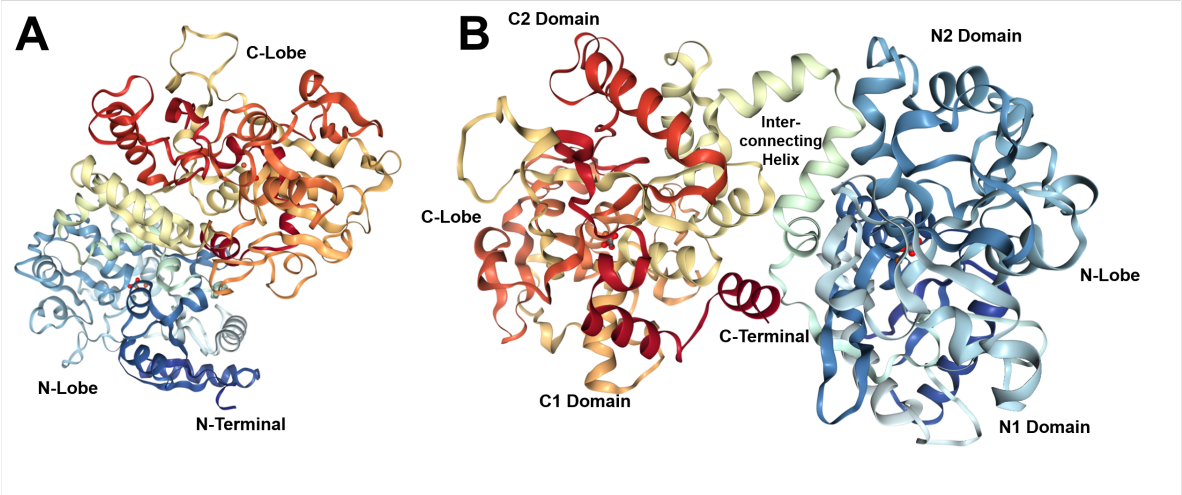

Figure 1. Structural model of human lactoferrin. Lactoferrin is a glycoprotein consisting of a single polypeptide chain containing 692 amino acids, which is folded into two symmetrical lobes: the N-lobe (in blue) and the C-lobe (in orange and yellow) with a hinge region (in green) connecting to two regions. Each lobe is able to bind one Fe^{3+} iron together with one CO_3^{2-} iron (red spheres) within the cleft of the lobe that contains a nonheme iron-binding site. Each lobe has two domains: the C1 and C2 domain within the C-lobe, and the N1 and N2 domain within the N-lobe. Structure was generated using PyMOL software by S.D. Townsend.

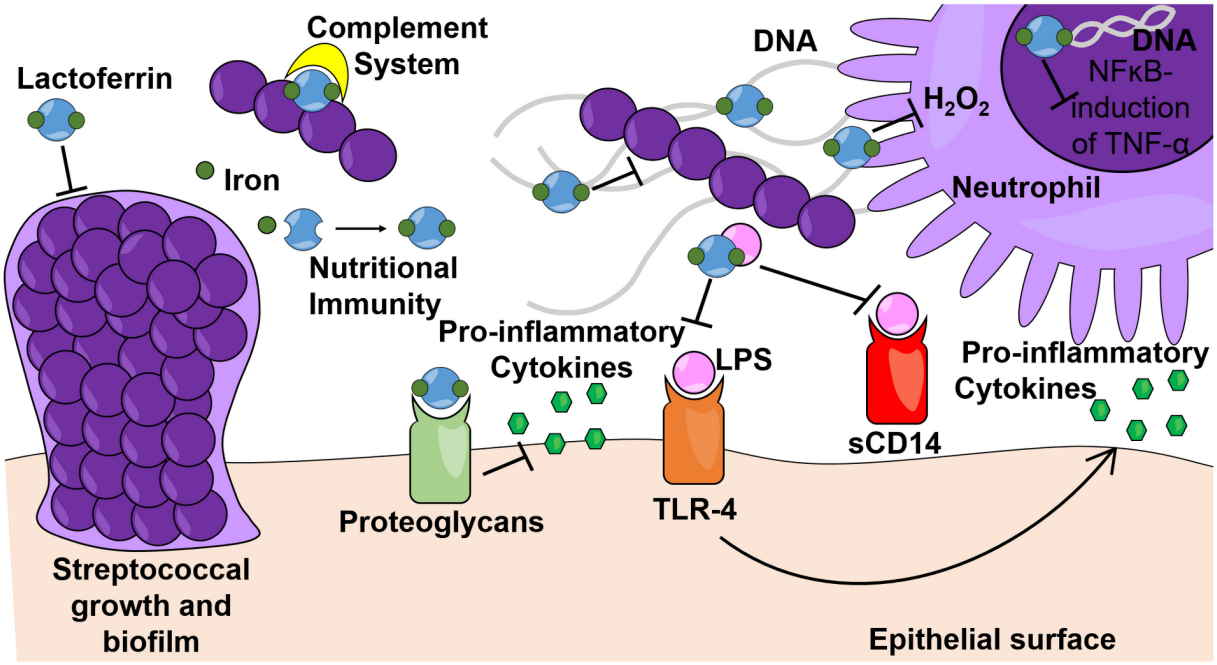

Figure 2. Conceptual model of the role of lactoferrin in host-pathogen interactions during *Streptococcus spp*. infections. Lactoferrin (also referred to as lactotransferrin) is produced by epithelial cells and innate immune cells such as granulocytes and participates in nutritional immunity by binding two molecules of iron. Lactoferrin interacts with cell surface membranes via proteoglycan receptors as well as pathogen-associated molecular patterns such as LPS to inhibit TLR4 and soluble CD14 receptors to ultimately repress pro-inflammatory signaling and cytokine production. Lactoferrin can bind to host DNA and repress NFκB-dependent induction of TNF-α. Lactoferrin can also inhibit hydrogen peroxide production by innate immune cells. Streptococcal cells induce neutrophil extracellular trap formation. Lactoferrin decorates the neutrophil extracellular trap, which is comprised of DNA and inhibits bacterial growth and biofilm formation. Lactoferrin also binds to the surface of streptococcal cells to promote complement-binding and opsonization. This model was produced by J.A. Gaddy and J. Lu.

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CHAPTER II

Antibacterial and antibiofilm activity of the human breast milk glycoprotein lactoferrin against Group B *Streptococcus*

Introduction

Group B *Streptococcus* (GBS), or *Streptococcus agalactiae*, is an encapsulated Gram-positive bacterium and one of the leading infection-related causes of adverse pregnancy and neonatal outcomes. Some consequences of GBS infection include chorioamnionitis, preterm prelabor rupture of membranes (PPROM), preterm birth (PTB), stillbirths, early-onset and late-onset sepsis, and neonatal meningitis (1,144). For most healthy people, GBS is part of the commensal microflora of the digestive tract. Although seemingly innocuous in this environment, GBS can also colonizes the vaginal tract which is the strongest risk factor for invasive infection of the newborn (145). This cross colonization likely occurs through ascending infection or ingestion/inhalation of infectious fluids through vertical transmission during childbirth (3,42). A longitudinal study of GBS colonization rates pregnant women in South Africa revealed that up to 50% of the cohort was transiently colonized at some point during the pregnancy (43). Currently, no commercial vaccine is available for GBS, although this avenue is being pursued (146). Treatment includes introduction of intrapartum antibiotic prophylaxis for mothers who screen positive for GBS prior to delivery. While treatment is available, the lack of improvement of late-onset neonatal sepsis (147), hypersensitivities to first-line antibiotics (148), and the rise of antibiotic resistant strains of GBS (149) call for novel treatments.

There is a wealth of evidence supporting the benefits of breastfeeding to improve infant health, including combating pathogenic bacterial infections. Laboratory and clinical studies have supported that human breast milk can prevent urinary tract infections (150,151), bacterial-related diarrhea (152), and other diseases associated with bacterial infection (153). More studies have revealed that components of human breast milk, such as maternal immunoglobulins (154), transforming growth factor beta (TGF-β) (155), and milk oligosaccharides (156) aid in improving infant health. Components of human breast milk also influence the infant microbiome to protect against invading pathogens (157),

and many of these components can directly interact with pathogenic bacteria. In fact, studies have shown that human milk oligosaccharides can suppress GBS growth and biofilm formation (158,159) and sensitize them to a wide assortment of antibiotics (160,161).

One antimicrobial glycoprotein secreted in high concentrations in human breast milk is lactoferrin, which can comprise up to 20% of human breast milk (162–164). However, the concentration of this antimicrobial peptide in milk varies with gestational age at delivery and time after childbirth (163). In general, there are higher concentrations of lactoferrin in colostrum, which decreases as the infant matures (163). On average, the estimated lactoferrin concentrations in the term colostrum are 7–9 g/L (163,164) and decrease to 1–3 g/L in mature milk. Lactoferrin levels in human breast milk from preterm infants are significantly higher than those from term deliveries (164,165). Concentration of lactoferrin in breast milk of mothers of low-birth-weight newborns is high and remains high for two months after delivery. However, neonatal male gender was associated with lower lactoferrin levels (139). Interestingly, low-birth weight, prematurity, and male gender have all been implicated in postnatal risk for intestinal inflammation such as necrotizing enterocolitis (166).

Lactoferrin possesses antimicrobial activity against a wide range of bacterial, viral, and fungal pathogens (20). Lactoferrin is a glycoprotein with two iron binding sites that participates in the host defense strategy known as “nutritional immunity”, the biological phenomenon of starving bacteria of essential metals (20,21). Due to its intrinsic ability to bind iron, lactoferrin can exist in an apo isoform (lacking iron within its binding sites) or a holo isoform (where iron is present in the binding sites). Iron is crucial for bacterial survival within the host niche by operating as a cofactor for enzymes driving bacterial DNA replication, transcription, and central metabolism (167). In addition to iron-scavenging activity, lactoferrin interacts directly to bacterial cell walls causing destabilization in an iron-independent mechanism (24). Thus, due to its potent broad antimicrobial activity, different groups have studied lactoferrin for therapeutic use. In a systematic review of available literature, enteral lactoferrin supplementation decreases late-onset sepsis, and in combination with probiotics, may decrease necrotizing enterocolitis in preterm infants (141). Lactoferrin also shows promise as a therapeutic

candidate against streptococcal infections (168), including a study demonstrating lactoferrin decreases biofilm formation in *S. mutans* (109). Biofilms are multicellular structures that are critical for many bacterial pathogens, including GBS, to circumnavigate host defenses and persist in the hostile environment (11,169). Here I test the hypothesis that human milk lactoferrin decreases bacterial growth, survival, and biofilm formation in GBS, and that this important host glycoprotein influences bacterial-host interactions in the context of human pregnancy.

In this study, we purified lactoferrin from donor human breast milk and I demonstrated that human milk lactoferrin decreases bacterial growth, survival, and biofilm formation by GBS *in vitro*. Furthermore, this work demonstrates that human milk lactoferrin has the capacity to inhibit GBS adherence to human gestational membrane tissues *ex vivo*. Finally, the antimicrobial properties are enhanced when the glycoprotein is capable of binding iron (in the apo-isoform).

Methods and Material

Bacterial strains and culture conditions

S. agalactiae strains GB00112 (GB112), a clinical isolate from the human reproductive tract, a sequence type 12, capsular serotype III strain isolated from a rectovaginal swab of a post-partum patient (170), GB00002 (GB2) a sequence type 23, capsular serotype Ia strain from a rectovaginal swab of a pregnant patient, GB00012 (GB12) a sequence type 23, capsular V serotype strain from a rectovaginal swab of a pregnant patient, and GB00291 (GB291) a sequence type 12, capsular II serotype strain from a rectovaginal swab of a pregnant patient generously provided by Dr. Shannon Manning from their strain collection, were cultured on tryptic soy agar plates supplemented with 5% sheep blood (blood agar plates) at 37°C in ambient air overnight. Bacteria were sub-cultured from blood agar plates into liquid medium (Todd Hewitt Broth; THB) and incubated in aerobic conditions (ambient air, shaking at 200 rpm) at 37 °C overnight. The following day, bacterial density was measured spectrophotometrically to determine the optical density at 600 nm (OD_{600}) and these bacterial cultures were used for growth, viability, biofilm, and co-culture assays.

Purification of lactoferrin from human breast milk

Expressed human breast milk was gathered with informed consent from 17 healthy donors between 3 days and 3 months post-partum and stored between - 80 and - 20 °C. De-identified human milk samples were provided by Dr. J. Hendrik Weitkamp from the Vanderbilt Department of Pediatrics, under a collection protocol approved by the Vanderbilt University Institutional Review Board (IRB #100897). Six liters of milk from 4 donors were pooled for the purification of lactoferrin. Milk samples were thawed and centrifuged at 8000 *g* for 45 min to separate milk fats from the soluble fraction. Following centrifugation, the resultant top lipid layer was removed. Subsequently, proteins were precipitated by the addition of ammonium sulfate to the soluble fraction and incubation at 4°C overnight. Precipitated proteins were fractionated by ion-exchange chromatography. Cation-exchange resin (CM Sephadex C-50, GE Healthcare) suspension was packed in a column (300×18mm). After sample loading, the column was washed with equilibration buffer until the absorbance at 280 nm was less than 0.05. The bound protein was then displaced from the resin by a stepwise elution protocol. For elution, 10 mM sodium phosphate buffer containing 0.4 M NaCl, 0.6 M NaCl and 0.8 M NaCl were used as elution buffer A, B, and C, respectively. First, elution buffer A was passed through the column. 5 mL fractions were collected and the OD280 value of each fraction was measured by a UV/Vis spectrophotometer. The elution was continued until the fractions showed a minimum OD of 0.03. Further elution of the bound protein was carried out with elution buffer B and C. The Identity of the fractions were determined by high resolution mass spectrometry analysis. Fractions containing greater than 99% lactoferrin were combined and used in the assays.

Confirmation of molecular weight of lactoferrin

The purified lactoferrin was analyzed by high-resolution data-dependent LC-MS/MS. Briefly, the peptide was autosampled on a 200 mm × 0.1 mm (Jupiter 3 µm, 300 A), self-packed analytical column coupled directly to a Q-exactive plus mass spectrometer (ThermoFisher) using a nanoelectrospray source and resolved using a 70 min aqueous to organic gradient. Both the intact masses (MS) and

fragmentation patterns (MS/MS) of the peptide were collected in a data-dependent manner using dynamic exclusion to maximize the depth of coverage. The tandem mass spectra were extracted by ProRata. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Sequest (Thermo Fisher Scientific, San Jose, CA, USA; version 27, rev. 12). Sequest was set up to search the uniprot-human-reference-canonical_20121108_rev database (unknown version, 136958 entries) assuming the digestion enzyme trypsin. Sequest was searched with a fragment ion mass tolerance of 0.00 Da and a parent ion tolerance of 2.5 Da. Oxidation of methionine and carbamidomethyl of cysteine were specified in Sequest as variable modifications.

Criteria for protein identification

Scaffold (version Scaffold_4.11.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 12.0% probability to achieve an FDR less than 5% by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 96.0% probability to achieve an FDR less than 5.0% and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Preparation of holo- or apo-lactoferrin

Iron-bound (holo-) or unbound (apo-) lactoferrin was prepared as previously described (23,171). Briefly, 10 mg/mL stock of purified lactoferrin was dialyzed against either 0.1M sodium citrate-bicarbonate buffer pH 8.2 alone to generate apo-lactoferrin, or buffer containing 70 mM ferric chloride to generate holo-lactoferrin. Both apo- and holo-lactoferrin were dialyzed against 1 × phosphate-buffered saline (PBS) containing Chelex Resin (Sigma Aldrich) to remove any unbound iron content.

Evaluation of bacterial growth and viability

To determine bacterial growth or viability as previously described (23,172), OD_{600} measurements were recorded. GBS cultures were grown to stationary phase and diluted at 1:10 in metal-limited THB medium (50 % THB with 50 % calprotectin buffer (100 mM NaCl, 3 mM CaCl2, 20 mM Tris pH 7.5 (171))). 100 µL of 1:10 diluted cultures were added to each well in a 96-well plate. The appropriate concentration of purified lactoferrin was added into its corresponding well. The plates were left to grow at 37°C in ambient air, and the OD_{600} was measured every hour for the first 10 h. The following day, bacterial density was determined by measuring OD_{600}. For the viability assay, each well was serially diluted at 1:10 up to 10^{-8} with PBS or liquid medium. Each dilution series was plated on blood agar plates and left to grow at 37°C in ambient air overnight. The following day, colonies were counted to determine colony-forming unites (CFU).

Quantification of bacterial biofilms

To evaluate bacterial biofilms, a crystal violet assay was used as previously described (173). Briefly, overnight GBS cultures were diluted 1:10 in THB medium in 96-well plates. To analyze the effect of lactoferrin on biofilm formation, lactoferrin was applied in increasing concentrations at the time of inoculation. Biofilms were allowed to form at 37°C in ambient air overnight. OD_{600} was determined using a spectrophotometer and supernatant was removed and replaced with 0.1% crystal violet stain for 30 min. Wells were washed with deionized water three times and dried. The retained Crystal Violet was resolubilized with a solution of 80 % ethanol and 20 % acetone. Plates were incubated for at least 30 min, and optical density was determined at 560 nm (OD_{560}). To quantify changes in biofilm independent of changes in cellular growth, biofilm quantification was normalized to cell density (OD_{600}) by using a ratio of OD_{560}/OD_{600}.

Gestational membrane-GBS co-culture assays

Following informed consent, gestational membrane tissue samples were procured from term, non-laboring caesarean section-delivery live births at Vanderbilt University Medical Center with

approval from the Vanderbilt University Medical Center Institutional Review Board (VUMC IRB #181998). 12 mm gestational membranes biopsy punches were isolated and cultured in RPMI 1640 medium (ThermoFisher) with 10 % charcoal stripped fetal bovine serum (ThermoFisher) and 1 % antibiotic/antimycotic solution (ThermoFisher) overnight at 37°C in room air supplemented with 5% carbon dioxide. The membranes were infected with 10^6 CFU/mL of GBS in RMPI 1640 medium without antibiotics in the absence of lactoferrin treatment or supplemented with either apo- or holo-lactoferrin at a concentration of 250 µg/mL. Co-cultured tissues were incubated at 37 °C in air supplemented with 5 % carbon dioxide overnight.

Human gestational membrane immunohistochemistry (IHC) staining

Twenty-four hours post infection, membranes were transferred to histological cassettes and fixed in 4% formalin (buffered). Tissues were cut into 5 µm sections, and multiple sections were placed on each slide for analysis. For immunohistochemistry, slides were de-paraffinized, and heat-induced antigen retrieval was performed on the Bond Max automated IHC stainer (Leica Biosystems, Buffalo Grove, IL) using their Epitope Retrieval 2 solution for 5 to 20 min. Slides were incubated with a rabbit polyclonal anti-GBS antibody (ab78846; Abcam) for 1 h. The Bond Polymer Refine detection system (Leica Biosystems) with a peroxide block, post primary, polymer reagent, 3,3'-diaminobenzidine (DAB) chromogen, and hematoxylin counterstain was used for visualization. Slides were dehydrated and cleared, and coverslips were added before light microscopy analysis was performed.

High-resolution electron microscopy analyses

Samples were fixed in 2.0 % paraformaldehyde and 2.5 % glutaraldehyde in 0.05 M sodium cacodylate buffer for at least 12 hours prior to sequential dehydration with increasing concentrations of ethanol. Samples were subsequently dried at the critical point, using a CO_2 drier (Tousimis, Rockville, MD), mounted onto an aluminum stub, and sputter coated with 80/20 gold-palladium. A thin strip of colloidal silver was painted at the sample edge to dissipate sample charging. Samples were imaged with a FEI Quanta 250 field-emission gun scanning electron microscope.

Inductively coupled plasma mass spectrometry analyses

To enumerate the iron molecules associated with lactoferrin isoforms 100 µg of each sample were digested in 0.9 mL 50 % nitric acid overnight at 37°C in sealed Teflon sample containers. The following day, samples were diluted in 9 mL of deionized water in metal-free tubes. Elemental quantification was performed using a Thermo- Element 2 HR-inductively coupled plasma mass spectrometry (ICP- MS) instrument.

Statistical analyses

Statistical analyses of biofilm formation and bacterial growth were performed using a one-way ANOVA with either Tukey's or Dunnett's *post hoc* correction for multiple comparisons. All reported *P* values are adjusted to account for multiple comparisons. Analysis of bacterial viability was performed using log transformation of CFU data and either Mann-Whitney U or one-way ANOVA with either Tukey's or Dunnett's *post hoc* correction for multiple comparisons analyses. *P* values of < 0.05 were considered significant. All data analyzed in this work were derived from at least three biological replicates. Statistical analyses were performed using GraphPad Prism 8 software.

Results

Purification of the glycoprotein, lactoferrin, from human breast milk

Previous reports have indicated that lactoferrin is a dominant glycoprotein in human breast milk with antimicrobial and immunomodulatory functions (162–165). To study the activity of human milk-derived lactoferrin against GBS, lactoferrin was isolated from donor milk and purified via ion-exchange chromatography **(Figure 3)**. High-resolution mass fingerprinting technique (MALDI-TOF MS/MS fragmentation) was employed in conjunction with trypsin digestion and *m/z* peaks revealed a fragmentation pattern and molecular mass that was consistent with that of human lactoferrin **(Figure 3B)**.

Analysis of iron associated with human lactoferrin samples

Purified lactoferrin from human breast milk, as well as preparations of the apo- and holo-isoforms of human breast milk lactoferrin were subjected to ICP-MS analysis to enumerate the iron ions associated with 100 µg of sample **(Figure 4)**. Freshly purified fractions of lactoferrin from expressed human breast milk (human Lf) exhibited a mean value of 11.52 ppb of iron ions, while apo-lactoferrin (human apo-Lf) preparations exhibited a mean value of 56.28 ppb of Fe ions, results that were statistically indistinguishable by one-way ANOVA analyses. Conversely, holo-lactoferrin preparations (human holo-Lf) exhibited a mean value of 243.33 Fe ions per 100 µg of sample, a significant increase compared to both the freshly purified fraction and the apo preparation ($P < 0.0001$, one-way ANOVA). Together, these results indicate that lactoferrin is likely in the apo isoform within human breast milk.

Lactoferrin inhibits GBS growth

Previous studies have shown that commercially available recombinant lactoferrin possesses antimicrobial activity against GBS (23). To verify the ability of our purified lactoferrin from human milk to inhibit bacterial growth, GBS growth was measured every hour for the first 10 hours and at 24 hours across increasing concentration of apo- and holo-lactoferrin. Spectrophotometric analysis of bacterial growth revealed that lactoferrin at concentrations at or above 500 µg/mL significance dampened bacterial growth between 3 to 10 hours post-inoculation, compared to the respective timepoint grown without lactoferrin treatment (**Figure 5A**; $P < 0.05$, Student's *t*-test). However, this phenotype was not observed when treated with holo-Lf **(Figure 5B)**. Expansion of these analyses against three additional clinical isolates of GBS, strains GB2 (capsular serotype 1a, sequence type 23), GB12 (capsular serotype V, sequence type 23), and GB291 (capsular serotype II, sequence type 12) indicate that apo-human lactoferrin has broad antimicrobial activity against diverse capsular serotypes and sequence types of GBS clinical isolates **(Figure 6)**.

To determine the effect of lactoferrin on cell viability, serial dilutions of the culture at 24 hours post-inoculation were plated to determine differences in bacterial viability across increasing

concentrations of lactoferrin. Both apo- and holo-Lf were able to inhibit bacterial viability at concentrations at or above 250 μg/mL, when compared to cultures grown without lactoferrin (**Figure 7**; $P < 0.05$, Student's *t*-test). In summary, apo-Lf purified from human breast milk was able to inhibit bacterial growth, while both isoforms were able to inhibit bacterial viability.

Lactoferrin inhibits GBS biofilm in vitro

To determine if lactoferrin possesses anti-biofilm activity, GBS was treated with a concentration of lactoferrin (250 μg/mL) that was subinhibitory to bacterial growth and biofilm formation was quantified using a crystal violet assay. At this concentration, biofilm formation, as measured by optical density at 560 nm and normalized by optical density at 600 nm, was significantly reduced by 50% when cultured in the presence of apo-lactoferrin (**Figure 8**; one-tailed Student's *t*-test, $P = 0.0201$). Culture in the presence of holo-lactoferrin resulted in a 42% decrease in biofilm formation, though this was statistically indistinguishable from the negative controls grown in medium alone (one-tailed Student's *t* test, $P = 0.0918$). Expansion of these analyses against additional clinical isolates of GBS indicate that apo-human lactoferrin has anti-biofilm activity against GB2 (which exhibited a 32% decrease in biofilm formation com- pared to medium alone, $P < 0.0001$, Student's *t* test) and GB291 (which exhibited a 29% decrease in biofilm formation com- pared to medium alone, $P < 0.01$, Student's *t* test), but not GB12 which was a low biofilm former **(Figure 9)**. High-resolution scanning electron microscopy (SEM) revealed results congruent with the quantitative analyses. GBS grown in medium alone formed robust biofilms on the abiotic substrate (glass cover- slips), and culture in the presence of 250μg/mL of apo-lactoferrin resulted in diminished GBS biofilm tertiary architectural structure. GBS grown in the presence of 250 μg/mL of holo-lactoferrin resulted in diminished GBS biofilm tertiary architectural structure. However, this phenotype was observed to a lesser extent than results obtained from cultures treated with apo-lactoferrin.

Lactoferrin inhibits GBS adherence to gestational membrane tissues ex vivo

GBS commonly adheres to and infects gestational membranes and leads to adverse pregnancy outcomes such as chorioamnionitis, premature rupture of membranes, and preterm birth (1,144). To investigate if purified human milk lactoferrin can disrupt GBS adherence to and biofilm formation on human tissues, gestational membrane biopsies were acquired and co-cultured with GBS in medium alone or medium supplemented with either apo or holo isoforms of lactoferrin at a concentration of 250 µg/mL. Co-cultures were incubated for 24hours to promote adherence of bacteria and interactions within the gestational membranes. The tissues were then fixed and processed for IHC staining for GBS. IHC revealed that GBS can adhere to, and form, microcolonies on the maternal decidual side of the membrane **(Figure 10)**. Treatment with apo-lactoferrin inhibited this phenotype while GBS was able to form biofilm-like micro-colonies when treated with the same concentration of holo-lactoferrin.

Discussion

In this study, we were successful in purifying human lactoferrin from donor breastmilk using ion-exchange chromatography. This technique has been used to purify lactoferrin from a host of mammalian breast milk (174) and we were able to assess the quality of the purification by chromatography and mass spectrometry **(Figure 3)**. Mass spectra obtained from these studies are comparable to previously published spectra collected from validated lactoferrin samples (175). At the time of this study, lactoferrin utility has been studied for a wild range of human diseases, many of which are particularly detrimental the neonates and infants. However, many of these studies focus on the utility of bovine lactoferrin.

One of these studies assessed the use of bovine lactoferrin in preventing neonatal sepsis and necrotizing enterocolitis found that lactoferrin supplementation was able to reduce late-onset neonatal sepsis but not necrotizing enterocolitis (141). Interestingly, GBS infection is strongly correlated to preterm birth and late-onset neonatal sepsis. In another randomized placebo-controlled clinical trial with bovine lactoferrin supplementation, neonates with low-birth rate exhibited lower rates of late-onset

sepsis (176). Meanwhile, another group sought to determine the impact of bovine lactoferrin in preventing diarrhea in children discovered that supplementation did not reduce the incidences of occurrence but showed that across time, the prevalence and severity of diarrhea was decreased (177). While there is some encouraging evidence of the benefits of bovine lactoferrin, another study did not observe any differences in the incidence of infant sepsis with birth weight of under 2000 grams between lactoferrin-treated and untreated groups (178). Similarly, another cohort observed no improvement in death or major comorbidities but consistent with other findings, they observed a reduction in late-onset sepsis with bovine lactoferrin supplementation (179).

While some benefits have been observed with bovine lactoferrin supplementation, even more success has been shown when lactoferrin is used in combination with other biological agents. For instance, children supplemented with lactoferrin and lysozyme, another component of breast milk observed to improve gut health, experienced reduced development of acute malnutrition and decreased hospitalization (180). In another study with lactoferrin in combination with *Lactobacillus* GG, infants with intervention exhibited a decreased incidence of severe necrotizing enterocolitis (181). Both trials with bovine lactoferrin showed improvement in diseases; however, it remains to be seen how human lactoferrin may affect outcomes.

With pertinence to lactoferrin and GBS disease, one study found that vaginal supplementation in pregnant people with bacterial vaginosis reduced the rates of preterm births (137). Interestingly, lactoferrin has been identified as a critical component of cervicomucosal defense against a variety of lower genital tract infections including *Chlamydia trachomatis, Neisseria gonorrhoeae*, and *Trichomonas vaginalis* (138). One potential mechanism through which lactoferrin protects against reproductive tract infections is by repressing *Gardnerella*, *Prevotella*, and *Lachnospira*, within the host microbiome and increasing the occurrence of *Lactobacillus* species to prevent dysbiosis (182). It is interesting to note that *Lactobacillus* species have been recognized as one of the few bacteria who lack a strict nutrient requirement for iron. As such, it is plausible that the antimicrobial activity of lactoferrin associated with its role in nutritional immunity would be largely ineffective against these important

commensals (183). As GBS can infect the fetus through vaginal ascending infections, there is merit in studying the use of human lactoferrin in the prevention of GBS-mediated preterm births and adverse pregnancy outcomes.

The antimicrobial activity of lactoferrin has been demonstrated against many bacterial species, as well as viruses, and fungi (168). Our previous work demonstrated that recombinant lactoferrin could inhibit GBS growth via iron chelation. Our current work shows that freshly isolated lactoferrin from human breast milk inhibits the growth of four clinical isolates of GBS across diverse capsular serotypes (Ia, II, III, and V). In addition to GBS, lactoferrin also displays antimicrobial effects on other neonatal pathogens including and *Escherichia coli* and *Staphylococcus epidermidis*, and the probiotic *Bifidobacterium breve* (184). Lactoferrin, and several peptides derived from the protein, can kill bacterial cells (in particular *S. aureus* and *E. coli*) by permeabilizing the bacterial membrane (185). Other similar studies of lactoferrin and its peptide-derivative demonstrated reduction of bacterial growth of *Vibrio cholerae* and other *Vibrio* species, attributing to its ability to disrupt bacterial membranes (186,187). Lactoferrin has also been shown to inhibit the growth of the intracellular pathogen *Salmonella* Typhimurium. Lactoferrin and other milk glycoproteins can inhibit the bacterium from adhering to mammalian cells, preventing them from entering the cell and replicating intracellularly. This reveals yet another mechanism by with lactoferrin can disrupt bacterial growth (188).

Lactoferrin can bind two iron molecules and starve microbes of this essential metal (168). In the study presented this chapter, we were able to show that human milk lactoferrin's antimicrobial activity against GBS is in largely due to the chelation of nutrient iron, a result that was attenuated when the molecule was pre-saturated with iron to form the holo isoform. This is consistent with previous studies with recombinant lactoferrin inhibiting GBS growth through an iron-dependent mechanism (23). However, our studies also reveal that holo-lactoferrin can inhibit bacterial viability, suggesting that both iron-dependent and independent mechanisms play a role in its antimicrobial activity against GBS.

The majority of the anti-biofilm properties of lactoferrin has been studied with *Pseudomonas aeruginosa*, a pathogen known for its biofilm forming capabilities and its detrimental effects to human

health. A group studying the effects of lactoferrin and peptide-derivatives showed that the protein is able to downregulate virulence factors (elastase and pyocyanin) and reduce biofilm formation in a type strain of *P. aeruginosa* (189). Meanwhile, a different group demonstrated that lactoferrin inhibits *P. aeruginosa* biofilm formation, especially under anaerobic conditions (190). Beyond laboratory strains, lactoferrin also exhibit anti-biofilm effects on clinical strains of *P. aeruginosa* and pretreatment with ferric iron eliminated some of the phenotype, suggesting that iron chelation is involved (191). This iron-dependent mechanism is consistent with our finding in which apo-lactoferrin decreased GBS biofilm formation at a subinhibitory concentration whereas holo-lactoferrin did decrease biofilm formation, though it did not achieve statistical significance.

There have also been other studies assessing the anti-biofilm effects of lactoferrin against a range of streptococcal species. Several studies have demonstrated that coating titanium surfaces with lactoferrin inhibited bacterial adhesion and subsequent biofilm formation in *S. sanguinis* (114) and *S. gordonii* (192) Coating of lactoferrin to other surfaces also resulted in limited adhesion of *S. mutans* (193). Finally, lactoferrin has been shown to inhibit biofilm formation in *S. mutans* (109). To our knowledge, our study is amongst the first to investigate the antimicrobial and anti-biofilm properties of human milk lactoferrin against GBS.

One recent study with *S. pneumoniae* shown that lactoferrin can inhibit biofilm formation (120). Another study of *S. pneumoniae* revealed that iron increases biofilm formation while iron chelation led to disruption of biofilm formation (194). The group further characterized the role of the mononuclear iron protein *S*-ribosylhomocysteine lyase (LuxS) and quorum sensing and biofilm formation. As we saw similar iron-chelation dependent disruption of biofilm formation through the activity of apo-lactoferrin, it is plausible that GBS possesses similar iron sensing pathways that govern biofilm formation.

We observed that lactoferrin possess antimicrobial and anti-biofilm properties against GBS in laboratory *in vitro* conditions on abiotic surfaces. To further our studies, we sought to investigate if the anti-biofilm and antimicrobial properties of lactoferrin could be determined on a relevant biotic surface. To achieve this, we acquired human fetal membranes and performed co-cultures with GBS *ex vivo* to

determine if lactoferrin could alter GBS adherence to relevant human tissues. Congruent with our *in vitro* studies, we observed that treatment with lactoferrin disrupted GBS biofilm formation, and that this phenotype was most pronounced with the apo-isoform of lactoferrin. Consistent with our *in vitro* results, holo-lactoferrin exhibited an intermediate biofilm suppression phenotype. Previous studies using primary human gestational tissue *ex vivo* models to investigate bacterial biofilm formation have shown that bacterial biofilm formation on gestational tissues can be correlated with enhanced production of pro-inflammatory cytokines such as IL-6, IL-1β, IL-2, IFN-γ, TNF-α, and GM-CSF (195). Thus, it is possible that lactoferrin could inhibit bacterial adherence and biofilm formation and could also influence immunological outcomes associated with inflammation during GBS infection.

Several studies have explored the protective properties of lactoferrin against a range of infectious bacterial diseases *in vivo.* Other groups have shown using lactoferrin knockout mice that the protein is important in the defense against *Aggregatibacter actinomycetemcomitans* (196) and *S. mutans* related bacteremia (197). Other studies have demonstrated the benefit of lactoferrin supplementation against a variety of bacterial diseases. Diseases outcomes of candidiasis, enterohaemorrhagic *E. coli* (198), uropathogenic *E. coli* (140), Methicillin-resistant *S. aureus* (199), and *Mycobacterium tuberculosis* (200) improved upon introduction of lactoferrin. Lactoferrin was also used in combination with antibiotics to improve outcomes of *Helicobacter pylori* disease (175). Future studies should therefore involve investigating lactoferrin as a therapeutic against GBS infection in relevant animal models of disease.

In summary, this study presented in this chapter has revealed that lactoferrin is able to inhibit GBS growth and biofilm formation by binding to free iron ions in the environment, thus starving the pathogen of this essential metal by the process of “nutritional immunity” **(Figure 11)**. The innovation of this work includes the utility of natural purified lactoferrin from human breast milk (previous work demonstrated the antimicrobial activity of recombinant lactoferrin), as well as the anti-biofilm activity of human milk lactoferrin. Furthermore, this study reveals that lactoferrin inhibits GBS adherence to human gestational membranes by limiting the availability of iron for GBS. The implications of this work

suggest that human milk lactoferrin could be used as a prebiotic therapeutic strategy. However, additional testing on a larger collection of GBS isolates that vary in biofilm production and other phenotypes (such as capsular serotype) (201) is needed to conclude the utility of lactoferrin against a broad range of GBS strains.

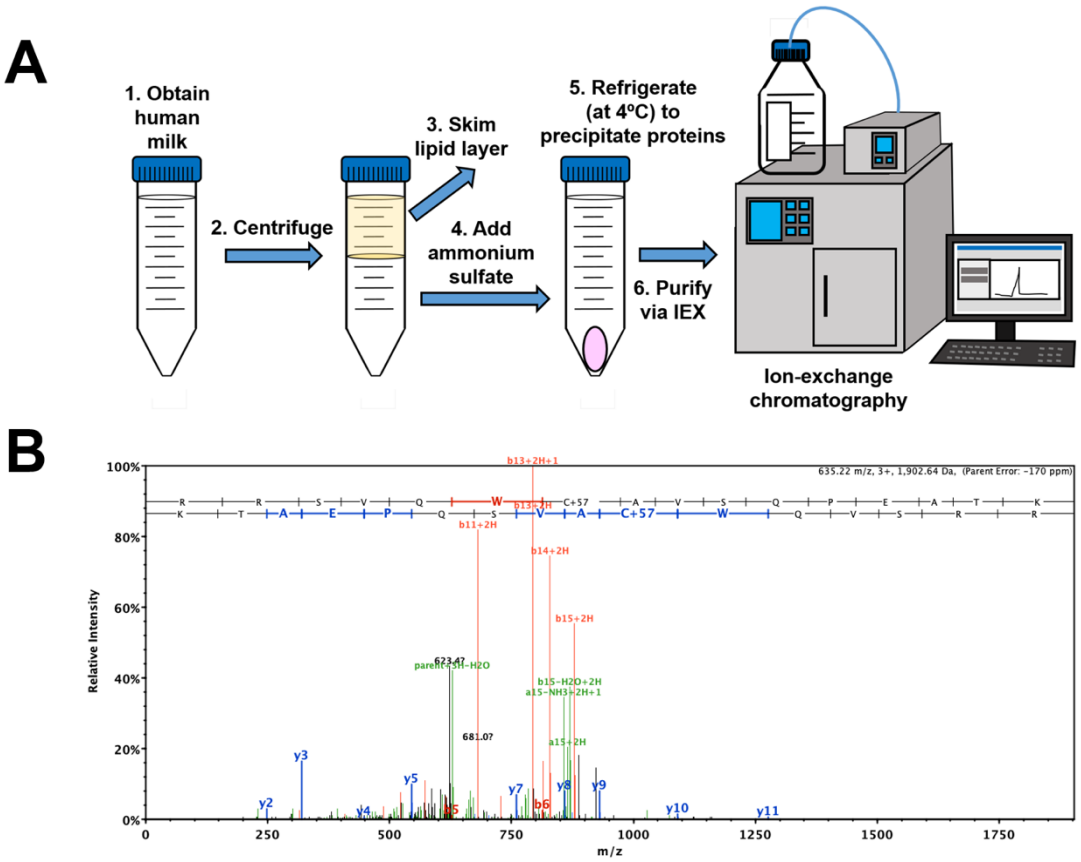

Figure 3. Purification of the innate immune antimicrobial glycoprotein, lactoferrin. A) Conceptual diagram of the protocol for purification of lactoferrin from human breast milk. Milk is centrifuged, lipid layer is removed, ammonium sulfate is added to precipitate milk proteins under refrigeration, and lactoferrin is purified by ion-exchange chromatography (IEX) techniques. B) Mass spectrometry results demonstrating the identity of lactoferrin within the sample. Our result matches previously published mass spectrometry fingerprints of human lactoferrin.

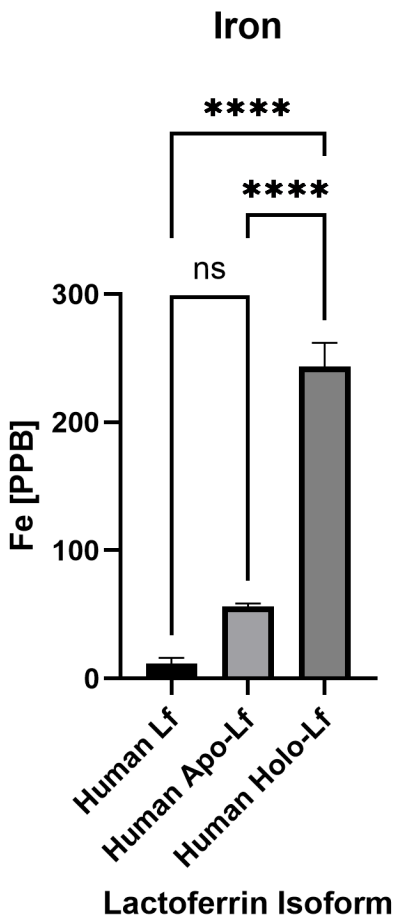

Figure 4. ICP-MS elemental analysis of lactoferrin preparations. Freshly purified fractions of lactoferrin from expressed human breast milk (Human Lf), apo-lactoferrin (Human Apo-Lf), and holo-lactoferrin (Human Holo-Lf) preparations were subjected to ICP-MS analysis to enumerate the iron (Fe) ions associated with 100 µg of sample. Bars indicate mean values of at least three biological replicates +/-SEM. (****P<0.0001, one-way ANOVA, *post hoc* Tukey's test). Results validate the saturation of holo-lactoferrin and indicate that human breast milk lactoferrin is largely in the apo- state.

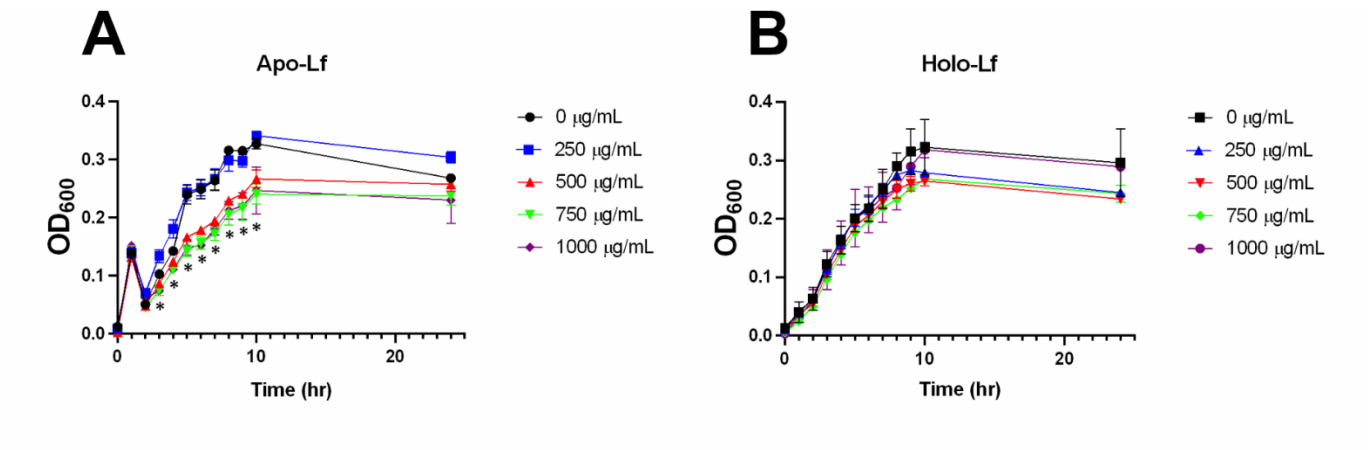

Figure 5. Analysis of bacterial growth in increasing concentrations of lactoferrin. GBS was grown in increasing concentrations (0, 250, 500, 750, 1000 µg/mL) of A) apo-lactoferrin (apo-Lf) or B) holo-lactoferrin (holo-Lf) over a 24-hour period. Bacterial growth was determined by measuring cellular density (optical density at 600 nm or OD_{600}). Points equal a mean +/- SEM, n=3-5. *P<0.05, Student's t test comparison to bacteria grown in medium alone at the same time point. Apo-lactoferrin significantly inhibits GBS growth at concentrations of 500, 750, and 1000 µg/mL at 3, 4, 5, 6, 7, 8, 9, and 10 hours post-inoculation, a result that was ablated by saturation of the glycoprotein with iron.

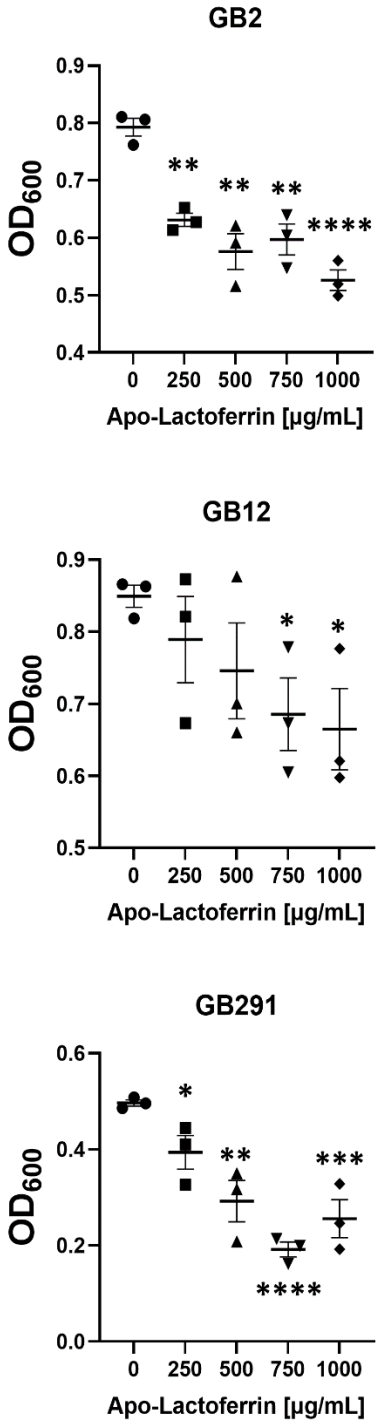

Figure 6. Evaluation of bacterial growth in increasing concentrations of apo-lactoferrin. GBS strains GB2, GB12, and GB291 were grown in increasing concentrations (0, 250, 500, 750, 1000 µg/mL) of A) apo-lactoferrin (apo-Lf) for 24-hours. Bacterial growth was determined by measuring cellular density (optical density at 600 nm or OD_{600}). Bars equal a mean +/- SEM, n=3. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, Student's t test comparison to bacteria grown in medium alone.

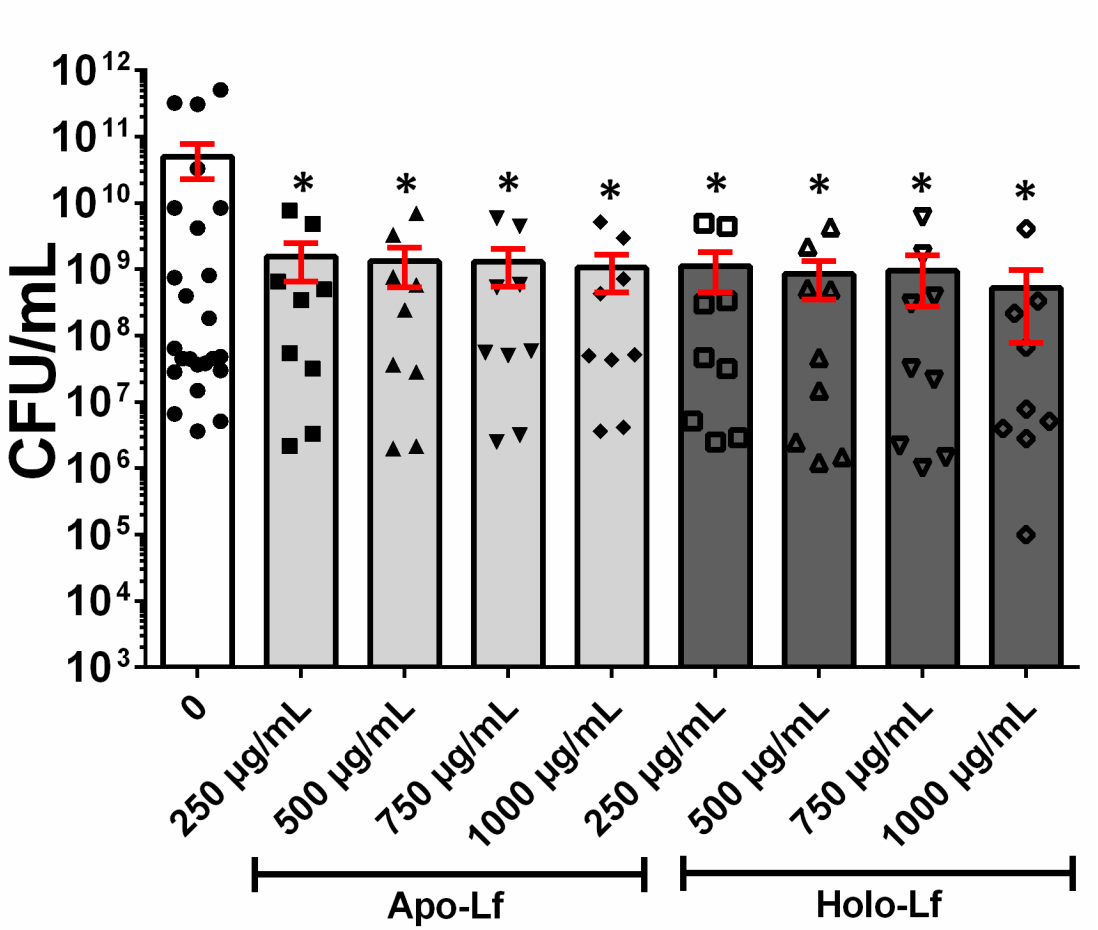

Figure 7. Quantitative culture analyses of bacterial viability. Bacterial viability was determined by serial dilution and culture on solid agar plates, and colony forming units per mL (CFU/mL) were determined. GBS bacterial cells were grown in medium alone (0, black circles), or medium supplemented with either holo-lactoferrin (Holo-Lf) or apo- lactoferrin (Apo-Lf) at concentrations of 250 µg/mL (black squares), 500 µg/mL (black triangle), 750 µg/mL (black inverted triangle), 1000 µg/mL (black diamond) for 24 hours prior to viability analyses. *P<0.05, Student's T test, compared to medium alone control. Apo-lactoferrin and holo-lactoferrin significantly inhibit bacterial viability at concentrations at or above 250 µg/mL.

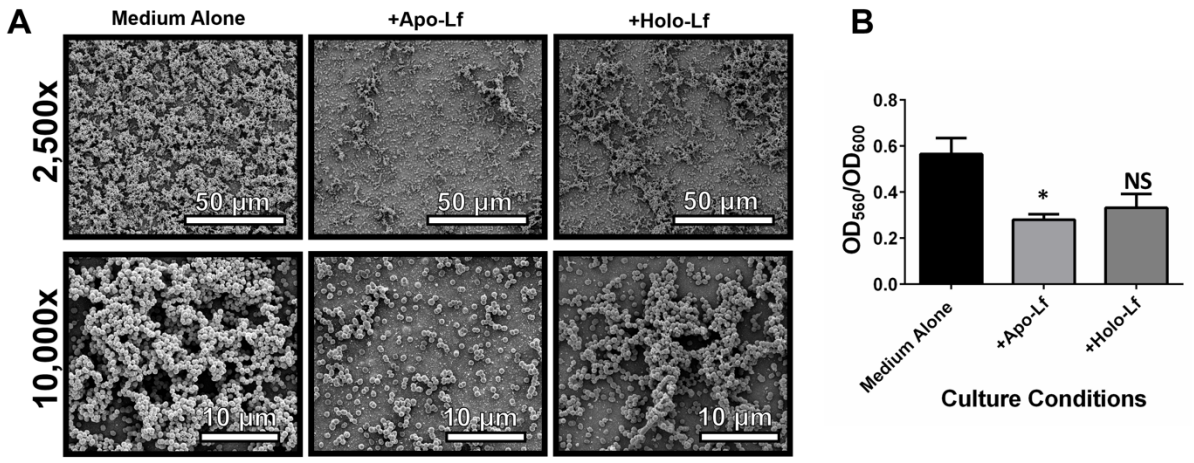

Figure 8. Analysis of *in vitro* bacterial biofilm formation on an abiotic surface in the presence or absence of lactoferrin. GBS was grown in medium alone or medium supplemented with 250 µg/mL of either apo-lactoferrin (apo-Lf) or holo-lactoferrin (holo-Lf). Bacterial biofilm was analyzed by A) high-resolution scanning electron microscopy (SEM) at low magnification (2,500x, magnification bar indicates 50 µm) and high magnification (10,000x, magnification bar indicates 10 µm) and by B) quantitative analysis of crystal violet staining at an optical density of 560 nm (OD_{560}) normalized to bacterial cell density (OD_{600}). Bars indicate mean values of at least three biological replicates +/-SEM. *P<0.05, One-way ANOVA, post hoc Tukey's test comparison to bacteria grown in medium alone. Apo-lactoferrin significantly inhibits GBS biofilm formation, while holo-lactoferrin has an intermediate phenotype that is statistically indistinguishable from the negative control.

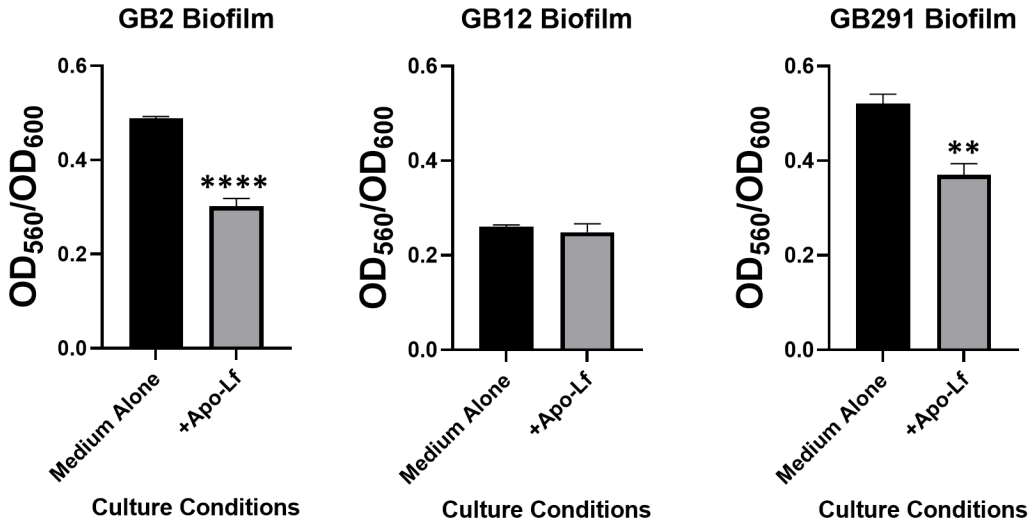

Figure 9. Analysis of bacterial biofilm formation in the presence or absence of apo-lactoferrin. GBS was grown in medium alone or medium supplemented with 250 µg/mL of apo-lactoferrin (apo-Lf). Bacterial biofilm was analyzed by quantitative analysis of crystal violet staining at an optical density of 560 nm (OD_{560}) normalized to bacterial cell density (OD_{600}). Bars indicate mean values of at least three biological replicates +/-SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, Student's t test comparison to bacteria grown in medium alone.

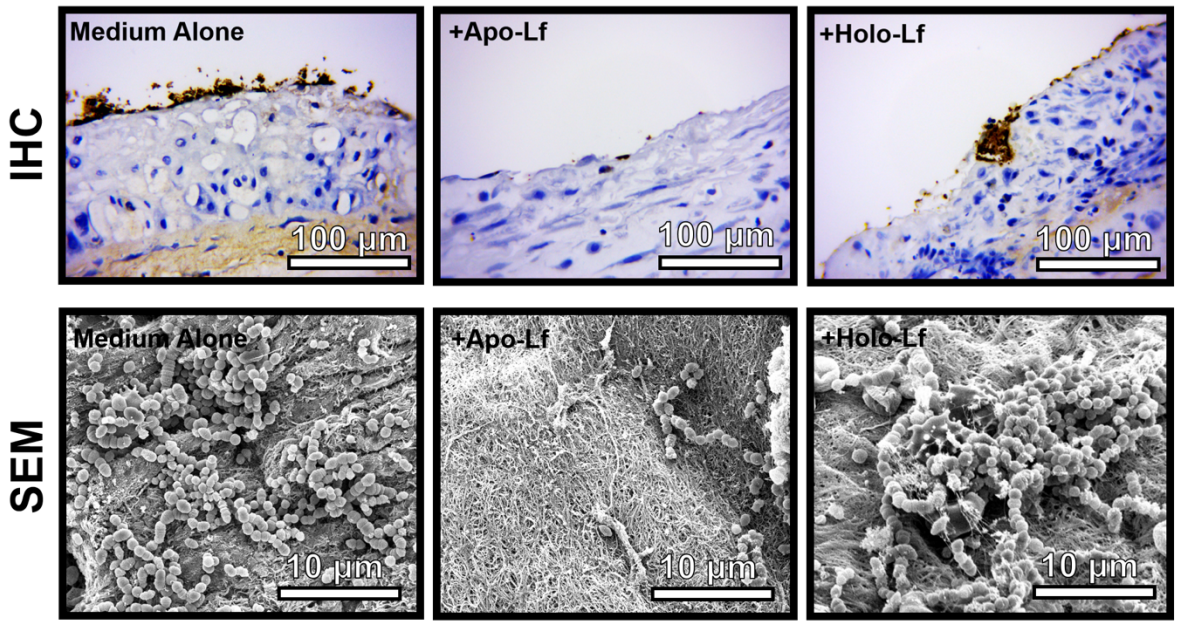

Figure 10. Analysis of *ex vivo* bacterial biofilm formation on a biotic surface in the presence or absence of lactoferrin. GBS was grown in co-culture with gestational membrane tissue in medium alone or medium supplemented with 250 µg/mL of either apo-lactoferrin (apo-Lf) or holo-lactoferrin (holo-Lf). Bacterial biofilm was analyzed by either immunohistochemical (IHC) staining with an antibody specifically to GBS (brown staining) and high-resolution scanning electron microscopy (SEM) at high magnification (10,000x, magnification bar indicates 10 µm). Apo-lactoferrin significantly inhibits GBS adherence to gestational membrane tissue, while holo-lactoferrin has an intermediate phenotype that is comparable to the control sample cultured in medium alone.

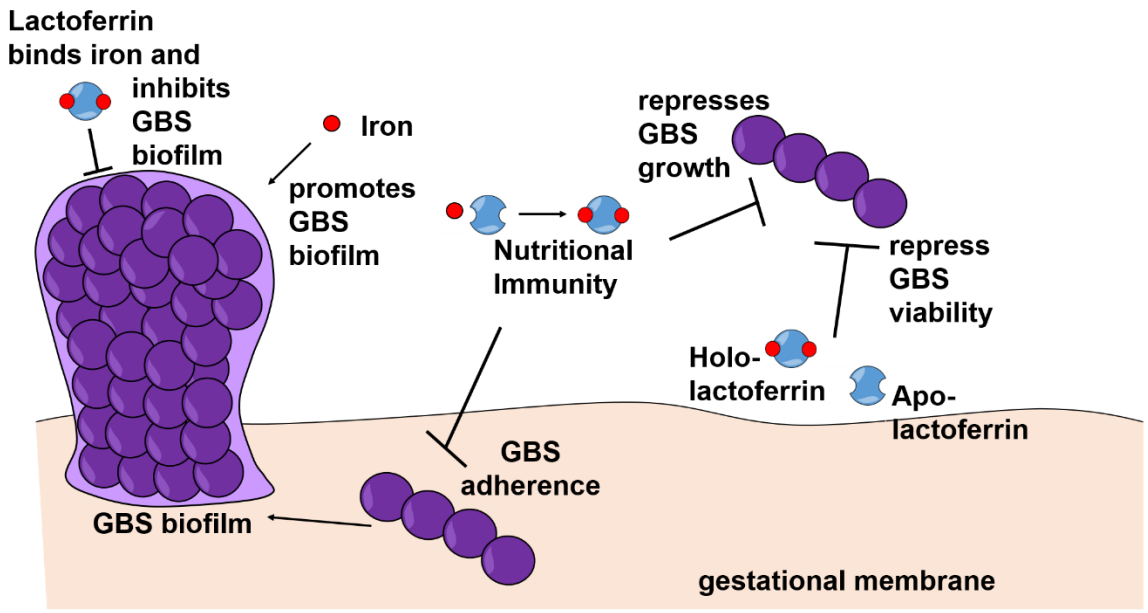

Figure 11. Model of lactoferrin iron binding-dependent inhibition of biofilm and growth. Free iron ions promote GBS biofilm formation. Apo-lactoferrin binds iron and inhibits biofilm formation and GBS growth by iron sequestration in a process termed “nutritional immunity”. Apo-lactoferrin binds iron and inhibits GBS adherence to gestational membranes. Both holo- and apo-Lf have the capacity to repress GBS viability.

A version of the following section (*Chapter III, Analysis of Susceptibility to the Antimicrobial and Anti-Biofilm Activity of Human Milk Lactoferrin in Clinical Strains of Streptococcus agalactiae With Diverse Capsular and Sequence Types*) was originally published in *Frontiers in Cellular and Infection Microbiology.* (Sep 2021).

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CHAPTER III

Analysis of Susceptibility to the Antimicrobial and Anti-Biofilm Activity of Human Milk Lactoferrin in Clinical Strains of *Streptococcus agalactiae* With Diverse Capsular and Sequence Types

Introduction

Streptococcus agalactiae, more commonly known as Group B *Streptococcus* (GBS), is amongst the leading infection-related causes of adverse pregnancy and neonatal outcomes (202). Adverse maternal complications include chorioamnionitis, preterm prelabor rupture of membranes (PPROM), preterm birth, stillbirth, and maternal sepsis (1,144). For the newborn, GBS infections can result in early- and late- onset neonatal sepsis, endocarditis, and meningitis. Early onset disease (EOD) occurs in neonates up to a week after birth (3). Neonates with EOD usually present with pneumonia and sepsis. In contrast, late onset disease (LOD) defines infection between 1-week and 3 months after birth and most commonly manifests as meningitis and sepsis. Furthermore, newborns who survive LOD frequently suffer from neurodevelopmental deficiencies (42).

GBS is a gram-positive encapsulated bacterium, and a commensal member of the human microflora in the gastrointestinal tract. While GBS asymptomatically colonizes 20-30% of adults, the bacterium may traverse from the lower gastrointestinal tract to the vagina and infect the neonate through ascending infection or ingestion/inhalation of infectious fluids during childbirth (3). Indeed, the primary risk factor for EOD is rectovaginal colonization of pregnant women with GBS during delivery (6). The ability of GBS to colonize and persist in the maternal urogenital tract to cause disease is related to the bacterium's ability to form biofilms (11). Colonization rates differ worldwide, spanning between 6.5-36% (202). Recent reports include colonization rates of 13.2% in a cohort in Ethiopia (203), 16.6% in the Western Cape region of South Africa (9), and 21.6% over a twelve-year span in North Carolina, USA (204).

GBS strains can be divided into 10 distinct serotypes (Ia, Ib, and II to IX) based on a serological reaction directed against the polysaccharide capsule (205). The streptococcal polysaccharide capsule

facilitates evasion of the innate immune response by protecting the bacterial cell from deposition of complement, opsonization, and phagocytosis (14,206,207). A recent study from our laboratory revealed that the GBS capsule aids in biofilm formation and ascending infection of the reproductive tract during pregnancy (208). Moreover, the capsule across all serotypes shares terminal sialic acid (Sia) residues that allow molecular mimicry of human cell surface sialic acids. This allows interaction with Sia-receptors, Siglecs, on innate immune cells serve to dampen inflammatory responses (209). Different capsular serotypes manifest in different range and severity in human disease. For instance, capsular serotype III strains are associated with higher rates of invasive neonatal disease (210) and account for the majority of late-onset meningitis cases in neonates (211). In contrast, serotype Ia and V predominate among invasive isolates in non-pregnant cases (8). However, dominate serotypes fluctuate between regions and across time (202).

Another method by which GBS strains are grouped and characterized is multi-locus sequence typing (MLST), which classifies strains into sequence types (STs) based on allelic variation within seven conserved housekeeping genes (212). Based on phylogenetic analysis, GBS STs can be further grouped into multiple clonal complexes (CCs), with most human isolates belonging to CC1, CC10, CC17, CC19, CC23, and CC26 (213). Similar to capsular type, ST diversity also results in different disease outcomes and severities. For instance, a study with GBS from multiple continents revealed that STs 1 and 19 have been linked to asymptomatic colonization, while ST-17 predominately related to invasive neonatal disease. Meanwhile, ST-23 was associated with both carriage and invasive GBS disease (212). All four STs, however, were found to colonize pregnant women at higher rates in different patient populations (214). ST-17 serotype III, alongside ST-291, belong to CC17, a group of GBS distinguished for its hypervirulence (211). ST-17 strains are strongly linked to both EOD and LOD, as well as meningitis (215–217). Meanwhile, ST-291 belonging to serotype IV is strongly associated with EOD and septicemia (218,219).

To combat GBS, the immune system deploys a repertoire of antimicrobial peptides. These peptides aid in combatting infection through the process of nutritional immunity, or the sequestration of

essential metals to starve bacteria (25). Bacteria require these trace elements as enzymatic cofactors for essential biological processes such as respiration. One important example of a protein expressed in defense against GBS is lactoferrin (23,220). Lactoferrin is a glycoprotein that contains two iron binding sites (20) and was shown to have antimicrobial activity against a wide range of bacterial, viral, and fungal pathogens (168). Indeed, our previous study from the previous chapter demonstrated that human breast milk lactoferrin has antimicrobial and anti-biofilm activity against GBS and inhibits some GBS strains from adhering to human gestational membranes (220). In the study presented this chapter, we advanced our findings by analyzing the antimicrobial and anti-biofilm effects of lactoferrin against a larger panel of clinical GBS strains that vary by capsular serotype, ST, isolation source, and clinical presentation. We observed broad antimicrobial and antibiofilm action by lactoferrin against most GBS strains, though the maternal colonizing strains were more susceptible to inhibitory effects than the neonatal invasive strains.

Materials and Methods

Bacterial strains and culture conditions

This study utilized a diverse set of 25 previously characterized *S. agalactiae* strains recovered from neonates with invasive disease (214) and colonized mothers sampled before and after childbirth (217); all strains were originally isolated by Dr. H. Dele Davies (221,222). GBS strains were cultured on tryptic soy agar plates supplemented with 5% sheep blood (blood agar plates) at 37°C in ambient air overnight. Bacteria were sub-cultured from blood agar plates into liquid medium (Todd Hewitt Broth; THB) and incubated in aerobic conditions (ambient air, shaking at 200 rpm) at 37°C overnight. The following day, bacterial density was measured spectrophotometrically to determine the optical density at 600 nm (OD_{600}). These bacterial cultures were used for growth, viability, biofilm, and co-culture assays.

Purification of lactoferrin from human breast milk

Human lactoferrin was isolated from breast milk as previously described (220). Briefly, expressed human breast milk was gathered from 17 healthy donors between 3 days and 3 months post-partum and stored between -80 and -20˚C. De-identified human milk samples were provided by Dr. J. Hendrik Weitkamp from the Vanderbilt Department of Pediatrics, under a collection protocol approved by the Vanderbilt University Institutional Review Board (IRB #100897). Milk samples were thawed and centrifuged at 8000 g for 45 minutes to separate milk fats from the soluble fraction. Following centrifugation, the resultant top lipid layer was removed. Subsequently, proteins were precipitated from the soluble fraction by the addition of ammonium sulfate to the soluble fraction and incubation at 4˚C overnight. Precipitated proteins were fractionated by ion-exchange chromatography. Cation exchange (CM Sephadex C-50, GE Healthcare) resin suspension was packed in a column (300 x 18 mm). After sample loading, the column was washed with equilibration buffer until the absorbance at 280nm was less than 0.05. The bound protein was then displaced from the resin by a stepwise elution protocol. For elution, 10 mM sodium phosphate buffer containing 0.4 M NaCl, 0.6 M NaCl and 0.8 M NaCl were used as elution buffer A, B, and C, respectively. First, elution buffer A was passed through the column. 5 mL fractions were collected and the OD_{280} value of each fraction was measured by a UV-vis spectrophotometer. The elution was continued until the fractions showed a minimum OD of 0.03. Further elution of the bound protein was carried out with elution buffer B and C. The Identity of the fractions were determined by high resolution mass spectrometry analysis. Fractions containing greater than 99% lactoferrin were combined and used in the assays. All lactoferrin used in this study was in the apo-form.

Evaluation of bacterial growth

Bacterial growth was determined by a spectrophometric reading as previously described (220). Briefly, optical density measurements at 600 nm (OD_{600}) were recorded to determine bacterial growth. GBS cultures were grown to stationary phase (OD_{600} between 0.2-0.3) and diluted at 1:10 in metal-

limited THB medium (50% THB with 50% calprotectin buffer (100 mM NaCl, 3 mM $CaCl_2$, 20 mM Tris pH 7.5 (171,172))). 100 µL of 1:10 diluted cultures were added to each well in a 96-well plate. The appropriate concentration of purified lactoferrin (0, 250, 500, 750, or 1000 µg/mL, concentrations which are physiologically relevant to the host-pathogen *in vivo* environment) was added into each corresponding well. The plates were incubated at 37ºC overnight. The following day, bacterial density was determined by measuring OD_{600}.

Quantification of bacterial biofilms

A crystal violet assay was utilized to evaluate bacterial biofilms as previously described (173,220). Briefly, overnight GBS cultures were diluted 1:10 in THB-CP medium in 96-well plates. To analyze the effect of lactoferrin on biofilm inhibition, lactoferrin was applied in increasing concentrations (0, 250, 500, 750, or 1000 µg/mL) at the time of inoculation. Biofilms were allowed to form at 37ºC in ambient air overnight. OD_{600} was determined using a spectrophotometer and supernatant was removed and replaced with 0.1% crystal violet stain for thirty minutes. Wells were washed with deionized water three times and dried. The retained crystal violet was resolubilized with a solution of 80% ethanol and 20% acetone. Plates were incubated for at least 30 minutes and optical density was determined at 560 nm (OD_{560}). Quantification was determined by using a ratio of OD_{560}/OD_{600}.

Statistical analyses

Statistical analyses of biofilm formation and bacterial growth were performed using Student's t-test or a one-way ANOVA with either Tukey's or Dunnett's *post hoc* correction for multiple comparisons. A Dunnett's test was performed when comparing to mean to a control (media alone). All reported *P* values are adjusted to account for multiple comparisons. *P* values of ≤0.05 were considered significant. All data analyzed in this work were derived from at least three biological replicates, data points reflect mean of technical replicates (1-3 technical replicates per biological replicate). Statistical analyses were performed using GraphPad Prism software (Versions 6 and 9, GraphPad Prism Software Inc., La Jolla, California).

Results

Human breast milk lactoferrin suppresses bacterial growth in many clinical GBS isolates

We previously reported that human breast milk lactoferrin possesses antimicrobial activity against three clinical isolates of GBS (219). To enhance the generalizability of these findings, we increased the number of GBS strains, thereby capturing more isolates across diverse capsular serotypes and STs. We investigated the effects of lactoferrin treatment across increasing concentrations against this panel of clinical isolates. Out of the 25 GBS strains screened, four strains exhibited inhibition of bacterial growth when treated with 250 μg/mL of human lactoferrin (**Table 1**; $P < 0.05$, Student's t-test). Growth of 9 total strains was inhibited when treated with a concentration of lactoferrin of 500 μg/mL ($P < 0.05$, Student's *t*-test). At 750 μg/mL of lactoferrin, 14 strains exhibited a decrease in bacterial growth as compared to media only ($P < 0.05$, Student's *t*-test). Finally, the growth of 14 strains was inhibited when treated with 1,000 μg/mL of lactoferrin ($P < 0.05$, Student's *t*-test). While there was a trending decrease in bacterial growth for 10 strains relative to the media control, the differences were not statistically significant. No significant differences in growth in medium alone were observed across diverse strains of GBS.

Human breast milk lactoferrin exhibits anti-biofilm activity against numerous clinical GBS strains

Because we previously described the iron-dependent anti-biofilm properties of lactoferrin against a limited number of GBS strains (220), we expanded the number of GBS strains to determine if lactoferrin can suppress other strains with diverse genetic backgrounds. Among the 25 strains assayed, 20 strains exhibited a significant decrease in biofilm formation when treated with 250 μg/mL (**Table 1**; $P < 0.05$, Student's t-test). When the concentration of lactoferrin was increased to 500 μg/mL, one additional strain showed susceptibility and a decrease in biofilm production. Of all the strains screened, only four had no reduction in biofilm formation across increasing concentrations of human lactoferrin.

Colonizing GBS strains are more susceptible to lactoferrin than invasive isolates

GBS strains were grouped into two groups - colonizing and invasive – and susceptibility to lactoferrin was compared to GBS grown in medium alone without lactoferrin supplementation. At 250 μg/mL, the 13 colonizing strains were more susceptible to lactoferrin compared to the 12 invasive strains in respect to bacterial growth with a 12.21% vs 1.76% mean reduction, respectively (**Figure 12A**; $P < 0.05$, Student's t-test). Increasing the concentration of lactoferrin to 500 μg/mL resulted in enhanced suppression of bacterial growth in the colonizing versus invasive strains (28.14% vs 17.48% mean reduction, respectively) ($P < 0.01$, Student's t-test). Although the addition of 750 μg/mL of lactoferrin decreased bacterial growth for both types of strains, the invasive strains were more resistant to inhibition than the colonizing strains (20.71% vs 33.17% mean reduction, respectively) ($P < 0.01$, Student's t-test). Lactoferrin at 1,000 μg/mL asserted antimicrobial activity against both types of strains but no differences were observed between the two strain types. With respect to biofilm formation, only the addition of lactoferrin at 250 μg/mL inhibited biofilm formation in the colonizing strains more than invasive ones (33.25% vs 22.01% mean reduction, respectively) (**Figure 12B**; $P < 0.05$, Student's t-test).

Treatment with human lactoferrin at 750 μg/mL reveals differences in susceptibility between GBS sequence types (STs)

Because different GBS STs are associated with maternal colonization and neonatal disease, it is possible that different strains have variable mechanisms to cope with iron starvation. To investigate this possibility, GBS strains were binned by ST and susceptibility to lactoferrin was analyzed between STs. No significant differences in bacterial growth were detected with treatment at 250, 500, and 1,000 μg/mL of lactoferrin between the different STs; however, differences were observed with 750 μg/mL of lactoferrin. Specifically, the ST-1 strains were more resistant to bacterial growth suppression compared to ST-12 strains (**Figure 13A**; $P < 0.05$, One-way ANOVA; *post hoc* Tukey's test). Similar differences in biofilm suppression were observed between STs while treating with 750 μg/mL of lactoferrin. At this

concentration, the ST-17 strains were more resistant to the antibiofilm activity of lactoferrin compared to both ST-19 and ST-23 strains (**Figure 13B**; $P < 0.01$ and $P < 0.05$, respectively, One-way ANOVA; *post hoc* Tukey's test).

Treatment with human lactoferrin at 250 µg/mL reveals differences in susceptibility across capsular types

It is plausible that capsular type may also influence resistance to lactoferrin. To test this, strains were grouped into cohorts based on capsular serotype and susceptibility to lactoferrin across capsule types were analyzed. No significant differences in resistance against the antimicrobial activity of lactoferrin were observed between capsular types across increasing concentrations of lactoferrin (**Figure 14A**; $P > 0.05$, One-way ANOVA; *post hoc* Tukey's test). However, treatment with lactoferrin at 250 µg/mL showed that capsular type III strains exhibited resistance to its antibiofilm activity compared to capsular type 1a isolates (**Figure 14B**; $P < 0.05$, One-way ANOVA; *post hoc* Tukey's test). This phenotype was ablated with the additional stress imposed by increasing concentrations of lactoferrin.

Lactoferrin asserts anti-biofilm effects against both high and low biofilm formers but enhances biofilm formation in low biofilm formers at higher concentrations

Because our group has previously observed a range of biofilm production across GBS strains (201), we sought to determine the geometric mean of biofilm produced by all isolates investigated in this study. GBS strains that form biofilms above the determined geometric mean ($OD_{560}/_{600} = 0.3965$) were designated as "high" biofilm formers, while those below were named "low". Treatment with 250 µg/mL of lactoferrin significantly inhibited biofilm formation in both high and low biofilm formers (**Figure 15**; $P < 0.0001$, Student's t-test; $P < 0.001$, Student's t-test, respectively), whereas 500 µg/mL of lactoferrin only inhibited the high biofilm formers ($P < 0.0001$, Student's t-test). When treated with 750 µg/mL of lactoferrin, the high biofilm formers exhibited a decrease in biofilm formation ($P < 0.05$, Student's t-test), however, the low biofilm formers (mean = 0.3183) showed increased biofilm

production (mean = 0.3501) ($P < 0.05$, Student's t-test). This discrepancy between high and low biofilm formers was further amplified at treatment with 1000 μg/mL of lactoferrin (mean = 0.3934) ($P < 0.001$, Student's t-test).

Discussion

In the study presented this chapter, we expanded upon our work from the previous chapter by increasing the panel of GBS strains to include phenotypically and genetically diverse clinical strains from diverse anatomical sites of isolation and assessing susceptibility to the antimicrobial and anti-biofilm activity of human milk lactoferrin. We revealed that lactoferrin possesses antimicrobial and antibiofilm properties against many diverse GBS strains. In particular, colonizing maternal strains were more susceptible to lactoferrin, compared to invasive neonatal strains.

Other studies have revealed that lactoferrin may contribute to improvement of reproductive tract infections and subsequent disease. For instance, vaginal lactoferrin supplementation in pregnant people with bacterial vaginosis reduced the rate of preterm birth (137). Furthermore, other groups have identified lactoferrin as a critical component of cervicomucosal defense against a variety of lower genital tract infections caused by *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis* (138). One plausible explanation for this phenomenon may be that lactoferrin protects by repressing *Gardnerella*, *Lachnospira,* and *Prevotella*, species within the host microflora, thus reducing competition for *Lactobacillus* species and allowing them to prevent dysbiosis by dominating the vaginal microbiota (182). Adding credence to this possibility is that *Lactobacillus* species have been recognized as one of the few bacterial populations that lack a strict nutrient requirement for iron (183). Thus, it is plausible that the antimicrobial activity of lactoferrin associated with its role in nutritional immunity would be largely ineffective against these important commensal members. Indeed, our previous work has shown various *Lactobacillus* strains and their secreted products modulate GBS interactions with cells of the extraplacental membranes. Specifically, *Lactobacillus* supernatants inhibited GBS growth, biofilm formation and invasion of host cells, though strain-dependent effects were observed. Notably,

supernatant from *L. reuteri* 6475 broadly inhibited growth in 36 distinct GBS strains and inhibited GBS growth to an average of 46.6% of each GBS strain alone (223). Thus, there is merit in studying the use of human lactoferrin alone or in combination with *Lactobacillus spp.* in the prevention of GBS-mediated preterm births and adverse pregnancy outcomes as maternal colonizing GBS strains can infect the fetus by ascending the gravid reproductive tract. Here, we have shown that maternal colonizing GBS strains are greatly susceptible to the antimicrobial and antibiofilm action of lactoferrin.

Our results suggest that certain STs are more susceptible than others at 750 μg/mL of lactoferrin treatment. The MLST scheme uses seven loci that encode enzymes involved in intermediary metabolism to distinguish GBS STs (212). Because iron is an important cofactor for many enzymes involved in bacterial metabolism and physiology (224), lactoferrin can help defend against invading bacteria by starving the prokaryotic cells of nutritional iron needed for optimal enzyme activity. However, some enzymes are promiscuous with their utilization of transition metal co-factors. As a result, it is plausible that we witnessed variable antimicrobial effects of lactoferrin because some of these housekeeping enzymes, or another enzyme up- or downstream of its respective pathways, require iron for full function while others may use other transitional metals under conditions of iron starvation (225). An alternate explanation is that other enzymes in some pathways with similar functions may be able to compensate for the absence of or limited iron-cofactors. Perhaps the number of loci used in the MLST were too limited. It is plausible that the effect of lactoferrin is indirect and does not involve the specific genes loci used in the MLST. Further examination at the whole genome phylogeny may better differentiate lactoferrin effects across close or distantly related genomes regardless of cps type or isolation source.

The capsule of GBS serves an important function in pathogenesis in humans (10). As different capsular types have been correlated to varying disease outcomes and susceptibility to antimicrobial agents (205), we hypothesized that different capsular serotypes might result in variable responses to the antimicrobial activity of lactoferrin. We observed differences in biofilm formation but not bacterial growth between different GBS capsular types under a lower treatment of lactoferrin. Given that the

capsule is an important virulence factor for pathogenesis and evasion of immune assault (226), it was not surprising that no differences were observed in our controlled *in vitro* studies free of immune stressors. However, we did observe differences in GBS biofilm formation. Our group previously described the role of capsule in biofilm formation in GBS (208), further bolstering our findings. It is plausible that iron starvation alters capsule-mediated biofilm formation in certain capsular types of GBS. Further *in vitro* work with host cells or *in vivo* experiments are needed to bridge our gap in knowledge of these relationships between capsular serotype and lactoferrin.

In our study, we further analyzed differences in biofilm inhibition by lactoferrin between strains that formed robust biofilms compared to those that formed low or weak biofilms. Lactoferrin inhibited biofilm formation in high biofilm forming isolates, which is consistent to with our previous study presented last chapter, in a smaller cohort of strains (220). The intersection between iron and biofilm formation has also been studied in other bacterial pathogens. One study of interest by Trappetti and colleagues described the role of the mononuclear iron protein S-ribosylhomocysteine lyase (LuxS) in quorum sensing and biofilm formation in *S. pneumoniae* (194). Consistent with their work, we also observed that iron starvation results in inhibition of biofilm formation. Thus, it is plausible that GBS possesses similar iron-sensing pathways that govern biofilm formation. More work, however, is needed to elucidate and confirm the function of these pathways.

Colonization of the maternal genitourinary tract is the most important risk factor for neonatal GBS disease (145). In a longitudinal study performed by Kwatra and colleagues, up to 50% of the study cohort was transiently colonized by GBS at some point during pregnancy, highlighting the dynamic nature of GBS colonization (43). Currently in the United States, pregnant individuals are screened for the presence of GBS between 35 and 37 weeks of gestation (227). If a mother tests positive for GBS, then intrapartum antibiotic prophylaxis (IAP) is administered during labor and delivery to prevent neonatal EOD (4). Though antibiotic treatment is the only current preventative strategy available, the efficacy of IAP against EOD is around 80% (228). Despite the availability of IAP, however, the rates of LOD have remained unchanged (147). Additional drawbacks associated with the use of IAP (229)

include hypersensitivities to first-line antibiotics (148), alteration of the neonatal microbiota (230), and emergence of antibiotic resistant strains (231). Hence, the discovery of alternative therapies is critical and may help overcome these IAP drawbacks. Breastfeeding has been associated with protection against infection and could be utilized as a risk-mitigation strategy. However, cases of GBS transfer by breast milk have been recorded (232). The protective effects of breast milk are likely derived from the antimicrobial and immunomodulatory molecules that comprise milk, including lactoferrin and human milk oligosaccharides (233). Thus, utilization of these specific molecules could be leveraged to mitigate the risk of GBS transmission. In the study presented this chapter, we found that maternal colonizing GBS strains are most susceptible to lactoferrin, suggesting that the antimicrobial peptide may be a viable candidate to aid in the prevention of GBS disease.

Table 1. Genetic and clinical characteristics of GBS strains in this study

Strain Number	Strain Type	Sequence Type	Capsular Serotype	Isolation Source	Growth MIC	Biofilm MIC
GB0002	Colonizing	ST-23	cpsIa	Vaginal/rectal colonization	250 µg/mL	250 µg/mL
GB0012	Colonizing	ST-1	cpsV	Vaginal/rectal colonization	750 µg/mL	250 µg/mL
GB0037	Invasive	ST-1	cpsV	EOD/sepsis	>1000 µg/mL	250 µg/mL
GB0064	Invasive	ST-17	cpsIII	EOD/sepsis	>1000 µg/mL	500 µg/mL
GB0066	Invasive	ST-19	cpsIII	EOD/sepsis	500 µg/mL	>1000 µg/mL
GB0069	Invasive	ST-17	cpsIII	EOD/sepsis	500 µg/mL	>1000 µg/mL
GB0079	Invasive	ST-19	cpsIII	EOD/sepsis	>1000 µg/mL	250 µg/mL
GB0083	Colonizing	ST-1	cpsVI	Vaginal/rectal colonization	>1000 µg/mL	250 µg/mL
GB0112	Colonizing	ST-12	cpsIII	Vaginal/rectal colonization	500 µg/mL	250 µg/mL
GB0115	Colonizing	ST-17	cpsIII	Vaginal/rectal colonization	250 µg/mL	<1000 µg/mL
GB0241	Colonizing	ST-23	cpsV	Vaginal/rectal colonization	>1000 µg/mL	250 µg/mL
GB0285	Colonizing	ST-12	cpsII	Vaginal/rectal colonization	750 µg/mL	250 µg/mL
GB0291	Colonizing	ST-12	cpsII	Vaginal/rectal colonization	500 µg/mL	250 µg/mL
GB0374	Invasive	ST-12	cpsIb	EOD/sepsis	>1000 µg/mL	250 µg/mL
GB0377	Invasive	ST-19	cpsIII	EOD/sepsis	>1000 µg/mL	250 µg/mL
GB0390	Invasive	ST-23	cpsIa	EOD/sepsis	>1000 µg/mL	250 µg/mL
GB0397	Invasive	ST-23	cpsIII	EOD/sepsis	750 µg/mL	250 µg/mL
GB0411	Invasive	ST-17	cpsIII	EOD/sepsis	750 µg/mL	<1000 µg/mL
GB0418	Invasive	ST-17	cpsIII	EOD/sepsis	750 µg/mL	250 µg/mL
GB0438	Invasive	ST-12	cpsIb	LOD/sepsis	>1000 µg/mL	250 µg/mL
GB0571	Colonizing	ST-19	cpsIII	Vaginal/rectal colonization	250 µg/mL	250 µg/mL
GB0590	Colonizing	ST-19	cpsIII	Vaginal/rectal colonization	>1000 µg/mL	250 µg/mL
GB0653	Colonizing	ST-12	cpsII	Vaginal/rectal colonization	500 µg/mL	250 µg/mL
GB0654	Colonizing	ST-17	cpsIII	Vaginal/rectal colonization	250 µg/mL	250 µg/mL
GB0663	Colonizing	ST-19	cpsIII	Vaginal/rectal colonization	>1000 µg/mL	250 µg/mL

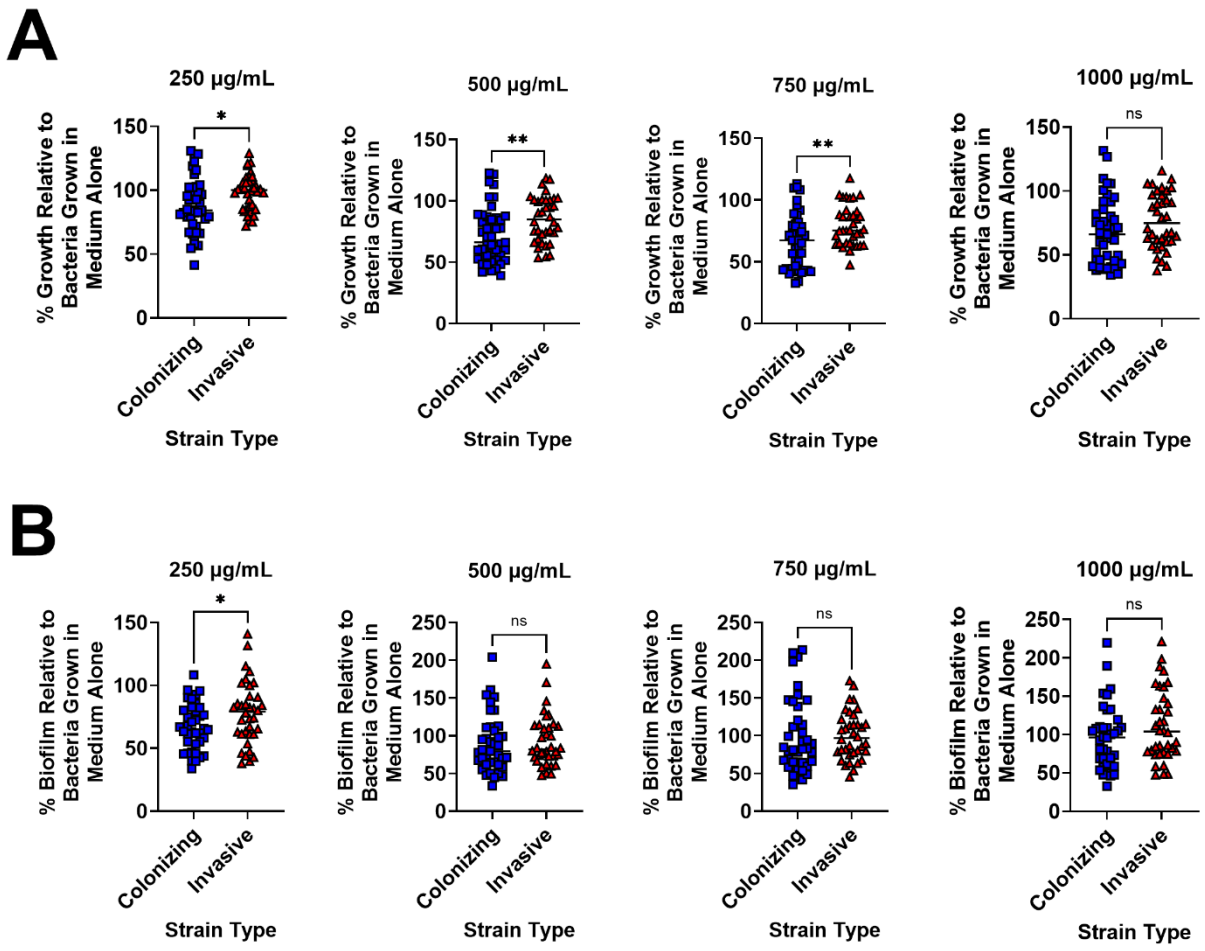

Figure 12. Analysis of susceptibility to lactoferrin-associated growth or biofilm inhibition in invasive vs. clinical isolates of Group B *Streptococcus* (GBS). GBS strains isolated from colonized patients, or patients experiencing invasive disease were grown in medium alone or increasing concentrations of lactoferrin. A) Bacterial growth was measured at 24 hr post-inoculation and percent growth was calculated with reference to growth observed in the medium alone negative control. At 250, 500, and 750 µg/mL, colonizing strains of GBS (blue squares) exhibited greater growth inhibition than invasive strains (red triangles) as determined by Student's t-test with Welch's correction (*P<0.05, and **P<0.01). B) Bacterial biofilm was measured at 24 hr post-inoculation and percent growth was calculated with reference to growth observed in the medium alone negative control. At 250 µg/mL, colonizing strains of GBS (blue squares) exhibited greater biofilm inhibition than invasive strains (red triangles) as determined by Student's t-test with Welch's correction (*P<0.05).

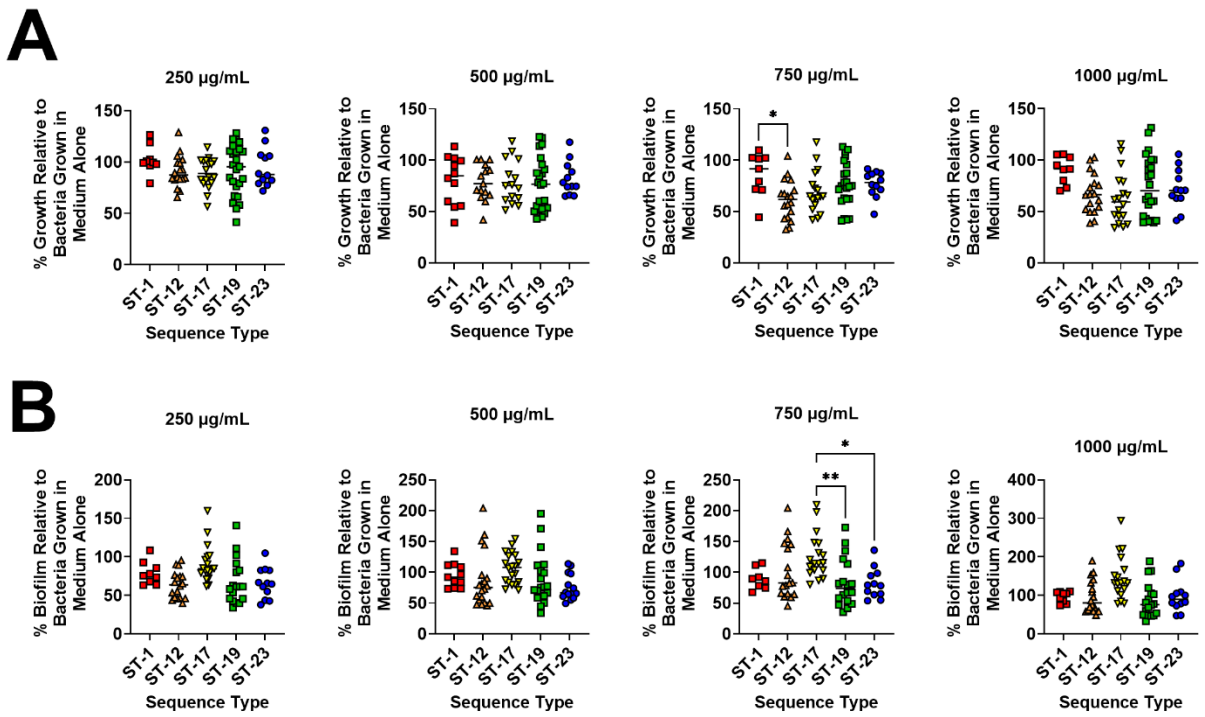

Figure 13. Analysis of susceptibility to lactoferrin-associated growth inhibition in diverse sequence types (STs) of Group B *Streptococcus* (GBS). GBS strains isolated with a variety of sequence type (ST-1, red; ST-12, orange; ST-17, yellow; ST-19, green; ST-23, blue) were grown in medium alone or increasing concentrations of lactoferrin. A) Bacterial growth was measured at 24 hr post-inoculation and percent growth was calculated with reference to growth observed in the medium alone negative control. At 750 µg/mL, ST-12 strains of GBS exhibited greater growth inhibition than ST-1 strains as determined by one-way ANOVA with *post hoc* Tukey's test (*P<0.05). B) Bacterial biofilm was measured at 24 hr post-inoculation and percent biofilm was calculated with reference to biofilm observed in the medium alone negative control. At 750 µg/mL, ST-19 and ST-23 strains of GBS exhibited greater biofilm inhibition than ST-17 strains as determined by one-way ANOVA with *post hoc* Tukey's test (*P<0.05, and **P<0.01).

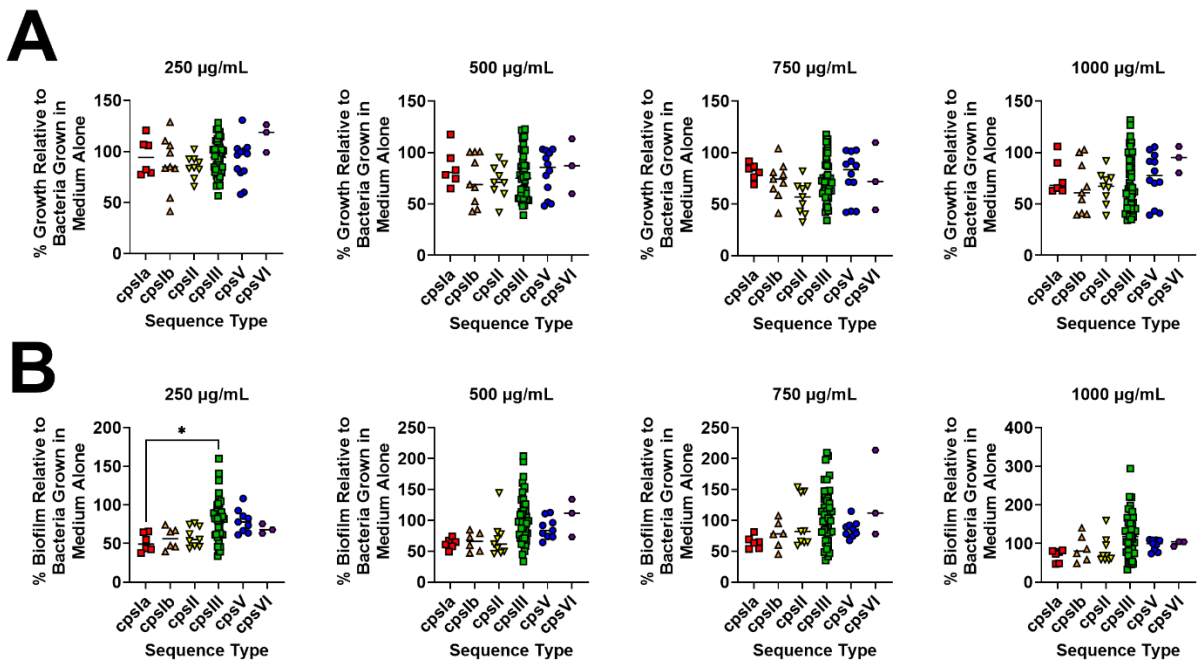

Figure 14. Analysis of susceptibility to lactoferrin-associated growth or biofilm inhibition in diverse capsular serotypes of Group B *Streptococcus* (GBS). GBS strains isolated with a span of capsular serotypes (cpsIa, red; cpsIb, orange; cpsII, yellow; cpsIII, green; cpsV, blue; cpsVI, purple) were grown in medium alone or increasing concentrations of lactoferrin. A) Bacterial growth was measured at 24 hr post-inoculation and percent growth was calculated with reference to growth observed in the medium alone negative controls. No differences in growth were detected across molecular serotype as determined by one-way ANOVA with *post hoc* Tukey's test (*P<0.05). B) Bacterial biofilm formation was measured at 24 hr post-inoculation and percent growth was calculated with reference to growth observed in the medium alone negative controls. At 250 µg/mL, cps1a strains of GBS exhibited greater biofilm inhibition than cpsIII strains as determined by one-way ANOVA with *post hoc* Tukey's test (*P<0.05).

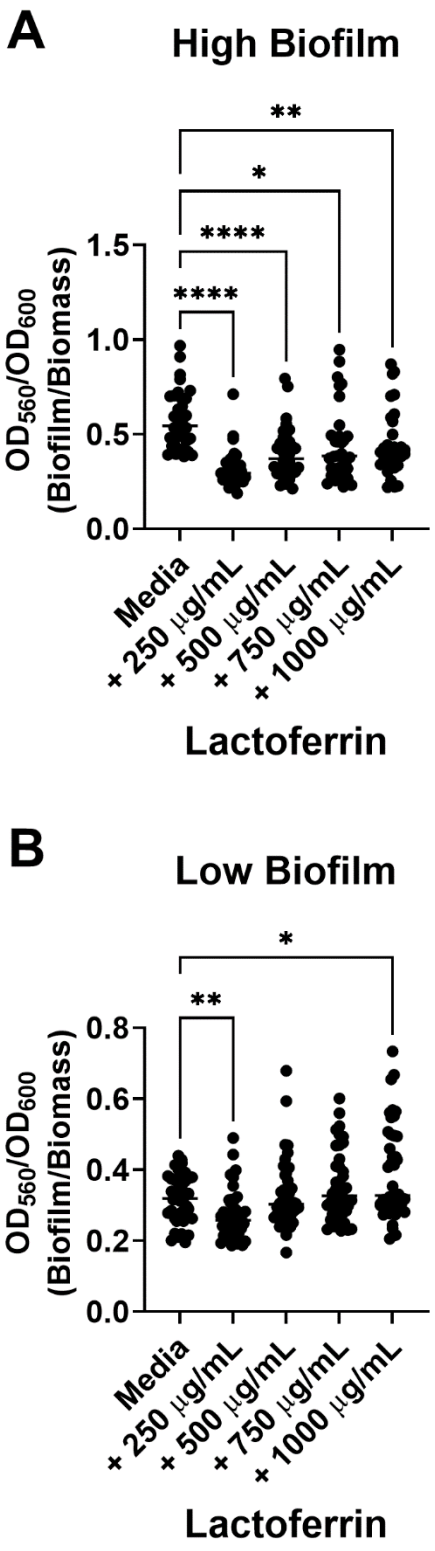

Figure 15. Lactoferrin-dependent inhibition of biofilm based on Group B *Streptococcus* (GBS) strain variation with respect to high or low biofilm production. GBS biofilm values (OD_{560}) were pooled, and the geometric mean was determined. Strains were divided between high (panel A) and low (panel B) biofilm formers. High biofilm formers were more susceptible to biofilm inhibition by lactoferrin across all concentrations of lactoferrin. At 250 μg/mL, low biofilm forming strains of GBS exhibited inhibition of biofilm formation compared to media alone. However, treatment with 750 μg/mL and 1,000 μg/mL of lactoferrin resulted in an increase in biofilm formation, gas determined by Student's t-test with Welch's correction (*P<0.05, **P<0.01, ***P<0.001, and ***P<0.0001).

CHAPTER IV

Lactoferrin interacts with placental macrophages to suppress cellular functions

Introduction

Group B *Streptococcus* (GBS), or *Streptococcus agalactiae*, is one of the leading causes of infection-related adverse pregnancy and neonatal outcomes, which includes stillbirth, chorioamnionitis, preterm birth, and neonatal sepsis and meningitis (1). The pathogen can infect by vertical transmission, either through ascending infection from the colonized mother, or exposure during vaginal birth (3). It is estimated that between 20-30% of pregnant women are colonized with GBS, making colonization the leading risk factor for disease. Generally, GBS is a commensal bacterium, but some strains can transition into an invasive pathogen, through a mechanism not well understood. For standard care, the CDC recommends routine screening for GBS at 36 weeks of pregnancy and administration of intrapartum antibiotic prophylaxis (IAP) to mothers who test positive (1). While recommended precautions and intervention have improved early-onset disease, IAP appears to have little effect on the rate of late-onset disease in newborns or preterm birth and stillbirths (6). Currently, it is estimated that these adverse outcomes impact 97,000 to 4 million pregnancies annually. Given this ineffective intervention against GBS and observed increases in antibiotic resistance, novel strategies must be developed to combat GBS-related adverse pregnancy outcomes.

The developing fetus is considered semi-allogenic to the mother so immune tolerance must be maintained for a successful pregnancy (12). Placental macrophages (PMs), or Hofbauer cells, are fetal-derived leukocytes that reside within the connective tissue core of the placental villous tree (13). PMs, representing around 20-30% of human gestational tissue leukocytes, play a key role in placental invasion, angiogenesis, tissue remodeling, and development (13,14). These phagocytes typically display a tolerogenic M2 or M2-like phenotype, constitutively express anti-inflammatory cytokines including IL-10 and TGF-β1, (15) and suppressors of cytokine signaling (13), which is crucial in supporting an optimal environment for fetal development (17). In contrast, a highly inflammatory

environment and macrophage polarization to classical pro-inflammatory M1 state are associated with abnormal pregnancies, including spontaneous abortions, preterm labor, and preeclampsia (6).

Upon contact with the GBS, PMs will phagocytose the pathogen and initiate a proinflammatory immune response (18). The release of pro-inflammatory cytokines and chemokines by the phagocytes and other resident immune cells leads to recruitment of neutrophils. Neutrophils aid in the clearance of GBS by a repertoire of defenses including reactive oxygen species (ROS), antimicrobial peptides (AMP), and other enzymes (19). One AMP produced by neutrophils is lactoferrin, a glycoprotein contains two iron binding domains, each of which chelates a single iron ion with high affinity (20), starving invading bacteria of this crucial metal (21). In addition to chelating iron, two regions of the protein exhibit potent antimicrobial activity against a range of bacteria, fungi, and viruses (168). Previous studies in our lab have demonstrated that lactoferrin level is increased with the influx of neutrophils in response to GBS infection in a mouse model (23). In addition to its antimicrobial properties, immune-modulatory properties have been described (24).

While most studies on lactoferrin have focused on iron chelation and antimicrobial activity, including in GBS infections (220,234), including my work described in the previous two chapters, the glycoprotein also possess immunomodulatory properties (63,235). Lactoferrin can bind to receptors of immune cells to dampen a pro-inflammatory response (90). The glycosaminoglycans of membrane proteoglycans on cell surfaces account for 80% of binding by lactoferrin with low affinity (10^{-5}-10^{-6} M) (91). One consequence of receptor binding is the downregulation of pro-inflammatory cytokines by the immune cells. Lactoferrin has been shown to bind to bacterial LPS, the ligand for TLR4, thus mitigating TLR4 mediated pro-inflammatory cytokine production by macrophages (92). Another study revealed that lactoferrin can bind to soluble CD14 (sCD14), which normally complexes with LPS to induce production of IL-8, resulting in the inhibition of IL-8 production by epithelial cells and macrophages, ultimately reducing recruitment of neutrophils to the site of infection (93). Lactoferrin is also known to

bind DNA, so internalization of the peptide into immune cells can inhibit NF-κB binding to the TNF-α promoter and downregulate LPS-induced cytokine production (92).

Because lactoferrin is highly abundant in GBS-infected reproductive tissues and a proinflammatory environment is associated to adverse pregnancy outcomes, we hypothesized that lactoferrin acts to balance inflammation to combat GBS infection while dampening the macrophage response to promote an environment suitable for fetal development. In this study presented this chapter, I demonstrated that lactoferrin interacts with placental macrophages to decrease bacterial kill by reducing production of reactive oxygen species. Furthermore, lactoferrin dampens production if proinflammatory cytokines by placental macrophages. Taken together, lactoferrin delivered by neutrophils aid in maintaining an immune tolerant environment during infection of the pregnant host.

Materials and Methods

Bacterial Strains and Culture Conditions

S. agalactiae strain GB00112 (GB112), which represent the wildtype or parental strain, was using in this study. GB112 is a clinical isolate from the human reproductive tract, a sequence type 12, capsular serotype III strain isolated from a rectovaginal swab of a post-partum patient generously provided by Dr. Shannon Manning from their strain collection (170,217). We have previously examined GB112 for interaction with host macrophages and fetal membranes (220,236). Isogenic mutants of this strain, including a *npx* deletion mutant (Δ*npx*) harboring the empty pLZ12 shuttle vector (*npx:EV*), and a complemented Δ*npx* mutant harboring a plasmid containing the *npx* locus (Δ*npx:C*) were previous used in a study with THP-1 macrophages and were used in this study (ref). Bacterial strains were grown on tryptic soy agar plates supplemented with 5% sheep blood (blood agar) plates or in Todd-Hewitt broth (THB) at 37˚C. Derivatives harboring the pLZ12 plasmid were grown in media supplemented with 3 μg/mL chloramphenicol. *E. coli* DH5α strains used for the mutation and complementation process were grown in LB broth or agar supplemented with either 150 μg/mL erythromycin or 20 μg/mL chloramphenicol when necessary.

Purification of Placental Macrophages

De-identified placental tissue was collected from non-laboring women who delivered healthy, full-term infants by Caesarian section at Vanderbilt University Medical Center with approval from the Vanderbilt University Medical Center Institutional Review Board (VUMC IRB #181998). Placental macrophages were isolated according to our previously published methods (237). Briefly, villous core tissue was macerated and enzymatically digested with hyaluronidase, collagenase, and DNase (Sigma-Aldrich) before being strained through a stainless-steel filter and suspended in RPMI with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), L-glutamine, and fetal bovine serum supplemented with antibiotic and antifungal factors. Cells were filtered and centrifuged, and $CD14^+$ cells were isolated using the magnetic MACS Cell Separation system with CD14 microbeads (Miltenyi Biotec). Cells were incubated in RPMI 1640 medium (ThermoFisher) with 10% charcoal stripped fetal bovine serum (ThermoFisher) and 1% antibiotic/antimycotic solution (ThermoFisher) overnight at 37°C in 5% carbon dioxide. The following day, PMs were suspended in RPMI 1640 medium without antibiotic/antimycotic and distributed into polystyrene plates. Cells were seeded at a density of 200,000 cells per well in a polystyrene, 24-well culture plate in RPMI with 1 % antibiotic/antimycotic solution and 10 % charcoal dextran FBS (RPMI +/+), and then incubated for 24 h in a humidified atmosphere at 37 °C and 5 % CO_2.

Co-culture of GBS with Placental Macrophages and Survival Assays

PMs were cultured in RPMI 1640 medium (ThermoFisher) with 10% charcoal stripped fetal bovine serum (ThermoFisher) and 1 % antibiotic/antimycotic solution (ThermoFisher) overnight at 37°C in room air supplemented with 5% carbon dioxide. at a multiplicity of infection (MOI) of 10:1. Cocultured cells were incubated at 37°C in air supplemented with 5% carbon dioxide for 1 to 24h. and macrophages were inoculated at a MOI of 10:1 bacteria to host cells for 1 hr. Co-cultures were washed with sterile media, resuspended in fresh medium containing 100 µg/mL of gentamicin (Sigma) to kill extracellular bacteria, and further incubated for 4 h at 37°C. Gentamicin kills extracellularly located GBS but is limited

in its ability to gain access to intracellular organisms. Subsequently, the samples were extensively washed with sterile PBS, and dislodged with trypsin (find out concentration and vendor). Following collection of the PMs, the cells were lysed by the addition of 1 mL of dH_2O. Cellular cytoplasmic contents were serially diluted in PBS and plated on blood agar plates to determine the number of viable intracellular bacteria. Samples containing only bacteria were used to estimate the efficacy of antibiotic killing as a control experiment (238).

ROS Assay

To determine differences in ROS production within placental macrophages, 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) was utilized. Following isolation, placental macrophages were pre-treated with 1 μM of the fluorescent dye. Cells were infected with GBS and treated with lactoferrin for 30 minutes in the dark. Intracellular fluorescence was measured at an excitation wavelength of 495 nm and emission at 522 nm to ascertain the fluorescence intensity as a proxy for intracellular reactive oxygen species within placental macrophages. Phorbol 12-myristate 13-acetate (PMA) treated cells were utilized as positive control.

Evaluation of cytokine response to GBS infection

Supernatant from infected and uninfected placental macrophages, with and without treatment of lactoferrin, were collected after 24 hours. Samples were frozen at -80˚C or on dry ice until analyses were performed. Samples were analyzed by Eve Technologies via multiplex cytokine array (Eve Technologies, Alberta, Canada) as previously described (239). Validation of host targets for specific cytokines (IL-1β, IL-6, KC, and TNF-α) were performed by sandwich ELISA (AbCam) as previously described (195). Briefly, supernatants from macrophages were added to wells pre-coated with capture antibodies. After a wash step, the detector antibody is added and binds to a second site on the target protein to form a sandwich complex. OD_{450} readings of known concentrations were used to generate a standard curve. Cytokine concentrations were determined with its respective standard curve.

Statistical analyses

Statistical analyses of biofilm formation and bacterial growth were performed using Student's t-test or a one-way ANOVA with either Tukey's or Dunnett's *post hoc* correction for multiple comparisons. All reported *P* values are adjusted to account for multiple comparisons. *P* values of ≤0.05 were considered significant. All data analyzed in this work were derived from at least three biological replicates, data points reflect mean of technical replicates (1-3 technical replicates per biological replicate). Statistical analyses were performed using GraphPad Prism software (Versions 6 and 9, GraphPad Prism Software Inc., La Jolla, California).

Results

Lactoferrin treatment suppresses placental macrophages to kill intracellular GBS

To investigate how lactoferrin interaction with placental macrophages to killing of GBS, a gentamicin protection assay was utilized **(Figure 16).** Lactoferrin treated placental macrophages had a reduce capacity to kill intracellular GBS, compared to media only control ($P < 0.0001$, Student's T-test). GBS survived up to a log-fold in placental macrophages treated with lactoferrin. This experimental result revealed that lactoferrin is interacting with the placental macrophages to decrease its ability to kill GBS.

Lactoferrin decrease production of reactive oxygen species by placental macrophages

One method macrophage employs to kill pathogens is by phagocytosis, followed by an assault with reactive oxygen species (240). Since we witnessed a defect in bacterial killing by placental macrophages treated with lactoferrin, we hypothesize that lactoferrin may be reducing killing by limiting the production of reactive oxygen species. To investigate this possibility, we measured intracellular ROS production by using the fluorescent dye H2DCFDA. GBS infected PMs treated with lactoferrin produced less ROS than untreated cells **(Figure 17A)** ($P < 0.01$, Student's T-test). Phorbol 12-myristate-13-acetate (PMA), which drives macrophages to an inflammatory M1 phenotype (241), was used as a positive control. In uninfected cells treated with PMA, cells treated with lactoferrin produced less ROS than PMA treated alone ($P < 0.01$, Student's T-test) **(Figure 17B)**. One possible explanation

is that chelation of iron by lactoferrin affects production of ROS through Fenton chemistry (242). To examine this possibility, ROS production in cells treated with apo- or holo-lactoferrin, which is iron bound and thus cannot further chelate more iron, was measured. Cells treated with either isoform of lactoferrin produced less ROS than untreated cells **(Figure 17C)** ($P < 0.001$, One-way ANOVA, *post hoc* Tukey's test). However, there was not a difference in ROS production between cells treated with apo- or holo-lactoferrin, indicating an iron-independent mechanism.

To further interrogate differences in ROS production, a *Δnpx* GBS mutant was utilized. NADH peroxidase (npx) is required for a full survival within macrophages and overcome oxidative stress (236). Consistent with our previous study, GBS *Δnpx*, displayed a defect in survival within PMs while restoration *in trans* restored survival (236) **(Figure 18A)**. If lactoferrin truly decreased ROS production within PMs, we hypothesized that GBS *Δnpx* will survive comparable to the WT parental strain and the complemented mutant. GBS *Δnpx* survived better than or similar to WT and GBS *Δnpx:c* in macrophages treated with lactoferrin **(Figure 18B)**. GBS *Δnpx* survival in lactoferrin-treated PMs increased by two-log fold **(Figure 18C)** ($P < 0.01$, Student's T-test). Taken together, lactoferrin inhibits GBS killing by PMs through decreased production of reactive oxygen species.

Lactoferrin decrease production of pro-inflammatory cytokines by placental macrophages

To determined differences in cytokine production by lactoferrin-treated PMs, immune phenotyping by multiplex cytokine analysis on co-culture supernatants was performed. Consistent with our previously reported study, GBS infection resulted expression of a few pro-inflammatory cytokines **(Figure 19A)**. GBS infection resulted in upregulation in CXCL-1, IL-1RA, IL-1α, IL-1 β, IL-6, IL-8, and TNF- α **(Figure 19B-H)**. Lactoferrin treatment suppressed expression of all seven pro-inflammatory cytokines ($P < 0.05$). Immune-phenotyping revealed that lactoferrin treatment suppressed expression of proinflammatory cytokines in GBS-infected PMs.

Discussion

When GBS encounters the human host, the bacterium encounters immune cells that lead to an inflammatory cascade that results in the recruitment of neutrophils to clear the infection. It has been shown that GBS interacts with macrophages by toll-like receptors signaling adaptor MyDD88 and IL-1R-assosiated kinase 1 further downstream. The Protein Kinase D1 (PKD) pathway is required for the activation of MAPKs and NF-κB, which results in the expression of proinflammatory mediators (243). This has been further studied in PMs, in which pharmacological inhibitor of PKD suppresses activation of NF-κB and subsequent release of TNFα, IL-1β, IL-6 (244). NF-κB activation leads to production of IL-1, IL-2, IL-6, IL-8, IL-12, and TNFα (245). Consistent with these studies, our multiplex cytokine analysis on PMs supports that GBS infection results in the upregulation of these pro-inflammatory cytokines **(Figure 19A)**. In addition to the activation of NF-κB, GBS infection also activates the NLRP3 inflammasome (246).

On the bacterial side, a host of virulence factors influences variation in the proinflammatory response. For instance, the capsule of GBS is crucial for limiting inflammation of the tissue (208). A survey revealed that GBS sequence and capsular types trigger different inflammatory profiles. In particular, ST-17 and CPS III, which represent some of the most virulence GBS strains, induced the stronger activation of NF-κB (247). IL1-β, IL-10 and MCP-2 levels also differed between strains by THP-1 macrophages (248). GBS possesses other virulence factors, including hyaluronidase (249), that aid GBS in evading the immune response and limit inflammation (10,202).

Regardless of the bacterium's intention to evade the immune system, a proinflammatory response will be initiation during GBS infection. The developing fetus is considered to be semi-allogenic, but a delicate balance of inflammation is needed to both protect the fetus from the mother and invading pathogens (250). Fetally-derived Hofbauer cells play a crucial role at maintaining tolerance at the maternal-fetal interface (251), generally having an "M2" phenotype that favors angiogenesis and immune tolerance (252). However, adverse pregnancy outcomes such as

chorioamnionitis, PPROM, and pre-term birth, all potential outcomes of GBS infection, are linked to inflammation (251).

Lactoferrin levels in tissues are increased with the influx of neutrophils during GBS infection (23). In addition to its antimicrobial properties, lactoferrin has also been shown to be immune-modulatory (24,168). Håversen and colleagues demonstrated that lactoferrin treatment with THP-1 resulted in the reduction of TNFα, IL-1β, IL-6, and IL-8 mRNA expression. Furthermore, they showed that lactoferrin reduced LPS-induced binding of NF-kappa B to the TNF-alpha promoter (92). Consistent with their results, we also overserved a decrease in a range of pro-inflammatory cytokines during GBS infection and lactoferrin treatment, supporting the immune-modulatory role of the peptide. Wisgill and colleagues conducted a similar study with lactoferrin and neonatal monocyte-derived macrophages. Similar to our study with PMs, they observed a decrease in proinflammatory cytokines upon the treatment with human lactoferrin. They also demonstrated that lactoferrin treatment decreased TLR-4 expression and TLR-4 signaling (253). To our knowledge, we are the first group to investigate the immune-modulatory property of lactoferrin with human PMs and demonstrate that lactoferrin reduced production of ROS by the cells. While we used the entire protein, other groups have identified a 17-kDa fragment of lactoferrin that associated with the termination of inflammation and promote inflammation (254,255). As lactoferrin comes in neutrophil, which brings along an arsenal of antimicrobials, lactoferrin may serve as a signal to limit inflammation, which is particularly delicate during pregnancy. While we, and other groups, have described an immune-modulatory interaction with lactoferrin and macrophages, other groups have found that lactoferrin treatment drives the immune cells to an inflammatory M1 phenotype (256–258). There are likely other cellular determinants that dictate the outcome of this interaction, and more research should be done in this area.

Our results from another study in our laboratory indicate that GBS can survive and proliferate within PMs and peripheral blood macrophages, and that depletion of host macrophages inhibits dissemination in the reproductive tract, supporting the hypothesis that macrophages could act as a Trojan Horse for GBS infections of the reproductive tract. In this study, I demonstrated that lactoferrin

interacts with the PM in an iron-independent manner to reduce production of reactive oxygen species, thereby allowing GBS to better survive inside these cells and utilize them as a reproductive niche to allow disease to progress. This result adds to our knowledge of the intricate interactions between host and microbes at the reproductive interface.

In this chapter, I have described the immune-modulatory interaction between lactoferrin and PMs. As a healthy pregnancy requires a delicate tolerance immune environmental for the developing fetus, I proposed that lactoferrin delivered to the site of infection by neutrophils serves as a signal to limit inflammation in order for the immune system to combat the infection while maintaining a tolerable level of inflammation. Lactoferrin interacts with resident PM to limit their production of reactive oxygen species and production of pro-inflammatory cytokines.

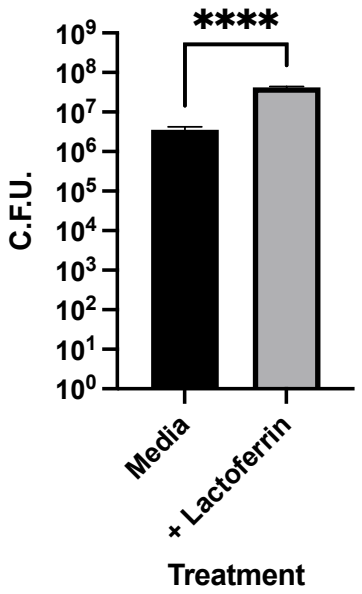

Figure 16. Lactoferrin treatment enhances GBS survival within placental macrophages. A) Gentamicin protection assay reveals that lactoferrin treatment with placental macrophages suppresses the cell's ability to kill intracellular GBS. Bars indicate mean +/- SEM. ****P< 0.0001, Student's t-test. B) Transmission electron microscopy analyses of human placental macrophages in co-culture with WT GB112. C) Quantification of GBS cells in macrophages by TEM analyses (n=3)

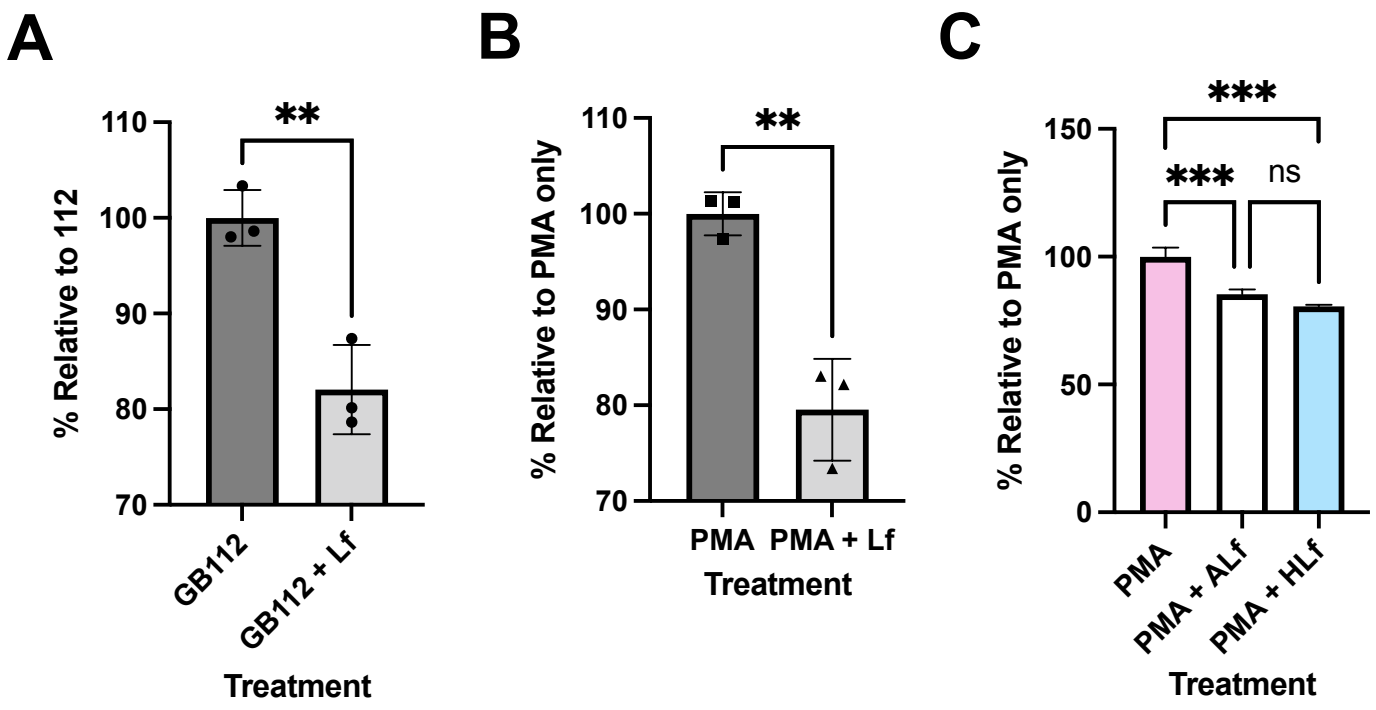

Figure 17. Lactoferrin treatment decreases production of reactive oxygen species within placental macrophages. A) H2DCFDA fluorescent dye revealed that lactoferrin treatment decreases production of reactive oxygen species within GBS-infected placental macrophages. B) Lactoferrin also reduce ROS levels in uninfected cells treated with Phorbol 12-myristate-13-acetate (PMA). **P<0.01, Student's t-test. C) Apo- and holo-lactoferrin both reduce production of ROS in PMA treated placental macrophages, indicating an iron independent mechanism. Bars indicate mean +/- SEM. ***P<0.001, ns = not significant, one-way ANOVA, post hoc Tukey's test. (n = 3).

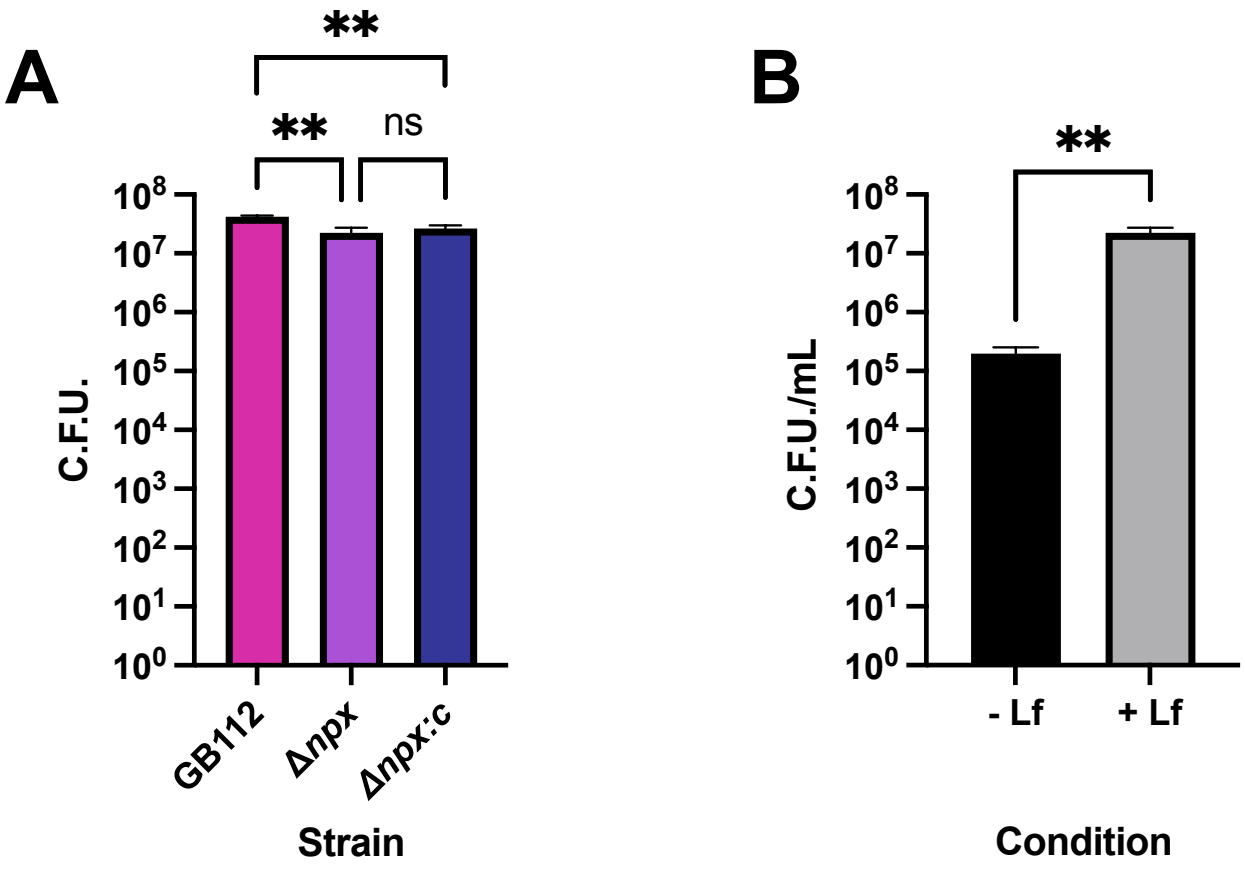

Figure 18. Lactoferrin treatment restores survival defect of GBS *npx* mutant within placental macrophages. GBS lacking NADH peroxidase are susceptible to ROS killing by macrophages. A) Lactoferrin treatment improves GBS npx survival within placental macrophages comparable to parental strain GB112 and complemented mutant GB112 Δnpx:c. **P<0.01, ns = not significant, one way ANOVA, post hoc Tukey's test. B) GB112 Δnpx survival is enhanced in placental macrophages treated with lactoferrin compared to untreated cells. Bars indicate mean +/- SEM. **P<0.01, Student's T-test. (n = 3).

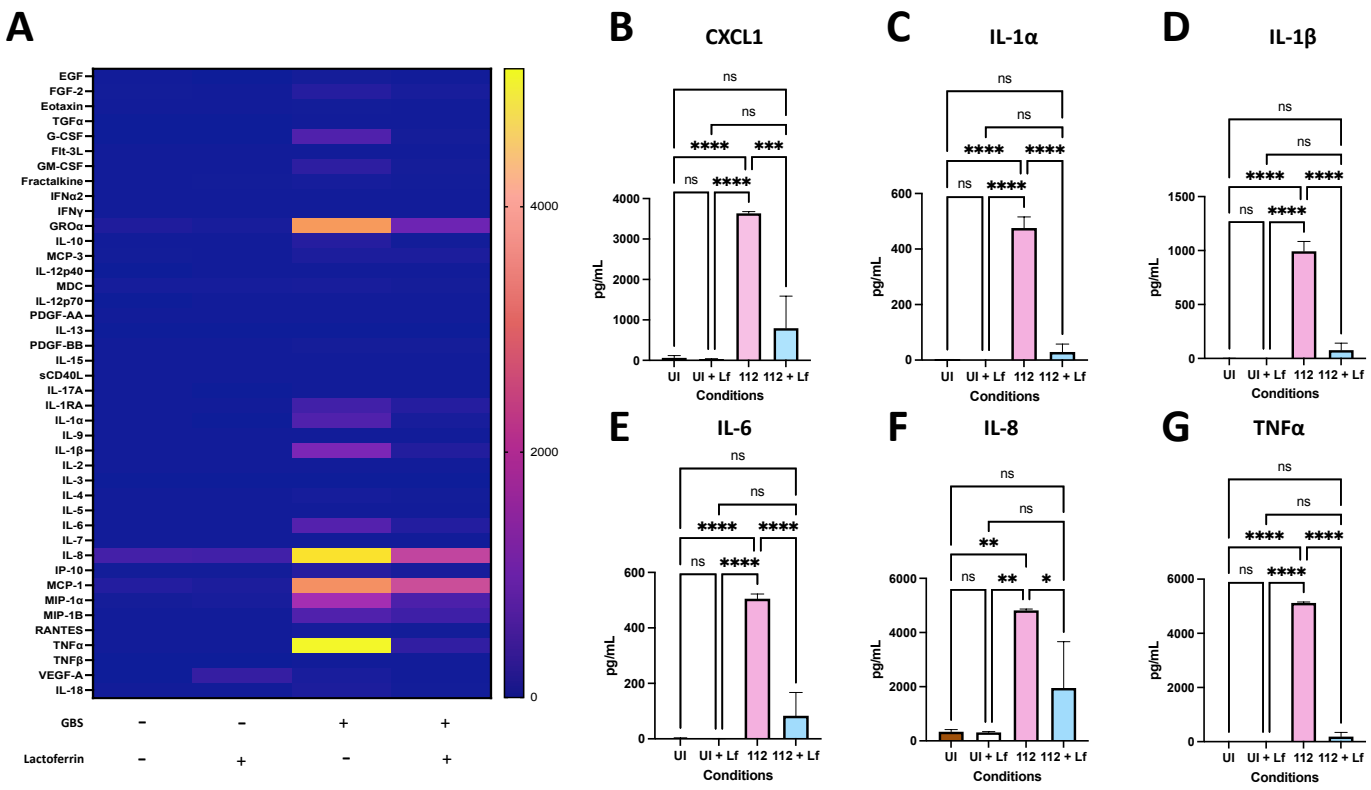

Figure 19. Lactoferrin suppresses expression of pro-inflammatory cytokines by placental macrophages during GBS infection. A) Heat map of multiplex cytokine analyses showing differential production of cytokines in response to GBS infection, with yellow indicating higher concentration and blue representing lower concentrations. B-H) Analysis of human placental macrophage secretion of cytokines (CXCL-1, IL-1RA, IL-1α, IL-1 β, IL-6, IL-8, and TNF- α) in response to GBS infection and lactoferrin treatment. GBS infection upregulates secretion of pro-inflammatory cytokines while treatment with lactoferrin suppresses expression. Bars indicate mean +/- SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, ns = not significant, One-way ANOVA, post hoc Tukey's test. I) Lactoferrin treatment increase secretion of VEGF-A in uninfected placental macrophages. ****P<0.0001, ns = not significant, One-way ANOVA, post hoc Tukey's test. (n = 3).

Chapter V

The antimicrobial and anti-biofilm properties of human breast milk lactoferrin against the ESKAPE pathogens - *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter species*

Introduction

The discovery of antibiotics revolutionized the field of infectious diseases and medicine, and advanced longevity in humans (259). However, the overuse of antibiotics has resulted in the emergence of pathogens that are multi-drug resistant (260). This is partly due to mutagenesis and horizonal gene transfer, in which previously susceptible strains acquire DNA material from resistant strains to overcome the antimicrobial challenge (261). Some common strategies activated by bacteria to resist chemical antimicrobials include the modification or degradation of antibiotics, expression of efflux pumps, sequestration of antimicrobial molecules, and protection of microorganisms by biofilms (262,263).

A group of pathogens that have emerged as clinically important organisms are bacteria known collectively as the ESKAPE pathogens. This group includes *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter species* (264). These pathogens are common and can cause debilitating and life-threatening hospital-acquired infections, where immunocompromised patients are at high risk for infection (265). A 2011 United States multi-state hospital-acquired infections survey revealed that of the 11,282 patients, 32.6% of the infections were caused by one or more of the ESKAPE pathogens (266). Many of these pathogens possess multi-drug resistant (MDR) mechanisms. Therefore, it is of utmost importance to develop novel therapies to overcome antibiotic resistance.

Microbes have evolved a range of mechanisms to obtain nutrients necessary for processes involved in survival and proliferation. Transitional metals, such as iron, are frequently incorporated into metalloproteins, storage proteins, and transcription factors (25). Eukaryotic organisms serve as an ideal habitat for many microbes in part because they possess rich reservoirs for these essential nutrients. In

response to pathogenic bacterial infections, a range of host defense mechanisms are deployed to aid and control the bacteria. One of these strategies, termed nutritional immunity, targets sequestration of essential metals to limit bacterial growth (21). Antimicrobial peptides involved in nutritional immunity, such as calprotectin and lactoferrin, have been studied against a plethora of bacterial pathogens (220,267–270).

Lactoferrin is an antimicrobial peptide expressed by mucosal epithelial cells and neutrophils (271). Additionally, it is found in high concentrations in mammalian breast milk (162). Lactoferrin is a glycoprotein with two iron binding domains (20) and has been demonstrated to possess antimicrobial activity against a wide range of bacteria, fungi, and viruses (168). Aside from sequestering iron, the protein can bind directly to bacterial cells causing destabilization of the cell membrane in an iron-independent mechanism (24). Due to its antimicrobial activity, lactoferrin has been a promising candidate for studies against microbial pathogens (272).

In the previous chapters, I demonstrated that purified human lactoferrin from breast milk exhibited potent antimicrobial and anti-biofilm activity against multiple clinical isolates of *Streptococcus agalactiae* (Group B *Streptococcus*, GBS)(234), largely due to an iron-dependent mechanism (220). In the present study, I sought to expand my studies to investigate the antimicrobial activity of human milk lactoferrin against other strains of pathogenic bacteria, particularly those that are public health threats.

Materials and Methods

Bacterial strains and culture conditions

Bacterial strains used in this study are shown in **Table 2**. All strains were grown on tryptic soy agar plates supplemented with 5% sheep blood (blood agar plates) at 37 °C in ambient air overnight. Strains were sub-cultured from blood agar plates into 5 mL Luria-Bertani broth (LB) and incubated under shaking conditions at 180 rpm at 37 °C in ambient air overnight. The following day, bacterial density was measured spectrophotometrically to determine the optical density at 600 nm (OD_{600}) using

a Promega GloMax-Multi Detection System plate reader and these bacterial cultures were used for growth and biofilm assays.

Purification of lactoferrin from human breast milk

Human lactoferrin was isolated from breast milk as previously described (220). Briefly, expressed human breast milk was gathered from 17 healthy donors between 3 days and 3 months post-partum and stored between -80 and -20˚C. De-identified human milk samples were provided by Dr. J. Hendrik Weitkamp from the Vanderbilt Department of Pediatrics, under a collection protocol approved by the Vanderbilt University Institutional Review Board (IRB #100897). Milk samples were thawed and centrifuged at 8000 g for 45 minutes to separate milk fats from the soluble fraction. Following centrifugation, the resultant top lipid layer was removed. Subsequently, proteins were precipitated from the soluble fraction by the addition of ammonium sulfate to the soluble fraction and incubation at 4˚C overnight. Precipitated proteins were fractionated by ion-exchange chromatography. Cation exchange (CM Sephadex C-50, GE Healthcare) resin suspension was packed in a column (300 x 18 mm). After sample loading, the column was washed with equilibration buffer until the absorbance at 280 nm was less than 0.05. The bound protein was then displaced from the resin by a stepwise elution protocol. For elution, 10mM sodium phosphate buffer containing 0.4 M NaCl, 0.6 M NaCl and 0.8 M NaCl were used as elution buffer A, B, and C, respectively. First, elution buffer A was passed through the column. 5 mL fractions were collected and the OD_{280} value of each fraction was measured by a UV-vis spectrophotometer. The elution was continued until the fractions showed a minimum OD of 0.03. Further elution of the bound protein was carried out with elution buffer B and C. The Identity of the fractions were determined by high resolution mass spectrometry analysis. Fractions containing greater than 99% lactoferrin were combined and used in the assays.

Preparation of holo- or apo-lactoferrin

Iron-bound (holo-) or unbound (apo-) lactoferrin was prepared as previously described (171,273). Briefly, 10 mg/mL stock of purified lactoferrin was dialyzed against either 0.1 M sodium

citrate-bicarbonate buffer pH 8.2 alone to generate apo-lactoferrin, or buffer containing 70 mM ferric chloride to generate holo-lactoferrin. Both apo- and holo-lactoferrin were dialyzed against 1X phosphate buffered saline (PBS) containing Chelex Resin (Sigma Aldrich) to remove any unbound iron content.

Evaluation of bacterial growth

Bacterial growth was determined by a spectrophometric reading as previously described (220). Briefly, optical density measurements at 600 nm (OD_{600}) were recorded to determine bacterial growth. Bacterial cultures were grown to stationary phase (OD_{600} between 0.2-0.3) and diluted at 1:10 in metal-limited LB-CP medium (50% LB with 50% calprotectin buffer (100 mM NaCl, 3 mM $CaCl_2$, 20 mM Tris pH 7.5 (171,273)). 100 µL of 1:10 diluted cultures were added to each well in a 96-well plate. The appropriate concentration of purified lactoferrin (0, 250, 500, 750, or 1000 µg/mL, concentrations which are physiologically relevant to the host-pathogen *in vivo* environment) was added into each corresponding well. For *A. baumannii* growth assays, the concentration of lactoferrin was increased to include 2.5 mg/mL and 5 mg/mL conditions as well. The plates were incubated at 37ºC overnight. The following day, bacterial density was determined by measuring OD_{600}.

Quantification of bacterial biofilms

A crystal violet assay was utilized to evaluate bacterial biofilms as previously described (173). Briefly, overnight cultures were diluted 1:10 in fresh LB-CP medium in 96-well plates. To analyze the effect of lactoferrin on biofilm inhibition, lactoferrin was applied in increasing concentrations (0, 250, 500, 750, or 1000 µg/mL) at the time of inoculation. Biofilms were allowed to form at 37ºC in ambient air overnight. OD_{600} was determined using a spectrophotometer and supernatant was removed and replaced with 0.1% crystal violet stain for thirty minutes. Wells were washed with deionized water three times and dried. The retained crystal violet was resolubilized with a solution of 80% ethanol and 20% acetone. Plates were incubated for at least 30 minutes and optical density was determined at 560 nm (OD_{560}).

Statistical analyses

Statistical analyses of biofilm formation and bacterial growth were performed using Student's *t*-test or a one-way ANOVA with either Tukey's or Dunnett's *post hoc* correction for multiple comparisons. All reported *P* values are adjusted to account for multiple comparisons. *P* values of ≤0.05 were considered significant. All data analyzed in this work were derived from at least three biological replicates, data points reflect mean of technical replicates (1-3 technical replicates per biological replicate). Statistical analyses were performed using GraphPad Prism software (Versions 6 and 9, GraphPad Prism Software Inc., La Jolla, California).

Results

The antimicrobial activity of human milk lactoferrin is dependent on iron chelation and induces biofilm formation in Enterococcus faecalis

We observed differences in *Enterococcus faecalis* growth across increasing concentrations of human apo-lactoferrin **(Figure 20)**. Apo-lactoferrin treatment suppressed bacterial growth across all concentrations tested (250, 500, 750, and 1000 µg/mL), with a 38%, 35%, 31%, and 28% decrease in growth, respectively, when compared to medium alone controls **(Figure 20A)**. When grown in the same concentrations of holo-lactoferrin, only treatment with 1 mg/mL resulted in a decrease of 21%. Comparing the two different isoforms of lactoferrin, apo-lactoferrin was able to significantly inhibit bacterial growth at 250 µg/mL and 500 µg/mL compared to holo-lactoferrin, suggesting an iron-dependent mechanism of growth inhibition. Biofilm formation was also assessed at the same concentrations **(Figure 20B)**. Unlike bacterial growth, both isoforms of lactoferrin triggered an increase of biofilm formation at all concentrations. Taken together, lactoferrin was able to suppress *E. faecalis* growth dependent on iron availability but stress induced by lactoferrin results in an increase in *E. faecalis* biofilm formation.

Human breast milk lactoferrin suppresses Staphylococcus aureus growth and biofilm formation

S. aureus was grown at 250, 500, 750, and 1,000 µg/mL of lactoferrin and bacterial growth was assessed **(Figure 21)**. Compared to grown in media alone, apo-lactoferrin suppressed *S. aureus* by 39%, 40%, 38%, and 33% respectively, when compared to medium alone controls **(Figure 21A)**. At the same concentrations of holo-lactoferrin, only treatment at 1000 µg/mL resulted in a significant growth decrease of 13% when compared to medium alone controls. At all concentrations, apo-lactoferrin inhibited bacterial growth significantly more than holo-lactoferrin, suggesting that the difference was due to iron-chelation. Both isoforms were able to decrease biofilm formation compared to cultures in media alone **(Figure 21B)**. *S. aureus* was sensitive to the antimicrobial effects of apo-lactoferrin and the antibiofilm effects of both isoforms.

Apo-lactoferrin suppresses Klebsiella pneumoniae growth and apo- and holo-lactoferrin isoforms inhibit biofilm formation

K. pneumoniae was exposed to apo- or holo-lactoferrin between 0 and 1000 µg/mL, at intervals of 250 µg **(Figure 22)**. At 250 and 500 µg/mL, apo-lactoferrin suppressed bacterial growth by 14% and 20%, respectively when compared to medium alone controls **(Figure 22A)**. At the same lower concentration of holo-lactoferrin, no differences in bacterial growth were observed. At higher concentrations of lactoferrin, both isoforms were able to reduce bacterial growth, with apo-lactoferrin being more potent. Both apo- and holo-lactoferrin were able to inhibit bacterial growth compared to media alone but no differences were observed between the two. With respect to the anti-biofilm activity of lactoferrin against *K. pneumoniae*, both isoforms were able to reduce biofilm formation above 500 µg/mL **(Figure 22B)**. At a high concentration of 1000 µg/mL, apo-lactoferrin exhibited more potent anti-biofilm activity compared to holo-lactoferrin. Overall, apo-lactoferrin was able to reduce bacterial growth while both isoforms decreased biofilm formation at lactoferrin concentrations above 500 µg/mL.

Lactoferrin suppresses biofilm formation in Acinetobacter baumannii

A. baumannii strain 19606 was treated with either apo- or holo-lactoferrin and optical density at 600 nm was observed **(Figure 23)**. Lactoferrin treatment of both isoforms did not change bacterial growth compared to *A. baumannii* grown in medium only **(Figure 23A)**. Growth inhibition of *A. baumannii* was observed at 2.5 mg/mL and 5 mg/mL of apo-lactoferrin, but not holo-lactoferrin; underscoring that the antimicrobial activity is likely due to iron-chelation. Both apo- and holo-lactoferrin inhibited *A. baumannii* biofilm formation in all concentrations observed. Apo-lactoferrin was more potent with reduction of 40%, 50%, 57%, and 56% (at 250, 500, 750, and 1,000 µg/mL), while holo-lactoferrin decreased biofilm formation by 14%, 24%, 27%, and 39% at the same concentrations, when compared to medium alone controls **(Figure 23B)**. Taken together, lactoferrin had no effects on *A. baumannii* growth but was able to decrease biofilm formation; a phenotype which was exacerbated by the iron-binding activity of lactoferrin.

Human milk lactoferrin exerts antibacterial and anti-biofilm activity against Pseudomonas aeruginosa

We investigated differences in bacterial growth in *P. aeruginosa* across increasing concentrations of human apo- and holo-lactoferrin **(Figure 24)**. Apo-lactoferrin was able to significantly inhibit bacterial growth at 250 µg/mL but at higher concentrations *P. aeruginosa* exhibited modest growth reductions that were not significant **(Figure 24A)**. Conversely, holo-lactoferrin was able to reduce bacterial growth at a higher dose of protein at 750 and 1,000 µg/mL (35 and 40% respectively, when compared to medium alone controls). Differences in bacterial biofilms were also assessed and apo-lactoferrin inhibited biofilm formation at concentrations above 250 µg/mL (500 µg/mL: 18%, 750 µg/mL 24%, and 1,000 µg/mL: 26%, when compared to medium alone controls) **(Figure 24B)**. Holo-lactoferrin had even more effective anti-biofilm properties, with 20%, 25%, 25%, and 19% reduction in *P. aeruginosa* biofilm formation at 250, 500, 750, and 1,000 µg/mL respectively, when compared to medium alone controls.

Human milk lactoferrin inhibits bacterial growth and biofilm by Enterobacter cloacae

E. cloacae was treated with increasing concentrations of apo- and holo- lactoferrin and bacterial growth, as well as biofilm formation, was assessed **(Figure 25)**. Both isoforms of lactoferrin were able to inhibit bacterial growth with apo-lactoferrin being more effective than holo-lactoferrin **(Figure 25A)**. At 250 µg/mL, apo-lactoferrin reduced bacterial growth by 36%, while holo-lactoferrin decreased growth by 20% compared to medium alone controls. The reduction for apo-lactoferrin ranged between 30-36% between the concentrations tested. Holo-lactoferrin was less potent but still achieved 20-29% growth reduction in the concentration range. Similar to bacterial growth, both isoforms of lactoferrin reduced *E. cloacae* biofilms when compared to medium alone controls, with apo-lactoferrin being slightly more potent than the holo- isoform **(Figure 25B)**. In the range of concentrations tested, apo-lactoferrin decreased biofilm formation by 26-34% while holo-lactoferrin achieved 20-27% reduction when compared to medium alone controls. Together, lactoferrin suppresses *E. cloacae* growth and biofilm formation with additional efficacy from chelating iron.

Discussion

Antibiotic resistance in many healthcare-associated infectious microbes is a prevailing threat. In order to resolve this issue, novel therapies are required. Previous studies in our lab have indicated that lactoferrin, an iron-chelating antimicrobial peptide, is able to inhibit *Streptococcus agalactiae* bacterial growth and biofilm formation (220,234), largely dependent on removing iron from the environment. Many bacterial species have evolved iron acquisition mechanisms to overcome starvation threat (274,275), indicating that limiting iron may be an effective antimicrobial strategy against many bacterial pathogens *in vivo*. In this present study, we investigate the efficacy of human lactoferrin in inhibiting bacterial growth and biofilm against the ESKAPE pathogens. We demonstrated that lactoferrin reduces bacterial growth in *E. faecalis*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *E. cloacae*, largely dependent on iron chelation. Additionally, treatment of lactoferrin decreased biofilm formation by *S. aureus*, *K. pneumoniae*, *A. baumannii, P. aeruginosa,* and *E. cloacae*.

Lactoferrin, as well as its peptide derivatives, has been shown to limit bacterial growth against many bacterial pathogens. One such peptide is lactoferricin, a 14-amino-acid protein that comprises of amino acids positioned between 17 and 30 of lactoferrin. This lactoferrin derivative has been proposed to possess antimicrobial activity by membrane permeabilization (276). In a study performed by Wuersching and colleagues, lactoferricin possessed antimicrobial activity against oral facultative anaerobic bacteria (*S. mutans*, *S. sanguinis*, *Actinomyces naeslundii*) and obligate anaerobic bacteria (*Veillonella parvula*, *Parvimonas micra*, *Fusobacterium nucleatum*)(277). Lactoferricin also possesses antimicrobial activity against antibiotic resistant strains of *S. aureus* and *K. pneumoniae* (278), consistent with our findings in this study. In another study with neonatal pathogens, human breast milk, which has a high composition of lactoferrin, was able to suppress the growth of *Staphylococcus epidermidis* and *E. coli* (184). Lactoferrin has been shown to exhibit potent antimicrobial activity against *Streptococcus*, *Salmonella*, *Shigella*, *Staphylococcus*, and *Enterobacter* genera (103). Overall, lactoferrin possesses antimicrobial activity against a myriad of bacterial pathogens.

While several research groups have studied the effects of bovine milk lactoferrin or recombinant lactoferrin, and its peptide derivatives, our group has investigated the antimicrobial properties of purified lactoferrin (in holo- or apo-isoform) from human breast milk. While lactoferrin from different mammalian species shares a high degree of amino acid homology, unique glycosylation patterns between the protein that may be responsible for heterogeneity of the biological properties of the lactoferrins (279). Our studies demonstrated that human lactoferrin inhibits *S. agalactiae* growth and biofilm formation, both on abiotic and biotic surfaces. This activity of lactoferrin is largely dependent on the iron chelating properties of this glycoprotein (220). Furthermore, this antimicrobial and anti-biofilm activity was broadly effective against a panel of GBS isolates with diverse genetic background and clinical presentation (234). Our group also assessed the antimicrobial and anti-biofilm properties of human and bovine lactoferrin against a panel of *A. baumannii* clinical strains isolated in Nashville, Tennessee, many strains of which are multi-drug resistant (280). This study led by Avery *et al.* revealed that human lactoferrin limits bacterial growth and biofilm formation in many clinical strains of *A. baumannii* (268). In

a direct comparison between human and bovine lactoferrin, human lactoferrin exhibited greater antimicrobial activity, underscoring potential differences between human and bovine isoforms of this protein. Strain-dependent sensitively to lactoferrin was related to the anatomical isolation site. This present study assessed the antimicrobial effects against *A. baumannii* 19606, a laboratory type strain. Lactoferrin had negligible antimicrobial activity against this strain at concentrations below 1 mg/mL but exhibited modest antimicrobial activity at 2.5 and 5 mg/mL concentrations. Interestingly, lactoferrin did significantly inhibit biofilm formation at lower concentrations **(Figure 23)**. The resistance to relatively high concentrations (up to 1 mg/mL) of lactoferrin in the *A. baumannii* 19606 strain was similar to results observed in several strains in the clinical panel assessed by Avery, *et al.* which were also resistant to lactoferrin at these concentrations. It is plausible that these strains share genetic or phenotypic characteristics with the type strain 19606 which encodes virulence factors, such as the siderophore acinetobactin to overcome the iron chelation imposed by lactoferrin (281). As such, there is merit in assessing the antimicrobial and anti-biofilm properties against other clinical strains of the ESKAPE pathogens.

One potential utility of human lactoferrin against antibiotic resistant strains is the treatment of bacteria with antibiotics in combination with the antimicrobial peptide, as an antibiotic adjuvant. There is a growing interest in using breast milk components to restore the efficacy of conventional antibiotics. Craft and colleagues observed that human oligosaccharides (HMOs) purified from human breast milk potentiate the function of aminoglycosides, lincosamides, macrolides, tetracyclines, and antifolates on a strain specific basis in *S. agalactiae* (158,161,191). In the same study, the group also observed that HMOs enhanced the efficacy of aminoglycosides against both *S. aureus* and *A. baumannii*. Mechanistically, the group demonstrated that human milk oligosaccharides (HMOs) potentiate antibiotics by increasing the permeability of the cell membrane, allowing for enhanced penetration by the antibiotics. Interestingly, lactoferrin is also found in high concentrations in human breast milk and has been implicated in disrupting membrane integrity of cells due to its positively charged structure (105).

As such, lactoferrin may potentially exert antimicrobial activity against some of the ESKAPE pathogens in the same manner. Lactoferrin may also enhance other antimicrobial strategies by disrupting bacterial biofilms; biofilms provide a protective barrier against antibiotic assault as well as create a safe environment for horizontal transfer of antibiotic resistance genes to occur between the bacteria (79,282,283). In this present study, we demonstrated that human lactoferrin reduces biofilm formation in many of the ESKAPE pathogens. Studies investigating the use of lactoferrin, especially in the apo- isoform, in combination with antibiotics against bacterial pathogens is warranted.

Table 2. Bacterial Strains utilized in this study

Bacterial Strains	**Source**
Acinetobacter baumannii strain 19606	ATCC
Enterobacter cloacae strain 13042	ATCC
Enterococcus faecalis strain 19433	ATCC
Klebsiella pneumoniae strain 13883	ATCC
Pseudomonas aeruginosa strain PA14	ATCC
Staphylococcus aureus strain USA300	The *S. aureus* strain used was USA300 JE21 (284) a laboratory-adapted strain derived from the parental USA300 strain isolated from a skin and soft tissue infection (285)

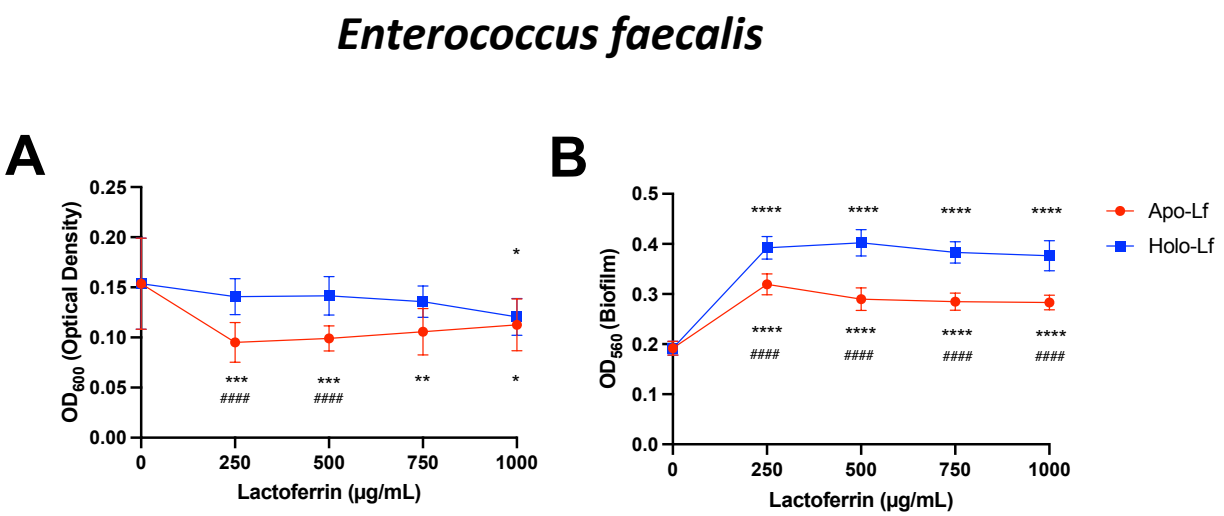

Figure 20. Analysis of bacterial growth and biofilm formation of *Enterococcus faecalis*. Exposure to human apo-lactoferrin (red line) reduced bacterial growth that was not observed with treatment of holo-lactoferrin (blue line). Both apo- and holo-lactoferrin increased biofilm formation. Each data point represents the mean reading ± SEM per concentration (N = 3 biological replicates). *P < 0.05, **P < 0.01, *** P <0.001, **** P < 0.0001, One-way ANOVA, post hoc Dunnett's multiple comparison test compared to growth or biofilm formation in media alone. #P < 0.05, ##P < 0.01, ###P <0.001, ####P < 0.0001, Student's t-test between the two isoforms at the same concentration.

Staphylococcus aureus

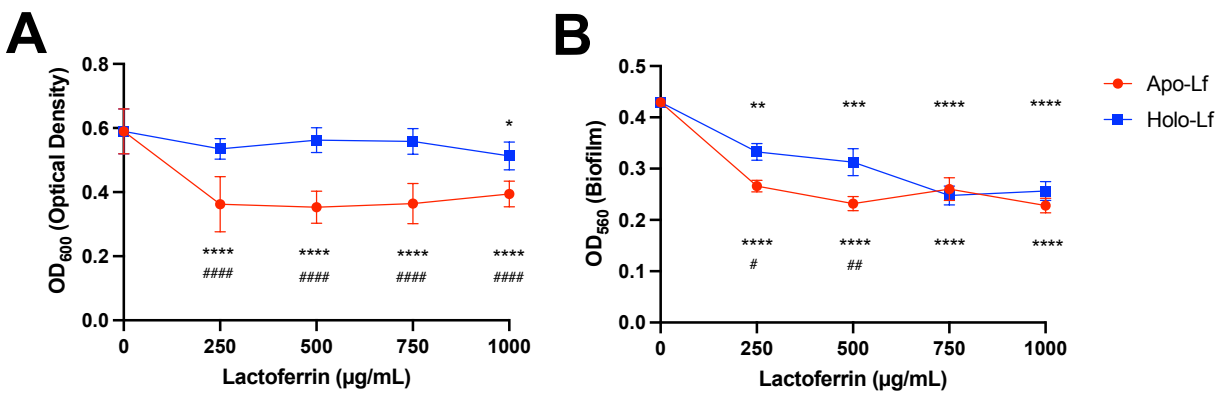

Figure 21. Analysis of bacterial growth and biofilm formation of *Staphylococcus aureus*. Exposure to human apo-lactoferrin (red line) reduced bacterial growth that was not observed with treatment of holo-lactoferrin (blue line). Both apo- and holo-lactoferrin reduced biofilm formation. Each data point represents the mean reading ± SEM per concentration (N = 3 biological replicates). *P < 0.05, **P < 0.01, *** P <0.001, **** P < 0.0001, One-way ANOVA, post hoc Dunnett's multiple comparison test compared to growth or biofilm formation in media alone. $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P <0.001, $^{\#\#\#\#}$P < 0.0001, Student's t-test between the two isoforms at the same concentration.

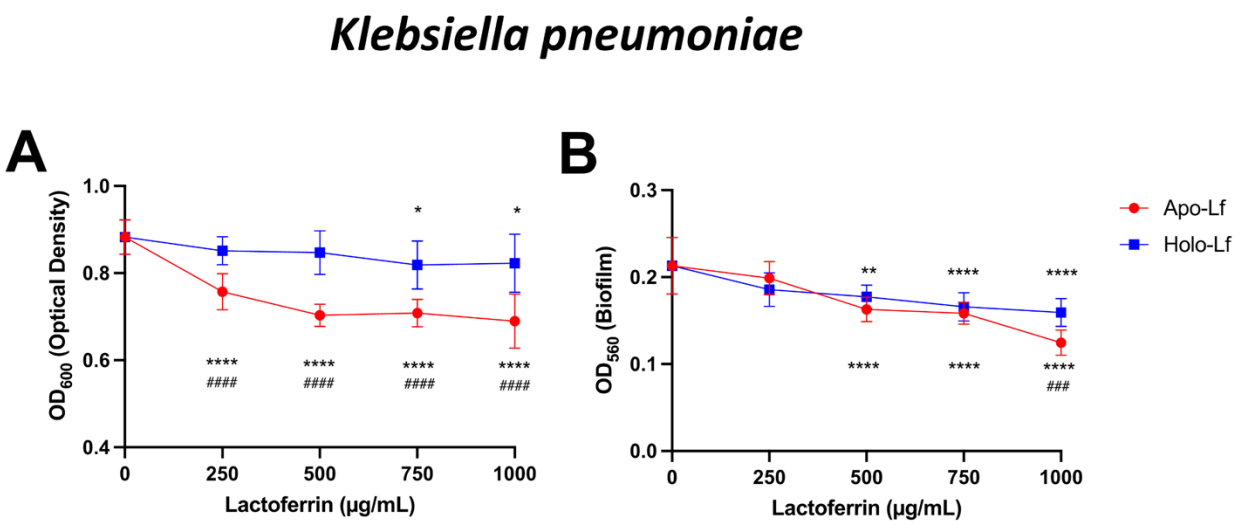

Figure 22. Analysis of bacterial growth and biofilm formation of *Klebsiella pneumoniae*. Exposure to human apo-lactoferrin (red line) reduced bacterial growth that was not observed with treatment of holo-lactoferrin (blue line). Both apo- and holo-lactoferrin reduced biofilm formation at higher concentrations. Each data point represents the mean reading ± SEM per concentration (N = 3 biological replicates). *P < 0.05, **P < 0.01, *** P <0.001, **** P < 0.0001, One-way ANOVA, post hoc Dunnett's multiple comparison test compared to growth or biofilm formation in media alone. $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P <0.001, $^{\#\#\#\#}$P < 0.0001, Student's t-test between the two isoforms at the same concentration.

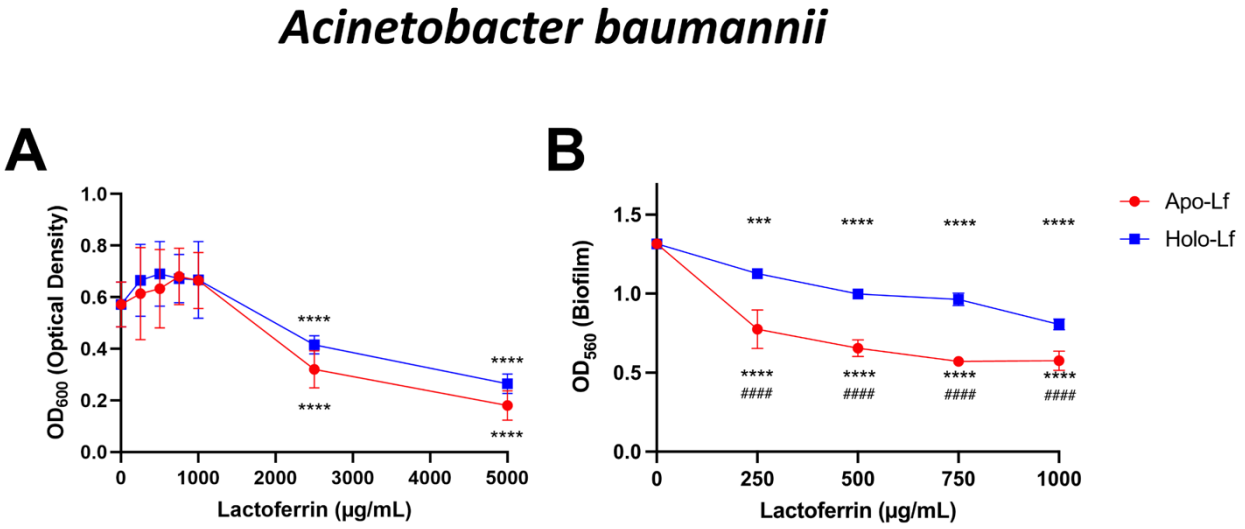

Figure 23. Analysis of bacterial growth and biofilm formation of *Acinetobacter baumannii*. Exposure to high concentrations of human apo-lactoferrin (red line) or holo-lactoferrin (blue line) inhibited bacterial growth. Both apo- and holo-lactoferrin reduced biofilm formation, with apo-lactoferrin more potent compared to holo-lactoferrin. Each data point represents the mean reading ± SEM per concentration (N = 3 biological replicates). *** P <0.001, **** P < 0.0001, One-way ANOVA, post hoc Dunnett's multiple comparison test compared to growth or biofilm formation in media alone. $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P <0.001, $^{\#\#\#\#}$P < 0.0001, Student's t-test between the two isoforms at the same concentration.

Pseudomonas aeruginosa

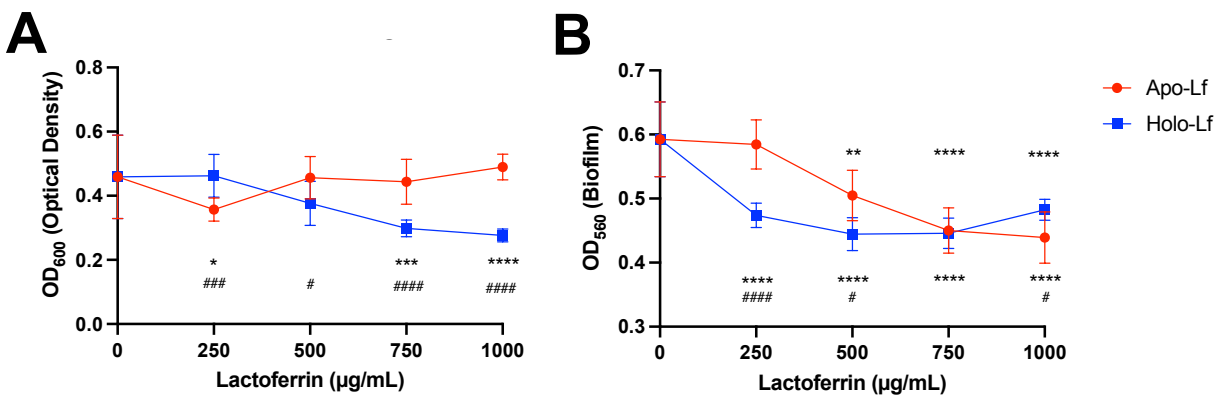

Figure 24. Analysis of bacterial growth and biofilm formation of *Pseudomonas aeruginosa*. Exposure to human apo-lactoferrin (red line) reduced bacterial growth at 250 µg/mL while treatment with holo-lactoferrin (blue line) decreased growth at higher concentrations. Both apo- and holo-lactoferrin reduced biofilm formation, with holo-lactoferrin more potent compared to holo-lactoferrin. Each data point represents the mean reading ± SEM per concentration (N = 3 biological replicates). *P < 0.05, **P < 0.01, *** P <0.001, **** P < 0.0001, One-way ANOVA, post hoc Dunnett's multiple comparison test compared to growth or biofilm formation in media alone. $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P <0.001, $^{\#\#\#\#}$P < 0.0001, Student's t-test between the two isoforms at the same concentration.

Enterobacter cloacae

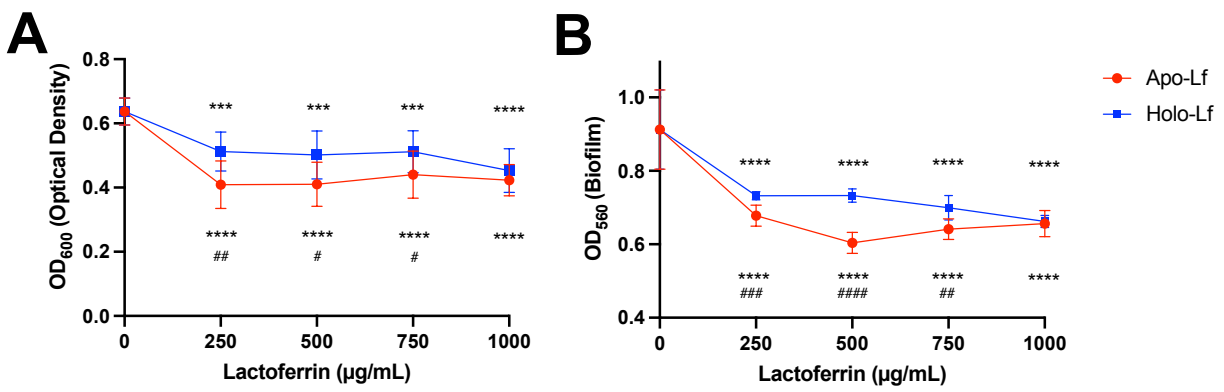

Figure 25. Analysis of bacterial growth and biofilm formation of *Enterobacter cloacae*. Exposure to human apo-lactoferrin (red line) and holo-lactoferrin (blue line) reduced bacterial growth, with apo-lactoferrin being more potent. Both apo- and holo-lactoferrin reduced biofilm formation, with apo-lactoferrin being more effective. Each data point represents the mean reading ± SEM per concentration (N = 3 biological replicates). *P < 0.05, **P < 0.01, *** P <0.001, **** P < 0.0001, One-way ANOVA, post hoc Dunnett's multiple comparison test compared to growth or biofilm formation in media alone. $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P <0.001, $^{\#\#\#\#}$P < 0.0001, Student's t-test between the two isoforms at the same concentration.

Chapter VI

Leveraging lactoferrin as an adjuvant to enhance antibiotic efficacy against Group B *Streptococcus* and other invasive bacterial pathogens

Introduction

Group B Streptococcus (GBS) infections are caused by the gram-positive, beta-hemolytic, encapsulated cocci, *S. agalactiae* (286). The bacterium colonizes up to 20-30% of all healthy adults (18). One study revealed that 18% of all healthy women are hosts to GBS, which is critical as GBS is one of the leading bacterial etiologies of adverse pregnancy outcomes (5,42). Though GBS is part of the commensal flora in most healthy individual, it can transition into an invasive pathogen in pregnant women and the elderly.

A systematic review found that invasive GBS disease in infants was 0.49 per 1000 live births, making the infection one of the leading causes of bacterial mediated adverse pregnancy outcomes (5). GBS is associated with chorioamnionitis, or inflammation of the fetal membrane, which may result in preterm premature rupture of membrane (PPROM) and preterm birth (144). In addition, GBS is implicated in early onset and late onset neonatal and maternal sepsis, which may result in death. Other complications include neonatal meningitis, pneumonia, and encephalopathy (1). In one longitudinal study in South Africa, the group found that up to 50% of pregnant women are infected at some point during pregnancy (43). Another study in the United States revealed that 65% of non-pregnant woman are colonized with GBS from rectal swabs and concluding that colonization in the gastrointestinal tract is a predictor for vaginal colonization. Another group susceptible to GBS infection is the elderly and this predominately manifests in soft tissue infections, although other complications may arise. An epidemiological study in the USA from 2008-2016 found that increasing incidences of GBS diseases in non-pregnant patients. Furthermore, they described up to 95% of the patients had at least one form of underlying condition, with obesity and diabetes being most common (287). Another study with a cohort of US veterans yielded similar results (288). GBS infection is treated with antibiotics but high levels of

resistances to antibiotics like clindamycin and erythromycin have been extensively described (149,289). Despite antibiotic treatment, there has not been a change in the incidence of late-onset neonatal sepsis (147). This observation, coupled with antibiotic resistances observed in the clinical setting, calls for novel therapies or strategies against GBS infections.

The human immune system's defense against GBS primarily takes form in a strong innate immune response. When the immune system senses a pathogen, neutrophils are recruited to the site of infection where they aid in the clearance of bacteria. One mode of defense is the excretion of extracellular traps loaded with antimicrobial peptides (75). One such peptide is lactoferrin, a glycoprotein with the ability to bind iron (76). Iron is an important transition metal involved in many biological processes such as the electron transport chain; starving bacterial of essential metals is a phenomenon termed "nutritional immunity" (25). In addition to killing bacteria by uptaking iron, lactoferrin has a very basic structure, which can interact with negatively charged molecules on cell surfaces to disrupt cell membranes (79,83). Aside from secondary granules of neutrophils, lactoferrin can be found abundantly in many mucosal fluids such as breast milk, saliva, and tears.

Many groups have begun to investigate lactoferrin's therapeutic potential. Patras *et al*. found that treatment with exogenous lactoferrin resulted in reduced adherence to epithelial cells by uropathogenic *E. coli*. Furthermore, lactoferrin enhanced effector functions of neutrophils, such as bacterial killing and production of neutrophil extracellular traps (140). Other groups have used transferrin, another iron chelating antimicrobial peptide, in combination with antibiotics to overcome resistance (142,143). As lactoferrin shares properties with transferrin, we sought to investigate if lactoferrin can improve the efficacy of a range of antibiotics against GBS.

In this study, we discovered that apo-bovine lactoferrin treatment enhanced antibiotic minimum inhibitory concentrations (MICs) against a clinical panel of GBS strains on a strain-specific basis. The effect was especially pronounced for chloramphenicol, in which we observed that high biofilm forming strains were particularly vulnerable to lactoferrin's adjuvant activity, suggesting that the anti-biofilm activity could contribute to this phenotype. Two strains were completely resistant to the antibiotic-

enhancing effects of lactoferrin: in these two GBS strains, lactoferrin enhanced biofilm formation. Additionally, these two strains also produced iron-chelating molecules, underscoring the role iron-homeostasis might play in this phenotype. We further our studies by investigating human lactoferrin as a potential adjuvant against selected antibiotics, including ones targeting the bacterial cell wall and protein synthesis. For some strains and antibiotics tested, we observed that human lactoferrin was able to enhance antibiotic MIC, a result that was not observed with treatment with bovine lactoferrin. Finally, we assessed the antibiotic-enhancing properties against other bacterial pathogens. This study suggests that there is merit is using lactoferrin as an adjuvant with antibiotics to enhance the antimicrobial utility against GBS and other bacterial pathogens with high incidences of antibiotic resistance.

Methods and Material

Bacterial strains and culture conditions

This study utilized a diverse set of 28 previously characterized *S. agalactiae* strains recovered from neonates with invasive disease (214) and colonized mothers sampled before and after childbirth (217); all strains were originally isolated by Dr. H. Dele Davies (221,222). Three common laboratory reference strains (A909, NEM316, and COH1; American Type Culture Collection) were also evaluated. This study also utilized *Acinetobacter baumannii* strain 19606 (ATCC), *Escherichia coli* strain 11775 (ATCC), *Pseudomonas aeruginosa* strain PA14 (ATCC), *Staphylococcus aureus* strain USA300 (284,290). Bacterial strains were cultured on tryptic soy agar plates supplemented with 5% sheep blood (blood agar plates) at 37°C in ambient air overnight. Bacteria were sub-cultured from blood agar plates into liquid medium (Todd Hewitt Broth; THB) and incubated in aerobic conditions (ambient air, shaking at 200 rpm) at 37°C overnight. The following day, bacterial density was measured spectrophotometrically to determine the optical density at 600 nm (OD_{600}). These bacterial cultures were used for growth, viability, biofilm, and co-culture assays.

Purification of lactoferrin from human breast milk

Human lactoferrin was isolated from breast milk as previously described (220). Briefly, expressed human breast milk was gathered from 17 healthy donors between 3 days and 3 months post-partum and stored between -80 and -20˚C. De-identified human milk samples were provided by Dr. J. Hendrik Weitkamp from the Vanderbilt Department of Pediatrics, under a collection protocol approved by the Vanderbilt University Institutional Review Board (IRB #100897). Milk samples were thawed and centrifuged at 8000 g for 45 minutes to separate milk fats from the soluble fraction. Following centrifugation, the resultant top lipid layer was removed. Subsequently, proteins were precipitated from the soluble fraction by the addition of ammonium sulfate to the soluble fraction and incubation at 4˚C overnight. Precipitated proteins were fractionated by ion-exchange chromatography. Cation exchange (CM Sephadex C-50, GE Healthcare) resin suspension was packed in a column (300 x 18 mm). After sample loading, the column was washed with equilibration buffer until the absorbance

at 280nm was less than 0.05. The bound protein was then displaced from the resin by a stepwise elution protocol. For elution, 10mM sodium phosphate buffer containing 0.4 M NaCl, 0.6 M NaCl and 0.8 M NaCl were used as elution buffer A, B, and C, respectively. First, elution buffer A was passed through the column. 5 mL fractions were collected and the OD_{280} value of each fraction was measured by a UV-vis spectrophotometer. The elution was continued until the fractions showed a minimum OD of 0.03. Further elution of the bound protein was carried out with elution buffer B and C. The Identity of the fractions were determined by high resolution mass spectrometry analysis. Fractions containing greater than 99% lactoferrin were combined and used in the assays. All lactoferrin used in study was in the apo-form, unless specified.

Preparation of holo- or apo-lactoferrin

Iron-bound (holo-) or unbound (apo-) lactoferrin was prepared as previously described (23,171). Briefly, 10 mg/mL stock of purified human lactoferrin or bovine lactoferrin (FrieslandCampina) was dialyzed against either 0.1M sodium citrate-bicarbonate buffer pH8.2 alone to generate apo-lactoferrin, or buffer containing 70 mM ferric chloride to generate holo-lactoferrin. Both apo- and holo-lactoferrin were dialyzed against 1 × phosphate-buffered saline (PBS) containing Chelex Resin (Sigma Aldrich) to remove any unbound iron content.

Evaluation of bacterial growth

Bacterial growth was determined by a spectrophometric reading as previously described (220). Briefly, optical density measurements at 600 nm (OD_{600}) were recorded to determine bacterial growth. GBS cultures were grown to stationary phase (OD_{600} between 0.2-0.3) and diluted at 1:10 in metal-limited THB medium (50% THB with 50% calprotectin buffer (100 mM NaCl, 3 mM $CaCl_2$, 20 mM Tris pH 7.5 (171,172))). 100 µL of 1:10 diluted cultures were added to each well in a 96-well plate. The appropriate concentration of purified lactoferrin (0, 250, 500, 750, or 1000 µg/mL, concentrations which are physiologically relevant to the host-pathogen *in vivo* environment) was added into each corresponding well. The plates were incubated at 37ºC overnight. The following day, bacterial density was determined by measuring OD_{600}.

Broth microdilution method for determination of minimum inhibitory concentrations and antibiotic sensitization

Minimum inhibitory concentrations of antibiotics were determined as previously described (160), with a few modifications. All strains were grown overnight as described above and diluted in THB at 5 × 10^6 CFU/mL. For lactoferrin treated condition, 500 µg/mL was added to the diluted cultures. 50 µL of diluted culture was added to each well, with the top concentration wells remaining empty. The highest desired concentration of the antibiotics was determined and added to 100 µL of culture. 100 µL was added to the top wells and cultures were diluted 1:2 (50 µL) to achieve a final volume of 50 µL. Bacteria grown in media in the absence of any compounds served as the control. The plates were incubated under static conditions at 37 °C in ambient air for 24 h. Bacterial growth was quantified through absorbance readings (OD_{600}). The minimum inhibitory concentrations were assigned at the lowest concentration of compound at which no bacterial growth was observed.

Quantification of bacterial biofilms

A crystal violet assay was utilized to evaluate bacterial biofilms as previously described (173,220). Briefly, overnight GBS cultures were diluted 1:10 in THB-CP medium in 96-well plates. To analyze the effect of lactoferrin on biofilm inhibition, lactoferrin was applied at increasing concentrations (0, 250, 500, 750, or 1000 µg/mL) at the time of inoculation. Biofilms were allowed to form at 37ºC in ambient air overnight. OD_{600} was determined using a spectrophotometer and supernatant was removed and replaced with 0.1% crystal violet stain for thirty minutes. Wells were washed with deionized water three times and dried. The retained crystal violet was resolubilized with a solution of 80% ethanol and 20% acetone. Plates were incubated for at least 30 minutes and optical density was determined at 560 nm (OD_{560}). Quantification was determined by using a ratio of OD_{560}/OD_{600}.

Bacterial Membrane Permeabilization Assay

To assess bacterial cell membrane integrity after exposure to lactoferrin, a LIVE/DEAD BacLight assay (Invitrogen, ThermoFisher) was employed. GBS were grown overnight as described above to OD of 0.3-0.4. Microcentrifuge tubes of 1 mL of cultures were pelleted and resuspending in minimal

media (50:50 THB:CP Buffer) with 500 µg/mL of lactoferrin. Following incubation under static conditions at 37 °C in ambient air for 24 h, cells were pelted and stained with propidium iodide (PI) and SYTO 9 (10 mg/mL, 8 µL of each in 5 mL of PBS) for 1 hour in the dark. Following staining, cells were pelleted and washed with PBS three times. 100 µL was transferred to a 96-well plate and absorbance was measured by a plate reader for excitation/emission 475 nm/520 nm (green, SYTO 9) and 493 nm/636 nm (red, PI). The ratio of green to red fluorescence was calculated and compared.

Determining of production of bacterial chelator on microplate

CAS reagent was prepared as per Schwyn and Neilands (291), with modifications from Arora *et al.* (2017)(292). Briefly, 121 mg CAS (Chrome azurol sulfonate, Sigma-Aldrich), was dissolved in 100 mL distilled water and 20 mL of 1 mM ferric chloride ($FeCl_3 \cdot {}_6H2O$) solution prepared in 10 mM HCl. This solution was added to 20 ml hexadecyl trimethyl ammonium bromide (HDTMA, Millipore-Sigma) solution under stirring. HDTMA solution was prepared by mixing 729 mg HDTMA in 400 mL of distilled water (5 mM). The CAS-HDTMA solution was sterilized before further use. GBS cultures were grown overnight to OD_{600} = 0.3 and 1 mL was transferred to a microcentrifuge tube and pelleted. 100 µL of supernatant was added to a 96-well plate and combined with 100 µL CAS reagent, with a final volume of 200 µL per well. Wells with THB media serve as a negative control. The plate was incubated shaking in the dark for 20 min at room temperature. Following incubation, absorbance at 630 nm was recorded. Iron chelator produced by strains was measured in percent iron chelating molecule unit (psu) which was calculated according to the following formula (293):

Iron chelating molecule production (psu) = $\frac{(A_r - A_s)\ x\ 100}{A_r}$

A_r = absorbance of reference (CAS solution and THB)

A_s = absorbance of sample (CAS solution and cell-free supernatant of sample)

Statistical analyses

Statistical analyses of biofilm formation and bacterial growth were performed using Student's *t*-test or a one-way ANOVA with either Tukey's or Dunnett's *post hoc* correction for multiple comparisons.

All reported *P* values are adjusted to account for multiple comparisons. Comparison of MICs fold changes were determined using Mann-Whitney U Test. *P* values of ≤0.05 were considered significant. All data analyzed in this work were derived from at least three biological replicates, data points reflect mean of technical replicates (1-3 technical replicates per biological replicate). Statistical analyses were performed using GraphPad Prism software (Versions 6 and 9, GraphPad Prism Software Inc., La Jolla, California).

Results

Lactoferrin acts as an adjuvant to enhance cell-wall targeting antibiotics in a strain specific basis

Ampicillin and vancomycin MICS were compared when treated with bovine lactoferrin across multiple clinical GBS strains with diverse genetic and clinical presentation **(Table 3)**. Out of the 31 strains tested, 2 strains witnessed impressive decrease in MICs for ampicillin when treated with lactoferrin. Ampicillin had an MIC of 0.125 µg/mL against GB0064 and 0.25 µg/mL for GB0112. With the addition of lactoferrin, the MIC of ampicillin against both strains were reduced to 0.0625 µg/mL. Compared to ampicillin, lactoferrin enhanced the MICs of vancomycin against more strains. Lactoferrin treatment reduced the MIC of vancomycin in the following strains: GB0079, GB0115, GB0438, GB590, COH1, and NEM16. In contrast, lactoferrin increased the MIC of vancomycin in GB0241, GB0291, and GB0374. Taken together, lactoferrin treatment resulted in strain specific increase and decrease for ampicillin and vancomycin.

Lactoferrin acts as an adjuvant to enhance 30s ribosomal subunit antibiotics in a strain specific basis

Gentamicin, kanamycin, spectinomycin, and tetracycline are 30s ribosomal subunit targeting antibiotics that were assessed in this study **(Table 4)**. Lactoferrin treatment increased the MIC of gentamicin in more GBS strains than the number of strains were with MIC reduction. However, treatment with lactoferrin notable reduced the MIC of GB0115 from 128 µg/mL to 16 µg/mL. Lactoferrin was more successful in enhancing kanamycin than gentamicin. In GB0653, lactoferrin treatment reduced the MIC from 1024 µg/mL to 16 µg/mL, rendering this strain much more vulnerable to kanamycin. Conversely, lactoferrin increase the MIC of kanamycin from 16 µg/mL to 1024 µg/mL in GB0418. In an assessment of spectinomycin, several strains witnessed moderate decrease in MIC in combination with lactoferrin. Treatment of lactoferrin with tetracycline notably reduced the MIC from 128 µg/mL to 4 µg/mL in GB0377.

Lactoferrin acts as an adjuvant to enhance 50s ribosomal subunit antibiotics in a strain specific basis

Four 50s ribosomal subunit targeting antibiotics were selected for this study: chloramphenicol, clindamycin, erythromycin, and puromycin **(Table 5)**. Out of the antibiotics assessed in this study, the largest changes in MICs were observed in chloramphenicol. Strikingly, lactoferrin treatment reduced the MIC of chloramphenicol from 1024 µg/mL to 16 µg/mL in COH1. Other notable chloramphenicol MIC decrease my lactoferrin include GB0079, from 1,024 µg/mL to 64 µg/mL, and GB1445, with a decrease from 256 µg/mL to 16 mg/mL. Only one strain was resistant; lactoferrin increased the MIC from 16 mg/mL to 32 µg/mL in GB0064. Lactoferrin moderately modulated the MICs of both clindamycin and erythromycin across the clinical strain. Interestingly in GB0653, the MIC of erythromycin was decreased from 0.5 µg/mL to 0.03125 µg/mL with lactoferrin treatment. Conversely, lactoferrin increased the MIC of erythromycin from 0.125 µg/mL to 1 µg/mL in GB0374. Finally, notable results from puromycin include a decrease from 64 µg/mL to 8 µg/mL in GB0377 while increase the MIC from 8 µg/mL to 64 µg/mL in GB0285. Taken together, lactoferrin treatment resulted in variable modulation of MICs across different clinical strains.

Bovine lactoferrin enhances antibiotic efficacy of certain antibiotics in a strain specific manner

Across the twelve antibiotics tested, lactoferrin treatment enhanced the antibiotic efficiency in chloramphenicol in the highest number of strains, with 13 GBS strains showing susceptibility **(Table 6)**, with MIC fold changes as high as 64 and two at 16. Only one strain was resistant for chloramphenicol. Tetracycline was the second most affected antibiotic, with 9 strains with decreased MICs with lactoferrin treatment. Kanamycin and puromycin MICs were lowered in 8 and 7 strains, respectively. Conversely, nine strains resistant to the MIC altering effect in gentamicin by lactoferrin. No changes were detected across all clinical strains with polymyxin B and nalidixic acid under lactoferrin treatment **(Table 7)**. There were no associations between susceptible strains and sequence type, clinical presentation, or capsular type.

High biofilm forming GBS strains are sensitive to the antibiotic enhancing property of lactoferrin in chloramphenicol

We observed that chloramphenicol was especially sensitive to the antibiotic enhancing property of lactoferrin. Previous studies have demonstrated that apo-lactoferrin reduced biofilm formation in a clinical bank of GBS strains (234). To fully probe this connection, the strains in this study were separated into high and low biofilm forming strains and MIC for chloramphenicol was compared between the two groups **(Figure 26A)**. Overall, chloramphenicol was more effective against low biofilm forming strains ($P < 0.05$, Mann Whitney U Test), suggesting that biofilms protect GBS against this antibiotic. Lactoferrin treatment resulted in higher fold changes in chloramphenicol in high biofilm forming strain **(Figure 26B)** ($P < 0.01$, Mann Whitney U Test). Taken together, lactoferrin may decrease the MIC of chloramphenicol by reducing GBS biofilms.

Bovine lactoferrin may enhance antibiotic efficacy by increasing bacterial membrane permeability

One potential mechanism by which lactoferrin may increase antibiotic efficacy is by increasing membrane permeability in GBS. Previous studies have demonstrated that lactoferrin disrupts bacterial membranes in *Escherichia coli* (79). To determine if lactoferrin can increase membrane permeability in GBS, a LIVE/DEAD BacLight assay was employed. GBS was treated with 500 μg/mL of bovine lactoferrin, and membrane permeability was assessed. Lactoferrin treatment resulted in a decrease in membrane integrity **(Figure 27)** ($P < 0.05$, Student's T-test), which may allow for more antibiotics to penetrate to cell and decreasing MICs in some strains.

Human lactoferrin decreases antibiotic MICs in some antibiotics in a strain specific manner

While both bovine and human lactoferrin possess iron-chelating activity, human lactoferrin possess unique glycosylation sites (294) that may have consequences in its activity against microbes. To determine of human lactoferrin also possess antibiotic enhancing potential, MICs of selected

antibiotics were compared with lactoferrin treatment in three GBS strains **(Table 7)**. In GB0037, human lactoferrin treatment enhanced the efficacy of clindamycin, gentamicin, and spectinomycin. In GB0112, human lactoferrin treatment enhanced the efficacy of kanamycin and spectinomycin. Finally, human lactoferrin treatment enhanced the efficacy of chloramphenicol and gentamicin in GB0590. Taken together, human lactoferrin enhances antibiotic efficacy in a strain specific manner.

Comparison of the antibiotic enhancing properties of human and bovine lactoferrin against selected antibiotics in three GBS strains

As human and bovine lactoferrin differ in its glycosylation pattern, its antibiotic enhancing properties were compared **(Table 8)**. For some antibiotics, human lactoferrin was able to decrease the MICs antibiotics that were not changed with human lactoferrin. These include clindamycin and spectinomycin in GB0037, kanamycin and spectinomycin in GB0112, and chloramphenicol and gentamicin in GB0590. Bovine lactoferrin increased the MICS for gentamicin and kanamycin in GB0037 and gentamicin in GB0112. Treatment with human lactoferrin ablate this phenotype. Conversely, bovine lactoferrin was effective in ampicillin in GB0112 and vancomycin in GB0590; this was not observed in treatment with human lactoferrin. Interestingly, both antibiotics target the cell wall.

Two clinical GBS strains are not only resistant to the antibiotic effects, but also the antimicrobial and antibiofilm properties of the glycoprotein.

From the panel of 31 GBS strained in this assay, two strains were notable for its resistance to the antibiotic enhancing property of lactoferrin. Lactoferrin did not enhance any antibiotics in GB0561 or GB0651. In fact, lactoferrin reduced antibiotic efficacy of gentamicin, puromycin, and spectinomycin in GB0561 and erythromycin, gentamicin, kanamycin, and spectinomycin in GB0651. One possible explanation is its reaction to lactoferrin treatment. Treatment with increasing concentrations with bovine apo-lactoferrin did not alter bacterial growth in the two strains **(Figure 28A and 28B)**. Using a crystal violet assay, we observed treatment with 500 μg/mL of lactoferrin, the concentration used in our

antibiotic sensitivity assays in this study, did not affect biofilm formation in these two strains **(Figure 28C and 28D)**. In fact, treatment with higher concentrations of apo-lactoferrin induced biofilm formation (**P < 0.01, ***P < 0.001) (One-way ANOVA, *post hoc* Tukey's test). Thus, the resistances witnessed by these two strains may be related to endurance against biofilm disruption in response to lactoferrin treatment.

GB0561 and GB0651 possess some iron acquiring mechanism to overcome lactoferrin mediate pressure

As this study utilized apo-lactoferrin, or lactoferrin that can chelate iron, the phenotype we have observed so far can, in part, be accounted for in an iron-dependent manner. Other groups have implicated iron acquisition mechanisms in GBS (295). In the human host, GBS possesses an arsenal of tool to interact with heme and can acquire iron during infection. In fact, GBS possess mechanisms to export heme and its associated iron to prevent iron and respiratory stress-associated toxicity (296). However, our controlled system does not contain heme and GBS must have alternate iron acquisition mechanisms. To investigate if the GBS strains from our genetically diverse panel possess such iron-acquisition mechanism, we employed a microplate chrome azurol sulfonate (CAS) competition assay **(Figure 29)**. With the CAS assay, we discovered that the strains, GB0561 and GB0651, can produce an iron-chelating molecule, to compete with CAS for iron (P<0.0001, One-way ANOVA). Interestingly, these two strains are resistant to lactoferrin and is it plausible that these two strains can acquire iron and form biofilms in response to lactoferrin stress. Taken together, some GBS strains may possess resistance to the effects of lactoferrin by encoding for iron acquisition pathways.

Lactoferrin enhances antibiotic efficacy in other bacterial pathogens

We were encouraged by our results demonstrating the antibiotic enhancing effects of lactoferrin against GBS. As a result, we expanded our study to other gram-positive and gram-negative pathogens that are relevant to human health **(Table 9)**. Using bovine lactoferrin and the same twelve antibiotics

utilized in this study, we observed that lactoferrin sensitized clindamycin in *S. aureus*. Lactoferrin treatment decrease the MIC of erythromycin, gentamicin, nalidixic acid, polymyxin B, and tetracycline in *P. aeruginosa*. In a strain of uropathogenic *E. coli,* lactoferrin treatment improved gentamicin, kanamycin, polymyxin B, and tetracycline. Finally, lactoferrin improved chloramphenicol, polymyxin B, and vancomycin in *A. baumannii*. Together, this study demonstrates that lactoferrin may be useful as an adjuvant to enhance antibiotic efficacy

Discussion

There are three potential mechanisms that can explain the enhancing properties of lactoferrin. The experiments conducted in this study are all in the apo-form (268). As such, the efficacy improvement activity may be explained by iron chelation. Iron, in addition to other transitional metals, acts as cofactors for many enzymes involved in basic biological processes (297). By chelating iron, bacteria are starved of this metal, rendering many enzymes inactive, potentially including those active in pathways required for resistance against antibiotics. With these pathways inactive, it may require a lower concentration of antibiotics required to limit bacterial growth. However, studies using the holo-form of the protein, or iron addback into the system, would greatly enhance our understanding of this relationship.

A different mechanism to improve antibiotic efficacy is by reducing the integrity of bacterial membranes acting as a barrier against some antibiotics. A study by Ellison and colleagues revealed that both transferrin and lactoferrin are able to damage gram negative bacterial membranes (79). An alternate study by our group, led by Craft, *et al.* revealed that human oligosaccharides enhance antibiotics against GBS by increasing bacterial membrane permeability. Our study is novel in demonstrating that lactoferrin can also disrupt bacterial membranes in the gram-positive bacterium GBS **(Figure 27)**. By damaging the membranes, the antibiotics can enter the bacteria and lowering its minimum inhibitory concentrations.

One last potential explanation for lactoferrin decreasing antibiotics MICs is by the disruption of bacterial biofilms. Biofilms play an important role in the defense against antibiotics because these structures prevent access of the antibiotics to the bacteria (298,299). In fact, our study demonstrate that this may explain the relationship between GBS and chloramphenicol, specifically, stronger biofilm forming GBS strains require higher concentrations of chloramphenicol to inhibit bacterial growth **(Figure 26A)**. At the temporal scale, these colonies provide a community where antibiotic resistant genes can be passed between bacteria through horizontal transmission (300,301). Our group have previously demonstrated that lactoferrin possesses potent anti-biofilm activity against this same panel of clinical GBS strains, largely due to an iron-dependent manner (234). Lactoferrin may improve antibiotics by first disrupting biofilms and allowing better access to the bacteria. This mechanism is further bolstered by our finding that two of the clinical GBS strains are resistant to the antibiotic enhancing properties and antibiofilm properties by lactoferrin **(Figure 28C and 28D)**.

Other groups have assessed targeting iron starvation as a strategy to mitigate antibiotics. Similar to lactoferrin, transferrin is a related protein produced by the human host that bind iron (302). In a study led by Lin and colleagues, the group revealed that apo-transferrin was antimicrobial against *S. aureus*, *A. baumannii*, and *Candida albicans* through iron chelation and disruption of membrane potential (303). Furthermore, using transferrin as an adjuvant reduced the emergence of rifampin-resistant mutants of *S. aureus* in infected mice treated with the antibiotic. In a separate study led by Luna and colleagues, the group studied the antibiotic-enhancing effects of apo-transferrin (143). They revealed that treatment with apo-transferrin enhanced the efficacy of meropenem and ciprofloxacin in *Klebsiella pneumoniae* and *A. baumannii* in a time-kill assay. Furthermore, treatment with apo-transferrin prevented emergences of antibiotic resistance mutants, likely through sequestering iron. Iron also mediates antibiotic-induced, free-radical DNA damage through the Fenton reaction; this mechanism has previously been shown to promote the formation of antibiotic-resistant mutants (304,305). As lactoferrin share many characteristics and properties transferrin, it is plausible that lactoferrin may prevent emergence of antibiotic resistant mutants as well, though more research is warranted.

While the utility of intraperitoneal antibiotic prophylaxis in GBS colonized women has lowered the incidences of early on-set neonatal sepsis, the incidence of late-onset disease is still rising (306), calling for novel strategies. One potential strategy is to utilize human breast milk to improve antibiotic efficacy. A large component of human breast milk are human-milk oligosaccharides (HMOs). Treatment with HMOs sensitizes GBS to clindamycin, erythromycin, gentamicin, and minocycline on a strain specific basis (160). Considering our results that lactoferrin also potentiates antibiotics against GBS, there is merit in considering using feeding infected infants with human breast milk to serve as an adjuvant to improve antibiotic efficacy against GBS in late-onset disease.

Table 3. Cell wall targeting antibiotic profile for bovine lactoferrin against GBS

Antibiotics:	Ampicillin		Vancomycin	
Strain Number	MIC (μg/mL)	MIC w/Lf (μg/mL)	MIC (μg/mL)	MIC w/Lf (μg/mL)
GB0002	0.0625	0.0625	1	1
GB0012	0.0625	0.0625	2	2
GB0037	0.0625	0.0625	1	1
GB0064	0.125	0.0625	4	4
GB0066	0.125	0.125	4	4
GB0069	0.0625	0.25	4	4
GB0079	0.125	0.25	8	4
GB0083	0.125	0.125	4	4
GB0112	0.25	0.0625	1	1
GB0115	4	4	8	4
GB0241	0.125	0.125	4	8
GB0285	0.125	0.125	4	4
GB0291	0.0625	0.125	2	4
GB0374	0.125	0.125	4	8
GB0377	4	4	4	4
GB0390	0.125	0.125	4	4
GB0397	0.125	0.125	4	4
GB0411	0.125	0.25	4	4
GB0418	0.125	0.25	4	4
GB0438	0.0625	0.0625	2	1
GB0561	0.125	0.125	4	4
GB0571	0.125	0.125	4	4
GB0590	0.125	0.125	8	4
GB0651	4	4	4	4
GB0653	4	4	4	4
GB0654	0.0625	0.0625	4	4
GB0663	0.125	0.125	4	4
GB1445	4	4	4	4
A909	0.125	0.125	4	4
COH1	4	4	8	4
NEM316	0.125	0.125	8	4

Table 4. 30S ribosomal subunit targeting antibiotic profile for bovine lactoferrin against GBS

Antibiotics:	Gentamicin		Kanamycin		Spectinomycin		Tetracycline	
Strain Number	MIC (µg/mL)	MIC w/Lf (µg/mL)	MIC (µg/mL)	MIC w/Lf (µg/mL)	MIC (µg/mL)	MIC w/Lf (µg/mL)	MIC (µg/mL)	MIC w/Lf (µg/mL)
GB0002	8	8	128	128	128	64	32	128
GB0012	32	32	256	256	128	128	128	128
GB0037	8	32	128	256	64	64	128	128
GB0064	32	32	256	256	512	512	128	128
GB0066	16	16	512	256	64	64	128	64
GB0069	128	128	512	256	512	512	512	512
GB0079	64	256	256	1024	256	256	64	64
GB0083	16	16	128	128	512	512	4	2
GB0112	32	64	1024	1024	128	128	512	128
GB0115	128	16	1024	512	128	64	128	64
GB0241	128	256	1024	1024	512	512	64	128
GB0285	256	256	1024	1024	128	256	128	64
GB0291	256	256	512	512	128	128	128	128
GB0374	256	128	256	256	64	64	16	16
GB0377	16	16	1024	1024	512	512	128	4
GB0390	16	32	512	512	256	128	128	128
GB0397	128	128	512	256	128	128	128	128
GB0411	32	32	512	512	512	256	128	128
GB0418	16	32	128	1024	512	512	512	512
GB0438	16	16	256	256	128	128	64	32
GB0561	64	128	512	512	64	128	128	128
GB0571	16	16	512	256	128	128	64	64
GB0590	32	32	256	256	64	64	64	64
GB0651	32	64	512	1024	64	128	128	128
GB0653	4	8	1024	128	16	16	512	128
GB0654	8	8	256	128	32	32	128	128
GB0663	32	8	64	64	128	128	64	64
GB1445	16	16	128	128	128	128	32	64
A909	8	8	128	128	64	64	64	64
COH1	16	16	1024	1024	128	64	32	16
NEM316	16	16	512	256	32	32	16	32

Table 5. 50S ribosomal subunit targeting antibiotic profile for bovine lactoferrin against GBS

Antibiotics:	Chloramphenicol		Clindamycin		Erythromycin		Puromycin	
Strain Number	MIC (µg/mL)	MIC w/Lf (µg/mL)	MIC (µg/mL)	MIC w/Lf (µg/mL)	MIC (µg/mL)	MIC w/Lf (µg/mL)	MIC (µg/mL)	MIC w/Lf (µg/mL)
GB0002	128	128	0.03125	0.03125	0.125	0.125	8	4
GB0012	128	64	0.03125	0.0625	1	0.5	32	8
GB0037	4	4	0.125	0.125	0.0625	0.0625	4	4
GB0064	16	32	0.0625	0.0625	1	2	32	32
GB0066	16	16	0.03125	0.0625	0.125	0.125	1	2
GB0069	1024	1024	0.5	0.5	2	1	64	64
GB0079	1024	64	0.5	0.5	2	2	64	16
GB0083	32	16	0.0625	0.03125	0.125	0.125	8	8
GB0112	256	256	0.03125	0.03125	0.015625	0.015625	16	32
GB0115	256	256	0.015625	0.0078125	0.015625	0.015625	16	8
GB0241	1024	256	0.5	0.5	1	1	64	128
GB0285	32	32	0.0625	0.0625	0.125	0.125	8	64
GB0291	32	32	0.03125	0.0625	2	2	64	128
GB0374	32	16	0.0625	0.0625	0.125	1	4	8
GB0377	1024	256	0.5	0.5	0.5	0.5	64	8
GB0390	32	32	0.0625	0.0625	2	2	64	64
GB0397	32	32	0.03125	0.0625	0.125	0.125	32	16
GB0411	32	16	0.03125	0.0625	2	2	128	128
GB0418	1024	1024	0.5	0.5	2	1	128	128
GB0438	128	64	0.0625	0.03125	0.125	0.125	4	4
GB0561	16	16	0.0625	0.0625	0.0625	0.0625	2	4
GB0571	32	16	0.125	0.125	0.125	0.125	4	4
GB0590	512	512	0.03125	0.03125	0.0625	0.0625	4	2
GB0651	16	16	0.0625	0.0625	0.5	2	4	4
GB0653	1024	1024	0.015625	0.0078125	0.5	g	4	4
GB0654	16	16	0.03125	0.03125	0.125	0.125	4	4
GB0663	128	32	0.0625	0.0625	0.015625	0.015625	8	8
GB1445	256	16	0.0625	0.0625	0.125	0.125	2	4
A909	16	16	0.0625	0.0625	0.125	0.125	4	4
COH1	1024	16	0.125	0.125	0.125	0.125	0.03125	0.03125
NEM316	16	16	0.0625	0.0625	0.03125	0.03125	8	8

Table 6. Fold reduction in antibiotic minimum inhibitory concentration with bovine lactoferrin treatment against GBS.

Strain Number	Ampicillin	Chloramphenicol	Clindamycin	Erythromycin	Gentamicin	Kanamycin	Puromycin	Spectinomycin	Tetracycline	Vancomycin
GB0002	1	1	1	1	1	1	2	2	0.25	1
GB0012	1	2	0.5	2	1	1	4	1	1	1
GB0037	1	1	1	1	0.5	0.5	1	1	1	1
GB0064	2	0.5	1	0.5	1	1	1	1	1	1
GB0066	1	1	0.5	1	1	2	0.5	1	2	1
GB0069	0.25	1	1	2	1	2	1	1	1	1
GB0079	0.5	16	1	1	0.25	0.25	4	1	1	2
GB0083	1	2	2	1	1	1	1	1	2	1
GB0112	4	1	1	1	0.5	1	0.5	1	4	1
GB0115	1	1	2	1	8	2	2	2	2	2
GB0241	1	4	1	1	0.5	1	0.5	1	0.5	0.5
GB0285	1	1	1	1	1	1	0.125	0.5	2	1
GB0291	0.5	1	0.5	1	1	1	0.5	1	1	0.5
GB0374	1	2	1	0.125	2	1	0.5	1	1	0.5
GB0377	1	4	1	1	1	1	8	1	32	1
GB0390	1	2	1	1	0.5	1	1	2	1	1
GB0397	1	2	0.5	1	1	2	2	1	1	1
GB0411	0.5	1	0.5	1	1	1	1	2	1	1
GB0418	0.5	1	1	2	0.5	0.125	1	1	1	1
GB0438	1	2	2	1	1	1	1	1	2	2
GB0561	1	1	1	1	0.5	1	0.5	0.5	1	1
GB0571	1	2	1	1	1	2	1	1	1	1
GB0590	1	1	1	1	1	1	2	1	1	2
GB0651	1	1	1	0.25	0.5	0.5	1	0.5	1	1
GB0653	1	1	2	16	0.5	8	1	1	4	1
GB0654	1	1	1	1	1	2	1	1	1	1
GB0663	1	4	1	1	4	1	1	1	1	1
GB1445	1	16	1	1	1	1	1	1	0.5	1
A909	1	1	1	1	1	1	1	1	1	1
COH1	1	64	1	1	1	1	1	2	2	2
NEM316	1	1	1	1	1	2	1	1	0.5	2
# Strains Susceptible	2	13	4	4	3	8	7	5	9	6
# Strains Resistant	5	1	5	3	9	4	7	3	4	3

Table 7. Nalidixic acid and polymyxin B antibiotic profile for bovine lactoferrin against GBS

Antibiotics:	Nalidixic Acid		Polymyxin B	
Strain Number	MIC (µg/mL)	MIC w/Lf (µg/mL)	MIC (µg/mL)	MIC w/Lf (µg/mL)
GB0002	256	256	32	32
GB0012	256	256	32	32
GB0037	256	256	32	32
GB0064	256	256	32	32
GB0066	256	256	32	32
GB0069	256	256	32	32
GB0079	256	256	32	32
GB0083	256	256	32	32
GB0112	256	256	32	32
GB0115	256	256	32	32
GB0241	256	256	32	32
GB0285	256	256	32	32
GB0291	256	256	32	32
GB0374	256	256	32	32
GB0377	256	256	32	32
GB0390	256	256	32	32
GB0397	256	256	32	32
GB0411	256	256	32	32
GB0418	256	256	32	32
GB0438	256	256	32	32
GB0561	256	256	32	32
GB0571	256	256	32	32
GB0590	256	256	32	32
GB0651	256	256	32	32
GB0653	256	256	32	32
GB0654	256	256	32	32
GB0663	256	256	32	32
GB1445	256	256	32	32
A909	256	256	32	32
COH1	256	256	32	32
NEM316	256	256	32	32

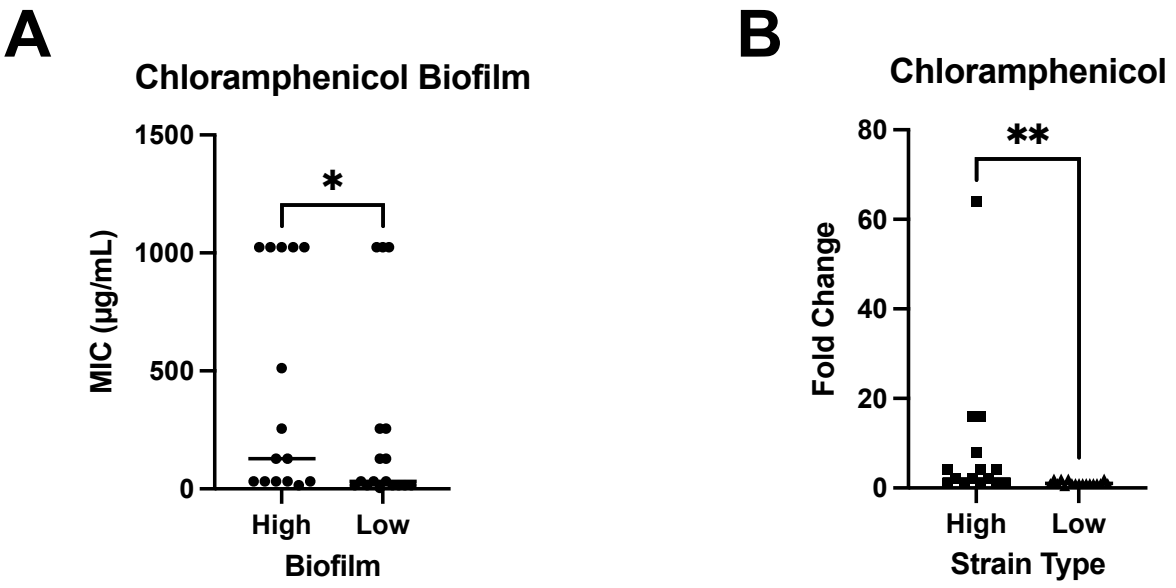

Figure 26. Treatment with lactoferrin inhibits biofilm formation and restores the utility of chloramphenicol against high biofilm forming strains of GBS A) MIC (µg/mL) of chloramphenicol against GBS separated by biofilm strength. Clinical GBS strains that form more biofilms are more protective against chloramphenicol. B) Fold change in chloramphenicol MIC with 500 µg/mL bovine lactoferrin treatment binned by GBS biofilm strength. The chloramphenicol enhancing properties of lactoferrin was more pronounced in high biofilm forming strains as determined by Mann-Whitney U test (*$P < 0.05$, ** $P < 0.01$).

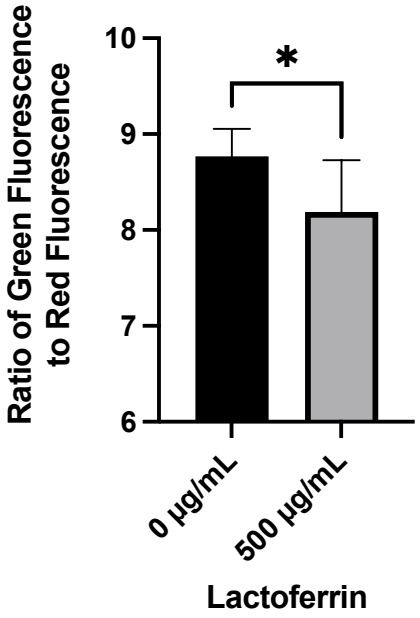

Figure 27. Bovine lactoferrin increases cell-permeability in GBS strain GB0037. LIVE/DEAD BacLight assay revealed that exposure to 500 µg/mL of bovine lactoferrin results in decreased cell integrity as determined by the ratio of green fluorescence (SYTO 9 stain of intact cells) to red fluorescence (PI stain of nonintact cells). *P < 0.05, Student's t test, N = 3.

Table 8. Antibiotic sensitization data of purified human lactoferrin against three GBS strains.

Strain Number	GB0037		GB0112		GB0590	
Antibiotic	MIC (µg/mL)	MIC w/Lf (µg/mL)	MIC (µg/mL)	MIC w/Lf (µg/mL)	MIC (µg/mL)	MIC w/Lf (µg/mL)
Ampicillin	0.0625	0.0625	0.25	0.25	0.125	0.125
Chloramphenicol	4	4	256	256	512	128
Clindamycin	0.125	0.0625	0.03125	0.03125	0.03125	0.03125
Erythromycin	0.0625	0.0625	0.015625	0.015625	0.0625	0.0625
Gentamicin	8	4	32	32	32	16
Kanamycin	128	128	1024	512	256	256
Spectinomycin	64	32	128	64	64	64
Vancomycin	1	1	1	1	1	1

Table 9. Comparison of selective antibiotic minimum inhibitory concentration against three strains of GBS when treated with human or bovine lactoferrin. Values indicate fold-reduction.

Strain Number	GB0037		GB0112		GB0590	
Antibiotic	Human	Bovine	Human	Bovine	Human	Bovine
Ampicillin	1	1	1	4	1	1
Chloramphenicol	1	1	1	1	4	1
Clindamycin	2	1	1	1	1	1
Erythromycin	1	1	1	1	1	1
Gentamicin	2	0.5	1	0.5	2	1
Kanamycin	1	0.5	2	1	1	1
Spectinomycin	2	1	2	1	1	1
Vancomycin	1	1	1	1	1	2

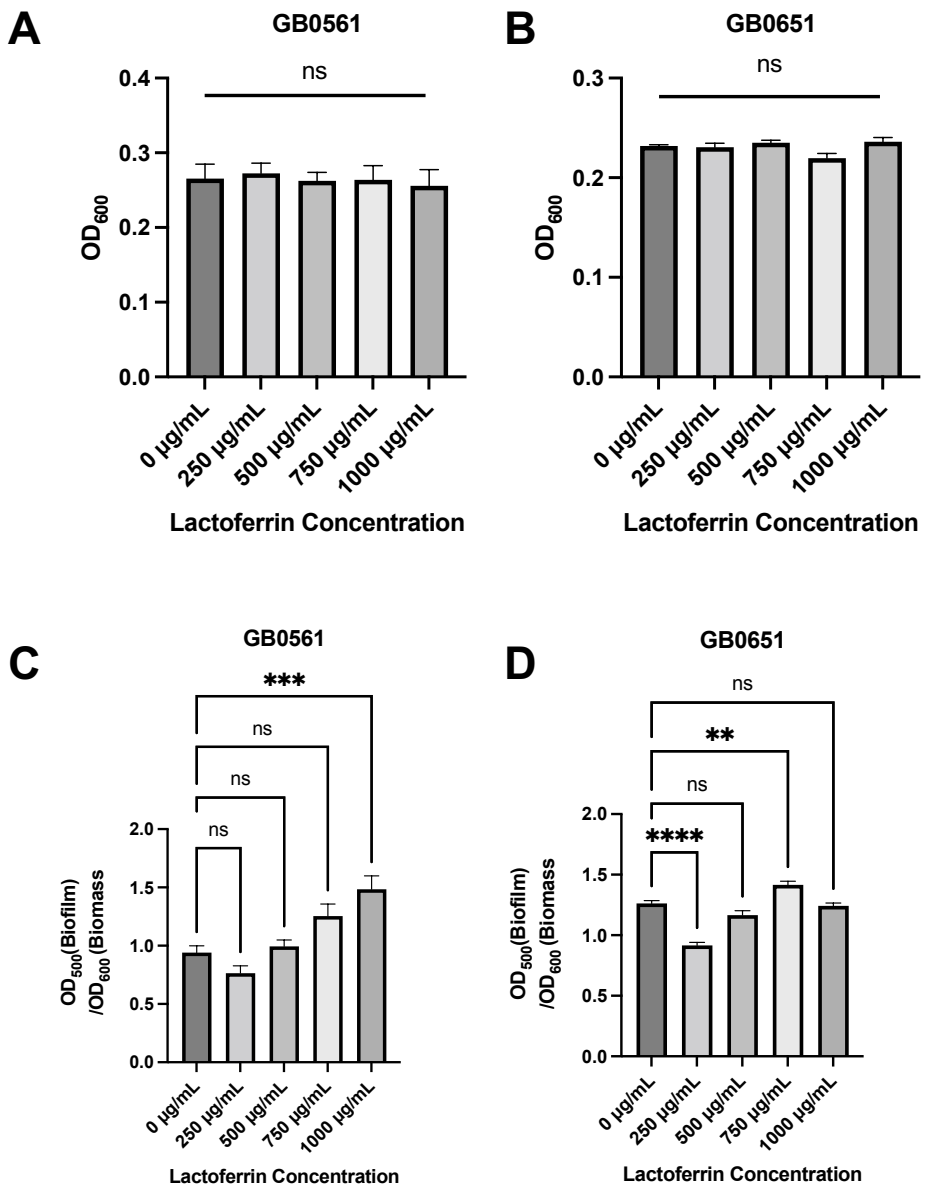

Figure 28. Apo-lactoferrin treatment induced biofilm formation in two clinical strains resistant to the antibiotic enhancing property of the antimicrobial peptide. A and B) GB0561 and GB651 is resistant to the antimicrobial properties of bovine lactoferrin as assessed by optical density. C and D) Lactoferrin did not prevent biofilm formation in the two resistant strains. Bacterial biofilm was analyzed by quantitative analysis of Crystal Violet staining at OD_{560} normalized to bacterial cell density (OD_{600}). Bars indicate mean values of at least three biological replicates +/- standard error of the mean. **P<0.01, ***P<0.001, ****P<0.0001. One-way ANOVA, post hoc Dunnett's test comparison to bacteria grown in medium alone.

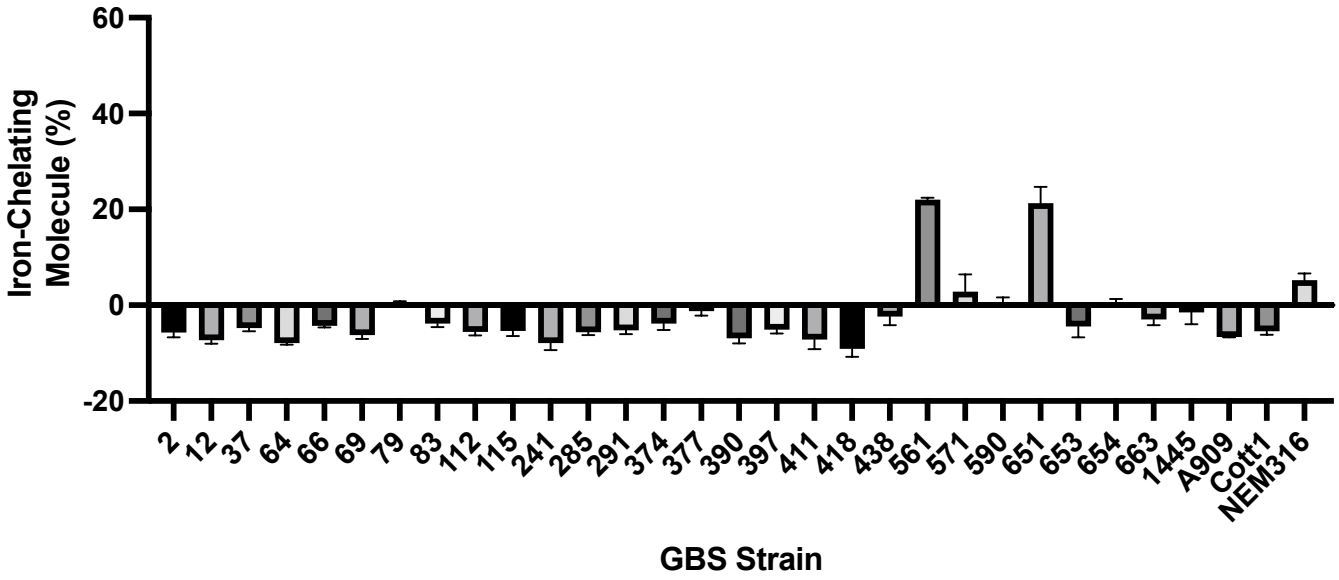

Figure 29. Two GBS strains resistant to the antibiotic enhancing properties of lactoferrin produces iron-chelating molecule GB561 and GB651 produces some iron-chelating molecules to overcome lactoferrin mediated iron-limitation as determined by a microplate CAS assay. One-way ANOVA, ****P < 0.0001, N = 3.

Table 10. Select Antibiotic Sensitization Data for Bovine Lactoferrin against Bacterial Pathogens

Bacteria	Antibiotics	MIC (µg/mL)	MIC w/ Lf (µg/mL)	Fold Reduction
USA300 (*Staphylococcus aureus*)	Clindamycin	32	16	2
PA14 (*Pseudomonas aeruginosa)*	Erythromycin	256	64	4
	Gentamicin	0.5	0.25	2
	Nalidixic Acid	512	256	2
	Polymyxin B	8	4	2
	Tetracycline	64	32	2
11775 (*Escherichia coli*)	Gentamicin	2	1	2
	Kanamycin	32	16	2
	Polymyxin B	2	1	2
	Tetracycline	2	1	2
19606 (*Acinetobacter baumannii*)	Chloramphenicol	128	64	2
	Polymyxin B	2	0.25	8
	Vancomycin	1024	512	2

CONCLUSIONS

There is a gap of knowledge between GBS and its interaction with the human host. In my research, I sought out to expand the relationship between our innate immune response and the bacterium. The field has studied the antimicrobial effects against a range of bacterial pathogens. Furthermore, there is evidence that lactoferrin can interact with the immune system to modulate inflammation. With this knowledge in mind, I hypothesized that lactoferrin possesses antimicrobial and antibiofilm against GBS. Additionally, I hypothesized that lactoferrin can interact with resident PMs and dampen their response during GBS infection to provide an environmental suitable for fetal development while combatting the pathogen. To address this gap in knowledge, my thesis research aimed to (I) review the current understanding of lactoferrin and streptococcal infection, (II) define the antimicrobial and antibiofilm properties of lactoferrin against GBS, (III) determine if these properties are broadly potent against a clinical bank of GBS strains, (IV) understand how lactoferrin interactions with PMs to dampen their function, (V) explore the antimicrobial peptide's protective properties against the ESKAPE pathogens, and finally (VI) assess the utility of lactoferrin to enhance antibiotic efficacy. My work has defined an aspect of the host-pathogen interaction and propose purified human lactoferrin as a chemotherapeutic strategy against GBS and other bacterial infections.

Lactoferrin possesses antimicrobial and antibiofilm properties against GBS is an iron dependent manner

GBS is an encapsulated Gram-positive human pathogen that causes invasive infections in pregnant hosts and neonates, as well as immunocompromised individuals. Colonization of the human host requires the ability to adhere to mucosal surfaces and circumnavigate the nutritional challenges and antimicrobial defenses associated with the innate immune response. Biofilm formation is a critical process to facilitate GBS survival and establishment of a replicative niche in the vertebrate host. Previous work has shown that the host responds to GBS infection by producing the innate antimicrobial glycoprotein lactoferrin, which has been implicated in repressing bacterial growth and biofilm formation.

Additionally, lactoferrin is highly abundant in human breast milk and could serve a protective role against invasive microbial pathogens. This study presented in chapter II demonstrated that human breast milk lactoferrin has antimicrobial and anti-biofilm activity against GBS and inhibits its adherence to human gestational membranes. Together, these results indicate that human milk lactoferrin could be used as a prebiotic chemotherapeutic strategy to limit the impact of bacterial adherence and biofilm formation on GBS-associated disease outcomes.

Lactoferrin is antimicrobial and reduce biofilm formation against a clinical bank of GBS strains

In Chapter II, I demonstrated that lactoferrin possesses antimicrobial and antibiofilm properties against several strains of GBS. This is largely due to the ability of lactoferrin to bind and sequester iron. We expanded upon that study by assessing the effects of purified human breast milk lactoferrin against a panel of phenotypically and genetically diverse isolates of GBS in Chapter III of this dissertation. Of the 25 GBS isolates screened, lactoferrin reduced bacterial growth in 14 and biofilm formation in 21 strains. Stratifying the data, we observed that colonizing strains were more susceptible to the growth inhibition activity of lactoferrin than invasive isolates at lactoferrin concentrations between 250-750 μg/mL. Treatment with 750 μg/mL of lactoferrin resulted in differences in bacterial growth and biofilm formation between discrete sequence types. Differences in bacterial growth were also observed between capsular serotypes 1a and III. Maternally isolated strains were more susceptible to lactoferrin with respect to bacterial growth, but not biofilm formation, compared to neonatal sepsis isolates. Finally, high biofilm forming GBS strains were more impacted by lactoferrin across all isolates tested. Taken together, this study demonstrates that lactoferrin possesses antimicrobial and antibiofilm properties against a wide range of GBS isolates, with maternally isolated colonizing strains being the most susceptible.

Lactoferrin interactions with placental macrophages to reduce reactive oxygen species and proinflammatory cytokine production during GBS infection

GBS is one of the leading bacterial-related causes of adverse pregnancy and neonatal outcomes. For the mother to support the developing fetus, they must maintain an immune-tolerant environment. Infection of the fetal tissues triggers inflammation that may result in these adverse pregnancy outcomes. Lactoferrin is an antimicrobial peptide delivered by the recruitment of neutrophils following infection. While its antimicrobial properties have been studies, there has been a gaining interest in its immune-modulatory action. In Chapter IV, I studied how lactoferrin interacts with PMs to alter cellular function. Lactoferrin treatment by reduce the PM's capability to kill GBS. This may have been a result of reduction of intracellular reactive oxygen species production. Lactoferrin treatment resulted in the rescue of the survival defect phenotype in the GBS *npx* mutant, which is hypersensitive to reactive oxygen stress. Treatment with lactoferrin also resulted in the reduced production of proinflammatory cytokines including CXCL-1, IL-1α, IL-1 β, IL-6, IL-8, and TNF- α. Taken together, lactoferrin transported by neutrophils may serve as a signal to dampen the immune response in order to clear the pathogen while maintaining an immune-tolerant environment for the developing fetus.

The utilize of lactoferrin as an adjuvant to improve antibiotic efficacy

Lactoferrin has been the interest for many groups to investigate its therapeutic potential. Patras *et al*. found that treatment with exogenous lactoferrin resulted in reduced adherence to epithelial cells by Uropathogenic *Escherichia coli*. Furthermore, lactoferrin enhanced effector functions of neutrophils, such as bacterial killing and production of neutrophil extracellular traps (140). Other groups have used transferrin, another iron chelating antimicrobial peptide, in combination with antibiotics to overcome resistance (142,143). As lactoferrin shares properties with transferrin, human lactoferrin should be investigated as an adjuvant to improve the efficacy of a range of antibiotics against GBS. Biofilms are play an important role in the defense against antibiotic assault (307). In chapter VI, I revealed that bovine lactoferrin possess antibiotic-enhancing activity against GBS. Lactoferrin was implicated in

sensitive high biofilm forming GBS strains to chloramphenicol. Two strains were resistant to the antibiotic-enhancing property of lactoferrin; both strains produce some iron-chelating molecules and is resistant to the antimicrobial and anti-biofilm properties of lactoferrin. Human lactoferrin was able to enhance some antibiotics that were not improved with lactoferrin co-treatment. Finally, lactoferrin was also enhance antibiotic efficacy against some invasive pathogens.

Future Directions

In the research I completed and presented in this dissertation, I have demonstrated that lactoferrin may be a viable chemotherapeutic strategy against GBS and other bacterial infections. However, further investigation into the biology behind this host-microbe interaction is warranted. I have completed *in vitro* and *ex vivo* groundwork and I propose the following research direction for the future.

Confirmation of iron-dependent antimicrobial activity by lactoferrin

From my data presented in Chapter II, I demonstrated that human breast milk lactoferrin is largely in the apo-form. As we witnessed the strongest phenotype in the apo-form against GBS growth and biofilm formation, this is suggestive of an iron chelation-dependent mechanism. I also witnessed similar bacterial growth and biofilm suppression by apo-lactoferrin against many of the ESKAPE pathogens. To fully confirm that this antimicrobial activity is iron-dependent, I propose two experiments. In this first experiment, similar assays would be performed but exogenous iron will be added to the environment alongside apo-lactoferrin. If the phenotype I described it truly iron dependent, I would expect the growth and biofilm phenotypes in this condition to mirror closely to holo-lactoferrin treatment. A more intensive experiment would involve site-directed mutagenesis to the iron binding domain to ablate the antimicrobial peptide's ability to chelate iron. As this mutated lactoferrin should not bind iron, I would perform ICP-MS to confirm its inability to bind iron. An alternate approach is to utilize isothermal titration calorimetry (ITC). Finally, I would repeat the assays showcased in Chapter II and I hypothesized that this mutated form will not display strong antimicrobial and antibiofilm properties against GBS and other bacterial pathogens.

Understanding the link between lactoferrin SNPs in the human population and preterm birth

Preterm deliveries are defined by those that occur at less than 37 week's gestational age, but the cutoff varies by location (144). The preterm delivery rate in the USA was around 12-13% in 2008; in Europe and other developed countries, the preterm delivery rates is between 5-9% (308). The most recent estimate for preterm delivery in the United States is one in every ten pregnancies (309). Despite advancing knowledge of risk factors and mechanisms relating to preterm births and introduction of many public health and medical interventions to reduce the occurrence of preterm births, the rate in the USA and other developed countries still hovers around 10% (310). Preterm births account for 75% of perinatal mortality and over 50% of the long-term morbidity (311). While most preterm babies survive, they are at an increased risk of neurodevelopmental impairments as well as respiratory and gastrointestinal complications (312). Microbiology studies have revealed that intrauterine infection, including GBS, may account for 25-40% of preterm births and the number is likely an underestimate (144).

Polymorphisms of the lactoferrin gene on chromosome three exists in our global population. A single nucleotide polymorphism (rs1126478) at amino acid position 47 is of particular interest. At this position, the arginine is substituted by a lysine (86). The K47 allele is only found at 1% frequency in individuals with African ancestry. In contrast, this variant is found at roughly 30-65% allele frequency in non-African populations, with the greatest frequency observed among Europeans. Globally, Black women at higher risk for preterm birth (144). In the USA and the UK, women who are classified as Black, African American, and Afro-Caribbean are consistently reported to have the higher risk of preterm delivery. Black women exhibit preterm rates in the range of 16-18% while their white counterparts exhibit preterm delivery rates of 5-9% (313). Additionally, Black women are three to four times more likely to have a very early preterm delivery compared to women from any other racial or ethnic groups (314). Given that majority of women of African ancestry are at a higher risk for preterm birth and many of them carry this allele, it is plausible that this SNP associated with increased microbial

susceptibility, such as to GBS, contributing to higher likelihood of preterm delivery. With the findings from such epidemiology studies, I hope to properly address the health disparities in Black mothers. One potential way to address these questions is to utilize electronic health record data and sequence banked DNA samples from biobanks such as BioVU at Vanderbilt University.

Evaluate the contribution of lactoferrin to immune response, ascending infection, and disease outcome of GBS infection in gravid mice

To investigate the role of lactoferrin in GBS ascending infection *in vivo*, I will propose the use an established mouse model of ascending infection (208,315). C57BL6/J WT or global lactoferrin-deficient (*Ltf*$^{-/-}$) mice (Jackson Laboratories) aged 8-11 weeks will be mated in harem overnight. Mice will be checked daily, and pregnancy will be confirmed by the presence of a mucus plug to establish the embryonic date. On embryonic day 13, pregnant mice will be anesthetized and infected intravaginally with WT GB112 and uninfected controls will also be maintained. Mice will be placed into the following treatment groups; untreated or lactoferrin treated. Lactoferrin will be administered intravaginally on the same day as infection. On embryonic day 15, mice will be sacrificed, and the following tissues will be isolated: uterus, placenta, fetuses, decidua, amnion, and vagina. Prior to dissecting tissues, the number of fetal reabsorptions will be assessed for pregnancy quality. Additionally, maternal blood and amniotic fluid will be collected. To assess ascending infection, each tissue will be homogenized, and bacterial burden will be quantified by plating serial dilutions on blood agar plates. To analyze the histopathology of each treatment group, tissue will be stained with hemolysin and eosin (H&E) staining and immunohistochemistry for GBS. The slides will be scored by a pathologist. To characterize the immune response, multiplex cytokine analysis on the homogenized tissue samples will be performed. Additionally, flow cytometry will be used with markers for immune cells involved in the response against GBS, such as macrophages (i.e. F4/80) and neutrophils (i.e. Ly6G), to assess changes in cell populations. Finally, tissue samples will be fixed and prepared for SEM to assess biofilms. I hypothesize

that lactoferrin-deficient animals will have severe adverse pregnancy outcomes associated with increased inflammation. These sets of experiments will illuminate the role of lactoferrin *in vivo*.

Determine the contribution of lactoferrin supplementation to GBS infection and disease progression a mouse model of ascending infection during pregnancy

In my graduate research, I have investigated the antimicrobial and antibiofilm properties of lactoferrin, with the ultimate goal of utilizing purified human breast milk lactoferrin as a chemotherapeutic strategy to combat GBS infection. To investigate the use of purified lactoferrin as a therapeutic treatment to treat GBS ascending infection, an established mouse model of ascending infection should be utilized (208). C57BL6/J WT 8-11 weeks will be mated in harem overnight. Mice will be checked daily, and pregnancy will be confirmed by the presence of a mucus plug to establish the embryonic date. On embryonic day 13, pregnant mice will be anesthetized and infected intravaginally with WT GB112 and uninfected controls will also be maintained. Mice will be placed into the following treatment groups; untreated or lactoferrin treated. 250-750 μg/mL of purified lactoferrin will be administered intravaginally on the day before or after infection. This range of concentrations exhibit antimicrobial effects against panel of GBS strains with genetic diversity, as described in chapter III. A potential potent dose is 250-750 ug/mL, a dose that is well beneath the range that has been utilized for human patients with reproductive infections such as refractory bacterial vaginosis (316). On embryonic day 15, mice will be euthanized, and the uterus, placenta, fetuses, decidua, amnion, and vagina will be dissected and isolated. Prior to dissecting tissues, the number of fetal resorptions will be counted to assess intrauterine fetal loss. Additionally, maternal blood and amniotic fluid will also be collected. To assess ascending infection, each tissue will be homogenized, and bacterial burden will be quantified by plating serial dilutions on blood agar plates. Multiplex cytokine analysis will be performed to evaluate the immunological responses within these tissue homogenates. This panel will include the hallmark proinflammatory cytokines expressed during bacterial infections, including IL1β, IL-6, IL-8, and TNFα. Additionally, flow cytometry will be used with markers for immune cells involved in the response against

GBS, such as macrophages (i.e. F4/80) and neutrophils (i.e. Ly6G), to assess changes in cell populations.

From this set of proposed experiments, I expect the utilization of lactoferrin will impede ascending infection, resulting in lower bacterial burdens in other reproductive tissues and improved disease outcome. One pitfall may be the timing of lactoferrin application. Currently, I propose administration of lactoferrin post-GBS infection, mirror human treatment and support clinical relevance. Rather than treating with lactoferrin after infection, mice may be treated with lactoferrin prior to infection to investigate if lactoferrin can be used to prevent establishment of infection. An alternative method for measuring bacterial burden is to perform qPCR on each reproductive tissue and using primers specifically for GBS.

I further hypothesize that animals treated with lactoferrin will display lower production of proinflammatory cytokines. Additionally, I predict that tissue from lactoferrin treated animals will display less PMN influx and inflammation will score lower. The cell population analysis with flow cytometry should reveal less immune infiltrate in lactoferrin-supplemented animals. Specifically, lactoferrin treated animals will have less inflammation in the fetal membrane, which would be indicative of decreased chorioamnionitis, a common outcome of GBS infection, as the inflammation compromises the integrity of the membrane and results in preterm birth. As such, it is possible that lactoferrin can protect against preterm birth as well. A pitfall of this experiment is that mice are sacrificed prior to birthing. To properly investigate if lactoferrin can protect against preterm birth, time of birth and weight of each pup should be recorded. I hypothesized GBS infected mice will give birth closer to term when supplemented with lactoferrin. Finally, the mode of lactoferrin administration may sway the outcome. Currently, lactoferrin will be supplemented intravaginally but alternative modes of introduction to investigate include orally and intravenously. Taken together, this set of experiments is the logical next step to determine if purified lactoferrin is a strong candidate to prevent GBS ascending infection.

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