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

Jacky Lu[1], Miriam A. Guevara[1], Jamisha D. Francis[1], Sabrina K. Spicer[2], Rebecca E. Moore[2], Schuyler A. Chambers[2], Kelly M. Craft[3], Shannon D. Manning[4], Steven D. Townsend[2*], Jennifer A. Gaddy[1,5,6*]

[1]Department of Pathology, Microbiology, and Immunology, Vanderbilt University School of Medicine, Nashville, Tennessee, U.S.A.

[2]Department of Chemistry, Vanderbilt University, Nashville, Tennessee, U.S.A.

[3]Department of Chemistry, Harvard University, Cambridge, Massachusetts, U.S.A.

[4]Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, Michigan, U.S.A.

[5]Division of Infectious Diseases, Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee, U.S.A.

[6]Tennessee Valley Healthcare Systems, Department of Veterans Affairs, Nashville, Tennessee, U.S.A.

Corresponding Author Contact Information

Address correspondence to Jennifer A. Gaddy, Ph.D., jennifer.a.gaddy@vanderbilt.edu

Vanderbilt University Medical Center

Department of Medicine

Division of Infectious Diseases

A2200 Medical Center North

1161 21st Avenue South

Nashville, Tennessee, U.S.A. 37232

Telephone: (615)-873-7884

Fax: (615) 343-6160

-or-

Steven D. Townsend, Ph.D., steven.d.townsend@vanderbilt.edu

Vanderbilt University

Department of Chemistry

Stevenson Center

Nashville, Tennessee, U.S.A. 37232

Telephone: (615) 322-8171

Keywords: biofilm, lactoferrin, *Streptococcus agalactiae*, nutritional immunity, antimicrobial

Abstract

Background: Group B *Streptococcus* (GBS) is one of the leading infection-related causes of adverse maternal and neonatal outcomes. This includes chorioamnionitis, which leads to preterm ruptures of membranes and can ultimately result in preterm or stillbirth. Infection can also lead to maternal and neonatal sepsis that may contribute to mortality. Currently, treatment for GBS infection include a bolus of intrapartum antibiotic prophylaxis to mothers testing positive for GBS colonization during late pregnancy. Lactoferrin is an antimicrobial peptide expressed in human breast milk, mucosal epithelia, and secondary granules of neutrophils. **Methodology:** We previously 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. **Results:** Of the 25 GBS isolates screened, lactoferrin reduced bacterial growth in 14 and biofilm formation in 19 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 cps1a and cpsIII capsular serotypes. 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.

Introduction

Streptococcus agalactiae, more commonly known as Group B *Streptococcus* (GBS), is amongst the leading infection-related causes of adverse pregnancy and neonatal outcomes [1]. Adverse maternal complications include chorioamnionitis, preterm premature rupture of membranes (PPROM), preterm birth, stillbirth, and maternal sepsis [2, 3]. For the newborn, GBS infections can result in early- and late- onset neonatal sepsis, meningitis, and endocarditis. Early onset disease (EOD) occurs in neonates up to a week after birth [4]. 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 sepsis and meningitis. Newborns who survive LOD frequently suffer from neurodevelopmental impairments [5].

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 [4]. 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 its ability to form biofilms [7]. Colonization rates differ worldwide, spanning between 6.5-36% [1]. Some recent reports include colonization rates of 13.2% in a cohort in Ethiopia [8], 16.6% in the Western Cape region of South Africa [9], and 21.6% over a twelve-year span in North Carolina, USA [10].

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 [11]. The streptococcal polysaccharide capsule facilitates evasion of the innate immune response by protecting the bacterial cell from deposition of complement, opsonization, and phagocytosis [12–14]. A recent study from our laboratory revealed that the GBS capsule aids in biofilm formation and ascending infection of the reproductive tract during pregnancy [15]. 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 that serve to dampen inflammatory responses [16]. Different capsular serotypes result in different range and severity in human disease. For instance, capsular serotype III strains are associated with higher rates of invasive neonatal disease [17] and account for the majority of late-onset meningitis cases in neonates [18]. In contrast, serotype Ia and V are dominant invasive isolates in non-pregnant cases [19]. However, dominate serotype fluctuates between regions and across time [1].

Another method by which GBS strains are grouped and characterized is multi-locus sequence typing (MLST) or Sequence Types (STs). Several STs are grouped into clonal complexes (CC) when sharing six or seven matching alleles. The majority of human GBS isolates can be grouped into CC1, CC10, CC17, CC19, CC23, and CC26 [20]. Similar to capsular type, sequence type diversity also manifests in different disease outcomes and severities. For instance, STs 1 and 19 are significantly more associated

with asymptomatic colonization and ST-23 was common for carriage and invasive GBS and all three ST-types predominately colonize pregnant women at higher rates [21]. ST17 serotype III, ST23, and ST-24 strains are linked to EOD [22, 23] while ST17 serotype III is strongly associated with meningitis-prone LOD [22, 24, 25].

One method the immune systems uses to combat GBS is the deployment of 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 [26]. Bacteria require these trace elements as cofactors for essential biological processes. One of these proteins expressed in defense against GBS is lactoferrin [27, 28]. Lactoferrin is a glycoprotein that contains two iron binding sites [29] and has shown to have antimicrobial activity against a wide range of bacterial, viral, and fungal pathogens [27]. Our previous study 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 [30]. In this study, we advanced our findings by analyzing the antimicrobial and anti-biofilm effects of lactoferrin against a larger panel of clinical GBS isolates with diversity in strain type, capsular serotype, sequence type, isolation source, and clinical presentation. We observed broad antimicrobial and antibiofilm action by lactoferrin against the majority of the panel screened. In particular, maternal colonizing strains are more susceptible to lactoferrin compared to neonatal invasive strains.

Materials and Methods

Bacterial strains and culture conditions

This study utilized 25 previously characterized *S. agalactiae* strains, which were isolated from neonates with invasive disease [31] or colonized mothers before or after childbirth [32] by Dr. Shannon Manning's laboratory. 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 [30]. 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 this study was in the apo-form.

Evaluation of bacterial growth

Bacterial growth was determined by a spectrophometric reading as previously described [30]. Briefly, Optical density measurements at 600 nm (OD_{600}) were recorded to determined 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 [33, 34])). 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 [30, 35]. 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. 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. Statistical analyses were performed using GraphPad Prism 6 software (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 [30]. In order to expand our study, we increased the number of GBS strains, thereby capturing more isolates across diverse capsular serotypes and genetic sequence types. 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, the differences were not statistically significant.

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

Our group described the iron-dependent anti-biofilm properties of lactoferrin against a limited number of GBS strains. Here, we sought to expand the number of GBS strains to investigate if lactoferrin can suppress other strains with diverse genetic backgrounds. 25 strains were assayed, and 19 strains exhibited a 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. Of all the strains screened, four showed no differences 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. At 250 μg/mL, colonizing strains were more susceptible to lactoferrin compared to invasive strains in respect to bacterial growth (12.21% vs 1.76% mean reduction, respectively) (**Figure 1**; $P < 0.05$, Student's t-test). At 500 μg/mL, lactoferrin better suppressed bacterial growth in colonizing strains compared to invasive ones (28.14% vs 17.48% mean reduction, respectively) (**Figure 1**; $P < 0.01$, Student's t-test). 750 μg/mL of lactoferrin decreased bacterial growth for both types of strains but invasive strains were more resistant compared to colonizing ones (20.71% vs 33.17% mean reduction, respectively) (**Figure 1**; $P < 0.01$, 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, lactoferrin at 250 μg/mL inhibited biofilm formation in colonizing strains more than invasive ones (33.25% vs 22.01% mean reduction, respectively) (**Figure 2**; $P < 0.05$, Student's t-test).

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

Differences in GBS sequence type (ST) can result in different disease outcomes. As a result, it is possible that different STs may have variable mechanisms to cope with iron starvation. To investigate this possibility, GBS strains were binned by ST and susceptibility to lactoferrin between STs was analyzed. 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, treatment with 750 μg/mL of lactoferrin revealed that ST-1 strains were more resistant to bacterial growth suppression compared to ST-12 strains (**Figure 3**; $P < 0.05$, One-way ANOVA; *post hoc* Tukey's test). Similarly, differences in biofilm suppression between STs manifested at treatment with 750 μg/mL of lactoferrin. At this concentration, ST-17 strains were more resistant to the antibiofilm activity of lactoferrin compared to ST-19 (**Figure 4**; $P < 0.01$, One-way ANOVA; *post hoc* Tukey's test) and ST-23 (**Figure 4**; $P < 0.05$, One-way ANOVA; *post hoc* Tukey's test) isolates.

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

The capsule of GBS plays an important role in pathogenesis in humans [36]. As different capsular types can be correlated to varying disease outcome, it is plausible that capsular type may influence resistance to innate antimicrobial peptides. No differences in resistance against the antimicrobial activity of lactoferrin was observed between capsular types across increasing concentrations of lactoferrin (**Figure 5**; $P > 0.05$, One-way ANOVA; post hoc Tukey's test). However, capsular type III strains exhibited resistance to the antibiofilm activity of lactoferrin at 250 μg/mL, compared to capsular type 1a isolates

(**Figure 6**; $P < 0.05$, One-way ANOVA; post hoc Tukey's test). This phenotype was ablated with the additional stress imposed by increasing concentrations of lactoferrin.

Colonizing maternal GBS strains were more susceptible to the antimicrobial, but not anti-biofilm, properties of lactoferrin compared to invasive neonate isolates

Our panel of GBS strains can be separated from isolation source, which is linked to disease outcome. Each was either isolated from a rectal/vaginal swab from the mother or the blood of an invaded neonate with bacterial sepsis. Maternal rectal/vaginal strains were more susceptible to growth inhibition by lactoferrin at 250 µg/mL (**Figure 7**; $P < 0.05$, Student's t-test). Similar differences were also observed at 500 µg/mL (**Figure 7**; $P < 0.01$, Student's t-test) and 750 µg/mL (**Figure 7**; $P < 0.01$, Student's t-test). Bacterial growth of both strain types was equally affected by lactoferrin at 1,000 µg/mL. No differences in anti-biofilm resistance were observed between strains grouped by isolation source/disease outcome (**Figure 8**; $P > 0.05$, Student's t-test).

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

There is a range of biofilm production across GBS strains [37]. To fully probe the anti-biofilm feature of lactoferrin, we determined 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 9**; P

< 0.0001, Student's t-test; P < 0.001, Student's t-test, respectively). At 500 µg/mL of lactoferrin, only high biofilm formers were susceptible to the antimicrobial peptide (**Figure 9**; P < 0.0001, Student's t-test). When treated with 750 µg/mL of lactoferrin, high biofilm formers exhibited a decrease in biofilm formation (**Figure 9**; P < 0.05, Student's t-test). However, low biofilm formers (mean = 0.3183) increased biofilm production upon treatment of 750 µg/mL (mean = 0.3501) (**Figure 9**; 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) (**Figure 9**, P < 0.001, Student's t-test).

Discussion

Colonization of the maternal vaginal canal is one of the leading risk factors for GBS disease [38]. 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 [39]. Currently in the United States, pregnant individuals are screened for the presence of GBS between 35 and 37 weeks of gestation [40]. If a patient tests positive for GBS, intrapartum antibiotic prophylaxis (IAP) is administered during labor and delivery [41]. Though antibiotic treatment is the only current preventative strategy available, the efficacy of IAP against early onset neonatal disease is around 80% [42]. Despite available treatment, the rates of late-onset disease continue to rise [43]. There has been recent evidence for drawbacks with the use of IAP [44], including ineffectiveness in preventing late-onset GBS disease, hypersensitivities to first-line antibiotics [45], alteration of the neonatal microbiota [46], and emergence of antibiotic resistant strains [47]; and discovery of alternative therapies will overcome these IAP drawbacks. In our study, we found that colonizing maternal strains are especially susceptible to lactoferrin, which may suggest that the antimicrobial peptide may be a viable candidate to aid in the prevention of GBS disease.

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 [48]. Furthermore, other groups have identified lactoferrin as a critical component of cervicomucosal defense against a variety of lower genital tract infections caused by

Neisseria gonorrhoeae, *Chlamydia trachomatis*, and *Trichomonas vaginalis* [49]. One plausible explanation for this phenomenon may be that lactoferrin protects by repressing *Gardnerella*, *Prevotella*, and *Lachnospira* species within the host microbiome, thus decreasing competition for *Lactobacillus* species and allowing them to prevent dysbiosis by dominating the vaginal flora [50]. Adding credence to this possibility is that *Lactobacillus* species have been recognized as one of the few bacteria that lack a strict nutrient requirement for iron [51]. Thus, it is plausible that the antimicrobial activity of lactoferrin associated with its role in nutritional immunity would be largely ineffective against these important commensals. There is merit in studying the use of human lactoferrin 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 indicate that certain sequence types are more susceptible than others at 750 μg/mL of lactoferrin treatment. The multi-locus sequence typing (MLST) system uses seven loci that encode enzymes, or housekeeping genes, involved in intermediary metabolism to distinguish GBS sequence types [21]. Iron is an important cofactor for many enzymes involved in bacterial metabolism and physiology [52]. In fact, lactoferrin defends against many 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 [53]. 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.

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 [54], 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 [15], further bolstering our findings. It is plausible that iron starvation alters capsule-mediated biofilm formation in certain capsular types of GBS.

In our study, we further analyzed differences in biofilm inhibition by lactoferrin between strains that form robust biofilms (high biofilm) compared to those that do not (low biofilm). Lactoferrin inhibited biofilm formation in high biofilm forming isolates, which is consistent to with our previous study [30]. The intersection between iron and biofilm formation has also been studied in other bacterial pathogens. One study of interest by Trappetti and collagues described the role of the mononuclear iron protein S-ribosylhomocysteine lyase (LuxS) in quorum sensing and biofilm formation in *Streptococcus pneumoniae* [55]. 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.

In this present study, we expanded upon our previous work 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 discovered 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.

Declarations

Ethics approval to carry out this study was provided by the Vanderbilt University Institutional Review Board (IRB #100897 for human milk donation).

Acknowledgments

This work was funded by the National Institutes of Health grants HD090061 (to J.A.G. which supported the reagents for wet-bench work) from the National Institutes of Child Health and Human Development, 2T32HL007411-39 (to J.L), 2T32AI112541-06 (to J.F), which supported their time and efforts, XXX (to S.D.T.) which supported reagents for wet bench work. Additional support was provided by the Vanderbilt Institute for Clinical and Translational Research pro-gram supported by the National Center for Research Resources, Grant UL1 RR024975-01, and the National Center for Advancing Translational Sciences, Grant 2 UL1 TR000445-06 which supports access to Core Facilities and Biostatistics support.

Competing Interests

The authors declare no conflicts of interest. The authors declare no competing interests.

Author Contributions

KC, JL, and ST purified lactoferrin for the studies. JL performed bacterial culture experiments. SM curated and validated the clinical strains for this study. JL, JF, MG, SC, RM, SS, KC, ST, and JG conceptualized and analyzed results and interpreted data, and

wrote and edited the manuscript for critical content. All authors have read and approved the manuscript and have given their consent to publish this work.

Availability of Data and Materials

The datasets used and/or analyzed during the current study available from the corresponding authors upon reasonable request.

References:

1. Shabayek S, Spellerberg B. Group B Streptococcal Colonization, Molecular Characteristics, and Epidemiology. Front Microbiol. 2018;9:437.

2. Koumans EHA, Rosen J, van Dyke MK, Zell E, Phares CR, Taylor A, et al. Prevention of mother-to-child transmission of infections during pregnancy: implementation of recommended interventions, United States, 2003-2004. Am J Obstet Gynecol. 2012;206:158.e1-158.e11.

3. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. Lancet. 2008;371:75–84. doi:10.1016/S0140-6736(08)60074-4.

4. Verani JR, McGee L, Schrag SJ, Division of Bacterial Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention (CDC). Prevention of perinatal group B streptococcal disease--revised guidelines from CDC, 2010. MMWR Recomm reports Morb Mortal Wkly report Recomm reports. 2010;59 RR-10:1–36. http://www.ncbi.nlm.nih.gov/pubmed/21088663. Accessed 9 Apr 2018.

5. Russell NJ, Seale AC, O'Sullivan C, Le Doare K, Heath PT, Lawn JE, et al. Risk of Early-Onset Neonatal Group B Streptococcal Disease With Maternal Colonization Worldwide: Systematic Review and Meta-analyses. Clin Infect Dis an Off Publ Infect Dis Soc Am. 2017;65 suppl_2:S152–9.

6. Seale AC, Blencowe H, Bianchi-Jassir F, Embleton N, Bassat Q, Ordi J, et al. Stillbirth With Group B Streptococcus Disease Worldwide: Systematic Review and Meta-analyses. Clin Infect Dis an Off Publ Infect Dis Soc Am. 2017;65 suppl_2:S125–32.

7. Rosini R, Margarit I. Biofilm formation by Streptococcus agalactiae: influence of environmental conditions and implicated virulence factors. Front Cell Infect Microbiol.

2015;5:6.

8. Ali MM, Asrat D, Fenta DA, Chaka TE, Woldeamanuel Y. Group B Streptococcus colonization rate and serotype distribution among pregnant women and their newborns at Adama Hospital Medical College, Ethiopia. Sci Rep. 2020;10:9301.

9. Africa CWJ, Kaambo E. Group B Streptococcus Serotypes in Pregnant Women From the Western Cape Region of South Africa. Front public Heal. 2018;6:356.

10. Edwards JM, Watson N, Focht C, Wynn C, Todd CA, Walter EB, et al. Group B Streptococcus (GBS) Colonization and Disease among Pregnant Women: A Historical Cohort Study. Infect Dis Obstet Gynecol. 2019;2019:5430493.

11. Teatero S, Ferrieri P, Martin I, Demczuk W, McGeer A, Fittipaldi N. Serotype Distribution, Population Structure, and Antimicrobial Resistance of Group B Streptococcus Strains Recovered from Colonized Pregnant Women. J Clin Microbiol. 2017;55:412–22.

12. Winkelstein JA, Abramovitz AS, Tomasz A. Activation of C3 via the alternative complement pathway results in fixation of C3b to the pneumococcal cell wall. J Immunol. 1980;124:2502–6.

13. Brown EJ, Joiner KA, Cole RM, Berger M. Localization of complement component 3 on Streptococcus pneumoniae: anti-capsular antibody causes complement deposition on the pneumococcal capsule. Infect Immun. 1983;39:403–9.

14. Abeyta M, Hardy GG, Yother J. Genetic alteration of capsule type but not PspA type affects accessibility of surface-bound complement and surface antigens of Streptococcus pneumoniae. Infect Immun. 2003;71:218–25.

15. Noble K, Lu J, Guevara MA, Doster RS, Chambers SA, Rogers LM, et al. Group B

Streptococcus cpsE Is Required for Serotype V Capsule Production and Aids in Biofilm Formation and Ascending Infection of the Reproductive Tract during Pregnancy. ACS Infect Dis. 2021.

16. Carlin AF, Lewis AL, Varki A, Nizet V. Group B streptococcal capsular sialic acids interact with siglecs (immunoglobulin-like lectins) on human leukocytes. J Bacteriol. 2007;189:1231–7.

17. Alhhazmi A, Pandey A, Tyrrell GJ. Identification of Group B Streptococcus Capsule Type by Use of a Dual Phenotypic/Genotypic Assay. J Clin Microbiol. 2017;55:2637–50.

18. Bellais S, Six A, Fouet A, Longo M, Dmytruk N, Glaser P, et al. Capsular switching in group B Streptococcus CC17 hypervirulent clone: a future challenge for polysaccharide vaccine development. J Infect Dis. 2012;206:1745–52.

19. Phares CR, Lynfield R, Farley MM, Mohle-Boetani J, Harrison LH, Petit S, et al. Epidemiology of invasive group B streptococcal disease in the United States, 1999-2005. JAMA. 2008;299:2056–65.

20. Sørensen UBS, Poulsen K, Ghezzo C, Margarit I, Kilian M. Emergence and global dissemination of host-specific Streptococcus agalactiae clones. MBio. 2010;1.

21. Jones N, Bohnsack JF, Takahashi S, Oliver KA, Chan M-S, Kunst F, et al. Multilocus sequence typing system for group B streptococcus. J Clin Microbiol. 2003;41:2530–6.

22. Martins ER, Pessanha MA, Ramirez M, Melo-Cristino J. Analysis of group B streptococcal isolates from infants and pregnant women in Portugal revealing two lineages with enhanced invasiveness. J Clin Microbiol. 2007;45:3224–9.

23. Poyart C, Réglier-Poupet H, Tazi A, Billoët A, Dmytruk N, Bidet P, et al. Invasive group B streptococcal infections in infants, France. Emerg Infect Dis. 2008;14:1647–9.

24. Musser JM, Mattingly SJ, Quentin R, Goudeau A, Selander RK. Identification of a high-virulence clone of type III Streptococcus agalactiae (group B Streptococcus) causing invasive neonatal disease. Proc Natl Acad Sci U S A. 1989;86:4731–5.

25. Lin F-YC, Whiting A, Adderson E, Takahashi S, Dunn DM, Weiss R, et al. Phylogenetic lineages of invasive and colonizing strains of serotype III group B Streptococci from neonates: a multicenter prospective study. J Clin Microbiol. 2006;44:1257–61.

26. Hood MI, Skaar EP. Nutritional immunity: transition metals at the pathogen–host interface. Nat Rev Microbiol. 2012;10:525–37. doi:10.1038/nrmicro2836.

27. Lu J, Francis J, Doster RS, Haley KP, Craft KM, Moore RE, et al. Lactoferrin: A Critical Mediator of Both Host Immune Response and Antimicrobial Activity in Response to Streptococcal Infections. ACS Infect Dis. 2020;6:1615–23.

28. Kothary V, Doster RS, Rogers LM, Kirk LA, Boyd KL, Romano-Keeler J, et al. Group B Streptococcus Induces Neutrophil Recruitment to Gestational Tissues and Elaboration of Extracellular Traps and Nutritional Immunity. Front Cell Infect Microbiol. 2017;7:19. doi:10.3389/fcimb.2017.00019.

29. Weinberg ED. Nutritional immunity. Host's attempt to withold iron from microbial invaders. JAMA. 1975;231:39–41. http://www.ncbi.nlm.nih.gov/pubmed/1243565. Accessed 9 Apr 2018.

30. Lu J, Francis JD, Guevara MA, Moore RE, Chambers SA, Doster RS, et al. Antibacterial and Anti-biofilm Activity of the Human Breast Milk Glycoprotein Lactoferrin against Group B Streptococcus. Chembiochem. 2021.

31. Manning SD, Springman AC, Lehotzky E, Lewis MA, Whittam TS, Davies HD.

Multilocus sequence types associated with neonatal group B streptococcal sepsis and meningitis in Canada. J Clin Microbiol. 2009;47:1143–8.

32. Manning SD, Lewis MA, Springman AC, Lehotzky E, Whittam TS, Davies HD. Genotypic diversity and serotype distribution of group B streptococcus isolated from women before and after delivery. Clin Infect Dis. 2008;46:1829–37. doi:10.1086/588296.

33. Senkovich O, Ceaser S, McGee DJ, Testerman TL. Unique host iron utilization mechanisms of Helicobacter pylori revealed with iron-deficient chemically defined media. Infect Immun. 2010.

34. Haley KP, Delgado AG, Piazuelo MB, Mortensen BL, Correa P, Damo SM, et al. The human antimicrobial protein calgranulin C participates in control of helicobacter pylori growth and regulation of virulence. Infect Immun. 2015.

35. Gaddy JA, Tomaras AP, Actis LA. The Acinetobacter baumannii 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. Infect Immun. 2009;77:3150–60.

36. Vornhagen J, Adams Waldorf KM, Rajagopal L. Perinatal Group B Streptococcal Infections: Virulence Factors, Immunity, and Prevention Strategies. Trends Microbiol. 2017;25:919–31.

37. Parker RE, Laut C, Gaddy JA, Zadoks RN, Davies HD, Manning SD. Association between genotypic diversity and biofilm production in group B Streptococcus. BMC Microbiol. 2016;16:86.

38. Benitz WE, Gould JB, Druzin ML. Risk factors for early-onset group B streptococcal sepsis: estimation of odds ratios by critical literature review. Pediatrics. 1999;103:e77.

39. Kwatra G, Adrian P V, Shiri T, Buchmann EJ, Cutland CL, Madhi SA. Serotype-

specific acquisition and loss of group B streptococcus recto-vaginal colonization in late pregnancy. PLoS One. 2014;9:e98778.

40. Carrillo-Ávila JA, Gutiérrez-Fernández J, González-Espín AI, García-Triviño E, Giménez-Lirola LG. Comparison of qPCR and culture methods for group B Streptococcus colonization detection in pregnant women: evaluation of a new qPCR assay. BMC Infect Dis. 2018;18:305.

41. Seale AC, Bianchi-Jassir F, Russell NJ, Kohli-Lynch M, Tann CJ, Hall J, et al. Estimates of the Burden of Group B Streptococcal Disease Worldwide for Pregnant Women, Stillbirths, and Children. Clin Infect Dis an Off Publ Infect Dis Soc Am. 2017;65 suppl_2:S200–19.

42. Fairlie T, Zell ER, Schrag S. Effectiveness of intrapartum antibiotic prophylaxis for prevention of early-onset group B streptococcal disease. Obstet Gynecol. 2013;121:570–7.

43. Nanduri SA, Petit S, Smelser C, Apostol M, Alden NB, Harrison LH, et al. Epidemiology of Invasive Early-Onset and Late-Onset Group B Streptococcal Disease in the United States, 2006 to 2015: Multistate Laboratory and Population-Based Surveillance. JAMA Pediatr. 2019;173:224–33.

44. Rao GG, Khanna P. To screen or not to screen women for Group B Streptococcus (Streptococcus agalactiae) to prevent early onset sepsis in newborns: recent advances in the unresolved debate. Ther Adv Infect Dis. 2020;7:2049936120942424.

45. Matteson KA, Lievense SP, Catanzaro B, Phipps MG. Intrapartum group B streptococci prophylaxis in patients reporting a penicillin allergy. Obstet Gynecol. 2008;111 2 Pt 1:356–64.

46. Nogacka A, Salazar N, Suárez M, Milani C, Arboleya S, Solís G, et al. Impact of intrapartum antimicrobial prophylaxis upon the intestinal microbiota and the prevalence of antibiotic resistance genes in vaginally delivered full-term neonates. Microbiome. 2017;5:93.

47. Hayes K, O'Halloran F, Cotter L. A review of antibiotic resistance in Group B Streptococcus: the story so far. Crit Rev Microbiol. 2020;46:253–69.

48. Miranda M, Saccone G, Ammendola A, Salzano E, Iannicelli M, De Rosa R, et al. Vaginal lactoferrin in prevention of preterm birth in women with bacterial vaginosis. J Matern neonatal Med Off J Eur Assoc Perinat Med Fed Asia Ocean Perinat Soc Int Soc Perinat Obstet. 2019;:1–5.

49. Pino A, Giunta G, Randazzo CL, Caruso S, Caggia C, Cianci A. Bacterial biota of women with bacterial vaginosis treated with lactoferrin: an open prospective randomized trial. Microb Ecol Health Dis. 2017;28:1357417.

50. Valenti P, Rosa L, Capobianco D, Lepanto MS, Schiavi E, Cutone A, et al. Role of Lactobacilli and Lactoferrin in the Mucosal Cervicovaginal Defense. Front Immunol. 2018;9:376.

51. Imbert M, Blondeau R. On the iron requirement of lactobacilli grown in chemically defined medium. Curr Microbiol. 1998;37:64–6.

52. Andrews SC, Robinson AK, Rodríguez-Quiñones F. Bacterial iron homeostasis. FEMS Microbiol Rev. 2003;27:215–37.

53. Foster AW, Osman D, Robinson NJ. Metal preferences and metallation. J Biol Chem. 2014;289:28095–103.

54. Gendrin C, Merillat S, Vornhagen J, Coleman M, Armistead B, Ngo L, et al. Diminished

Capsule Exacerbates Virulence, Blood-Brain Barrier Penetration, Intracellular Persistence, and Antibiotic Evasion of Hyperhemolytic Group B Streptococci. J Infect Dis. 2018;217:1128–38.

55. Trappetti C, Potter AJ, Paton AW, Oggioni MR, Paton JC. LuxS mediates iron-dependent biofilm formation, competence, and fratricide in Streptococcus pneumoniae. Infect Immun. 2011;79:4550–8.

Table 1.

Strain Number	Strain Type	Sequence Type	Capsular Serotype	Isolation Source	Growth MIC	Biofilm MIC
2	Colonizing	ST-23	cpsIa	Vaginal/rectal colonization	250 µg/mL	250 µg/mL
12	Colonizing	ST-1	cpsV	Vaginal/rectal colonization	750 µg/mL	250 µg/mL
37	Invasive	ST-1	cpsV	EOD/sepsis	>1000 µg/mL	250 µg/mL
64	Invasive	ST-17	cpsIII	EOD/sepsis	>1000 µg/mL	500 µg/mL
66	Invasive	ST-19	cpsIII	EOD/sepsis	500 µg/mL	>1000 µg/mL
69	Invasive	ST-17	cpsIII	EOD/sepsis	500 µg/mL	>1000 µg/mL
79	Invasive	ST-19	cpsIII	EOD/sepsis	>1000 µg/mL	250 µg/mL
83	Colonizing	ST-1	cpsVI	Vaginal/rectal colonization	>1000 µg/mL	250 µg/mL
112	Colonizing	ST-12	cpsIII	Vaginal/rectal colonization	500 µg/mL	250 µg/mL
115	Colonizing	ST-17	cpsIII	Vaginal/rectal colonization	250 µg/mL	<1000 µg/mL
241	Colonizing	ST-23	cpsV	Vaginal/rectal colonization	>1000 µg/mL	250 µg/mL
285	Colonizing	ST-12	cpsII	Vaginal/rectal colonization	750 µg/mL	250 µg/mL
291	Colonizing	ST-12	cpsII	Vaginal/rectal colonization	500 µg/mL	250 µg/mL
374	Invasive	ST-12	cpsIb	EOD/sepsis	>1000 µg/mL	250 µg/mL
377	Invasive	ST-19	cpsIII	EOD/sepsis	>1000 µg/mL	250 µg/mL
390	Invasive	ST-23	cpsIa	EOD/sepsis	>1000 µg/mL	250 µg/mL
397	Invasive	ST-23	cpsIII	EOD/sepsis	750 µg/mL	250 µg/mL
411	Invasive	ST-17	cpsIII	EOD/sepsis	750 µg/mL	<1000 µg/mL
418	Invasive	ST-17	cpsIII	EOD/sepsis	750 µg/mL	250 µg/mL
438	Invasive	ST-12	cpsIb	LOD/sepsis	>1000 µg/mL	250 µg/mL
571	Colonizing	ST-19	cpsIII	Vaginal/rectal colonization	250 µg/mL	250 µg/mL
590	Colonizing	ST-19	cpsIII	Vaginal/rectal colonization	>1000 µg/mL	250 µg/mL
653	Colonizing	ST-12	cpsII	Vaginal/rectal colonization	500 µg/mL	250 µg/mL
654	Colonizing	ST-17	cpsIII	Vaginal/rectal colonization	250 µg/mL	250 µg/mL
663	Colonizing	ST-19	cpsIII	Vaginal/rectal colonization	>1000 µg/mL	250 µg/mL

Figure Legends

Table 1. Isolation source, capsular type, and sequence type of clinical strains of *Streptococcus agalactiae* used in this study and the minimum inhibitory concentration (MIC) of lactoferrin required to suppress growth (as determined by OD_{600}) and biofilm (as determined by OD_{560}/OD_{600}).

Figure 1. Analysis of susceptibility to lactoferrin-associated growth inhibition in invasive vs. clinical isolates of GBS. GBS strains isolated from colonized patients, or patients experiencing invasive disease were grown in medium alone or increasing concentrations of lactoferrin. 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 exhibited greater growth inhibition than invasive strains as determined by Student's t-test with Welch's correction (*P<0.05, and **P<0.01).

Figure 2. Analysis of susceptibility to lactoferrin-associated biofilm inhibition in invasive vs. clinical isolates of GBS. GBS strains isolated from colonized patients, or patients experiencing invasive disease were grown in medium alone or increasing concentrations of lactoferrin. 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 exhibited greater biofilm inhibition than invasive strains as determined by Student's t-test with Welch's correction (*P<0.05).

Figure 3. Analysis of susceptibility to lactoferrin-associated growth inhibition in diverse sequence types of GBS. GBS strains isolated with a variety of sequence type were grown in medium alone or increasing concentrations of lactoferrin. 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).

Figure 4. Analysis of susceptibility to lactoferrin-associated biofilm inhibition in diverse sequence types of GBS. GBS strains isolated with a variety of sequence type were grown in medium alone or increasing concentrations of lactoferrin. 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 750 μg/mL, ST-19 and ST-23 strains of GBS exhibited greater growth 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 5. Analysis of susceptibility to lactoferrin-associated growth inhibition in diverse capsular serotypes of GBS. GBS strains isolated with a span of capsular serotypes type were grown in medium alone or increasing concentrations of lactoferrin. 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).

Figure 6. Analysis of susceptibility to lactoferrin-associated biofilm inhibition in diverse capsular serotypes of GBS. GBS strains isolated with a span of capsular serotypes type were grown in medium alone or increasing concentrations of lactoferrin. 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 growth inhibition than cpsIII strains as determined by one-way ANOVA with post hoc Tukey's test (*P<0.05).

Figure 7. Analysis of susceptibility to lactoferrin-associated growth inhibition based on maternal or fetal strain isolation. GBS strains isolated from mother or neonate were grown in medium alone or increasing concentrations of lactoferrin. 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. At 250, 500, and 750 μg/mL, strains of GBS isolated from colonized mothers exhibited greater growth inhibition than strains acquired from neonates with sepsis, as determined by Student's t-test with Welch's correction (*P<0.05, and **P<0.01).

Figure 8. Analysis of susceptibility to lactoferrin-associated biofilm inhibition based on maternal or fetal strain isolation. GBS strains isolated from mother or neonate were grown in medium alone or increasing concentrations of lactoferrin. 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 biofilm formation were observed across increasing concentrations of lactoferrin as determined by Student's t-test with Welch's correction (*P<0.05, and **P<0.01).

Figure 9. Lactoferrin-dependent inhibition of biofilm based on 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).