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Regulation of Cell Growth and Virulence Gene Expression in *Staphylococcus aureus* by the Iron-Binding Proteins Lactoferrin and Hemin

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ABSTRACT

Staphylococcus aureus is a leading cause of nosocomial infections in the United States, particularly in immune-suppressed patients and preterm infants. *S. aureus* requires free iron in its environment to thrive and reproduce. In mammals, free iron is sequestered away from pathogens by iron-binding proteins, including hemoglobin and lactoferrin. Lactoferrin has bacteriostatic properties and was shown in randomized controlled clinical trials and subsequent meta-analysis to reduced *S. aureus* septicemia in preterm infants. Herein, we tested the hypothesis that lactoferrin would reduce the growth of *S. aureus in vitro* and modulate the expression of iron-regulated surface determinate (Isd) proteins, which are used by *S. aureus* to cleave heme from the host's hemoglobin, and gyrA, a marker of cell oxidative stress. *S. aureus* S54F9 cells were cultured in sow serum in the absence (control; **Ctrl**) or presence of subphysiological (1 µM), physiological (3 µM), or superphysiological (6 µM) serum concentrations of hemin (HM), lactoferrin (LF), and both proteins (Cmb). Cell growth was assessed every 12 hr. by optical density and cells were harvested after 96 hr. to assess mRNA expression of *IsdG, IsdC,* and *gyrA*. LF at both 3 µM and 6 µM and Cmb at 6 µM inhibited cell growth compared to Ctrl. LF and Cmb at 6 µM increased mRNA expression of *IsdG* and *gyrA* supporting bacterial adaptations in response to iron sequestration. These findings suggest potential mechanisms whereby lactoferrin prevents *S. aureus* infection in clinical settings.

INTRODUCTION

Neonatal sepsis is the most common cause of neonatal death worldwide, affecting one to four infants per 1000 live births (Stoll 2004). Sepsis is a particular problem in preterm very low birth weight (VLBW) infants (<1500 g birth weight) (Stoll et al. 2002, 2005). For example, early-onset (EOS) and late-onset (LOS) sepsis occur in 1.5% and 21% of VLBW infants, respectively (Stoll et al. 2005). Most infections are caused by *Staphylococcus* and *Candida* species.

Staphylococcus aureus (*S. aureus*) is one of the leading causes of nosocomial infections in the United States (Ağca et al. 2014; Kapoor et al. 2014; Moran et al. 2006). It is has been estimated that 30-50% of healthy individuals are asymptomatic carriers for *S. aureus* and may contribute to the spread of the pathogen in hospitals and other public settings (Kapoor et al. 2014; Moran et al. 2006).

Both the host and bacterial pathogens require iron for metabolism and prevention of oxidative stress (Radtke & O'Riordan 2006). Bacterial pathogens, including *S. aureus*, require iron for replication and proliferation. Thus the host and the pathogen are in constant competition for iron. The level of free iron in the host is minimal as most iron is sequestered by host iron-binding proteins, including transferrin and lactoferrin (Radtke & O'Riordan 2006; Skaar 2010).

Lactoferrin is an iron-binding protein present at high concentration in human milk, which has been shown to be bacteriostatic in cultured cells (Arnold et al. 1980; Chen et al. 2013; Farnaud & Evans 2003) and animal models, including mice and piglets (Artyn et al. 2004; Venkatesh et al. 2007; Zagulski et al. 1989). Importantly, a recent meta-analysis identified four randomized controlled trials involving 678 VLBW infants that showed that dietary lactoferrin supplementation decreased the risk of LOS by ~50% (risk ratio [RR] 0.49, 95% confidence interval [CI] 0.32 to 0.73) (Pammi & Abrams 2015).

Staphylococcus. aureus has developed sophisticated mechanisms through the iron-regulated surface determinants (Isd) pathway to acquire iron from the host proteins, including hemoglobin and transferrin (Mazmanian et al. 2003; Moriwaki et al. 2013; Oogai et al. 2011; Skaar et al. 2003; Spirig et al. 2013), however, little is known about lactoferrin. Therefore the goal of this study was to examine the impact of lactoferrin at sub-, super- and physiological serum concentrations on the growth of *S. aureus in vitro* and the expression of Isd genes involved in the iron scavenging from the host (*IsdC* and *IsdG)* and virulence gene expression *(gyrA)*. Sow serum was used as the growth media and three concentrations of bovine hemin (HM) as an iron source, three concentrations of bovine lactoferrin (LF) and the combination (Cmb) of LF and HM were compared. Based on previous studies showing that serum concentrations of transferrin increased Isd expression in *S. aureus* (Oogai et al. 2011), we hypothesized that the presence of lactoferrin alone would up-regulate *IsdC* and *IsdG* expression relative to control and hemin-treated serum. Expression of *gyrA* was expected to show a similar pattern to *IsdC* and *IsdG* expression at each respective treatment, due to up-regulation of *gyrA* during chromosomal relaxation, as a result of virulent gene expression. The rationale for this research was to provide potential mechanistic evidence for bacteriostatic effect of dietary lactoferrin against *S. aureus,* which could provide additional support for its clinical use to prevent *S. aureus* infections in VLBW infants.

LITERATURE REVIEW

Neonatal sepsis: Late-onset sepsis (LOS), which occurs between day of life 4 and 120, may be caused by pathogens acquired at delivery or during the course of hospital care required by preterm infants (Stoll et al. 2002, 2004, 2005). A recent study examined the risk factors for sepsis, the causative organisms, and mortality following EOS and LOS infection in over 108,000 VLBW infants admitted to 313 neonatal intensive care units between 1997 and 2010 (Hornik et al. 2012). Late-onset sepsis occurred in 12,204 infants. Gram-positive organisms were isolated in 61% of cases of LOS, including coagulase-negative *Staphylococcus* and *S. aureus* in 28.3% and 15.4% of the cases of LOS. In addition, LOS increased risk of death by 30% (odds ratio 1.30 [95% CI 1.21, 1.40] after controlling for other confounders (Hornik et al. 2012). Furthermore, the emergence of antibiotic resistant strains of *S. aureus,* such as MRSA, in the neonatal intensive care unit (NICU) has resulted in higher mortality rates (Shane et al. 2012) and fewer treatment options. Therefore, identifying safe and effective means to prevent *S. aureus* infection in VLBW infants has become a priority for the care of these high-risk infants (Shane et al. 2012).

Iron Uptake from Host by S. aureus *S. aureus* requires an iron concentration of 0.4–4.0 μM to thrive and proliferate (Moriwaki et al. 2013; Skaar et al. 2003). Mammalian host proteins sequester iron away from pathogens, lowering

the concentration of free iron available to the pathogen. The mechanisms behind this will be further discussed below. To obtain iron needed for survival within the host, *S. aureus* utilizes several virulence factors. One of the key virulence factors that the pathogen utilizes to obtain iron sequestered by host proteins is the iron-regulated surface determinants (*Isd*) pathway (Skaar et al. 2003). *Isd* proteins anchored to the cell wall of *S. aureus* are activated in response to reduced free-iron concentrations in the environment (Moriwaki et al. 2013; Oogai et al. 2011). This suggests that in an environment low in free iron, expression of *Isd* proteins should increase. Additionally, *Isd* proteins within the cytoplasm take up heme-bound iron from cell wall anchored *Isd* proteins and process the iron for bacterial use (Oogai et al. 2011). *IsdA, IsdB,* and *IsdC* are three of the main *Isd* proteins anchored to the cell wall, and are responsible for binding hemoglobin in the host's blood; *IsdG* resides in the bacterial cytoplasm and breaks down heme-bound iron that is taken up by the cell-wall anchored proteins (Skaar et al. 2003). The *Isd* pathway consists of binding of hemoglobin and removal of heme by *IsdB*. Heme is then passed to *IsdA,* which relays it to the inter-membrane protein *IsdC*, which transports the heme across the membrane into the cytoplasm to *IsdG* (Spirig et al. 2013). In the *S. aureus* cytoplasm, *IsdG* acts as a heme oxygenase to break down heme into free iron for use by the bacterium (Skaar et al. 2003; Spirig et al. 2013). The *Isd* pathway represents a crucial virulence factor in *S. aureus*, allowing the pathogen to thrive in the free-iron replete environment of the mammalian host. Based on their important roles within the *Isd* pathway, *IsdC* and *IsdG* protein expression was used in this study to represent cell wall-bound and cytoplasmic *Isd* proteins.

DNA gyrA and Bacterial Chromosome Expression: DNA gyrase is an enzyme consisting of two subunits that is responsible for negative supercoiling in bacterial chromosomes (Schröder et al. 2014; Jaktaji & Mohiti 2010; Aligholi et al. 2011). Environmental stress, including oxygen concentration, antibacterial exposure, and temperature changes, can impact the extent of negative supercoiling in bacterial DNA (Schröder et al. 2014). Cell life phase also determines extent of supercoils and the resultant expression of *gyrA* and *gyrB* (Schröder et al. 2014). Mutations in *gyrA* and *gyrB* have been linked to antibiotic resistance in several methicillin resistant *S. aureus* strains (Jaktaji & Mohiti 2010; Aligholi et al. 2011). Up-regulation of *gyrA* and *gyrB* has been observed when the negative supercoils of DNA are relaxed, for example during times of gene expression or stress to the bacterial cell (Skaar et al. 2003). It can be inferred from previous studies that in cells up-regulating target genes, *gyrA* and *gyrB* expression should be up-regulated due to relaxation of supercoils (Schröder et al. 2014). Measurement of *gyrA* in this study was used to identify treatment groups with increased bacterial chromosome relaxation, either caused by environmental stress to the *S. aureus* cell or in correlation of chromosome relaxation for subsequent expression of *IsdG* and *IsdC* genes.

Host Protein Binding of Iron: Both host and bacterial pathogens require iron for metabolism and prevention of oxidative stress (Radtke & O'Riordan 2006). In addition, bacterial pathogens, including *S. aureus*, require iron for replication and proliferation. Free iron in the host is minimal as most iron is sequestered by host iron-binding proteins, including transferrin and lactoferrin (Radtke & O'Riordan 2006; Skaar 2003). For instance, during phagocytosis of a bacterial pathogen, lactoferrin released by neutrophils can be taken up by the phagocyte and bind to free iron that is present in the phagocyte (Radtke & O'Riordan 2006). This action prevents the pathogen from taking up the host's iron.

Bacteriostatic Properties of Transferrin and Lactoferrin:

Transferrin, the major iron-binding protein in serum, has been shown to starve *S. aureus* of iron *in vitro,* leading to growth inhibition, which was reversed with the addition of free iron (Lin et al. 2014). Furthermore, mice challenged with *S. aureus* had improved survivability when

human transferrin was administered intravenously, suggesting transferrin's potential therapeutic and antimicrobial role (Lin et al. 2014). Lactoferrin is an iron binding glycoprotein in the transferrin family, suggesting that similar results may be observed in studies using lactoferrin in place of transferrin. Lactoferrin is found at high concentration in milk, but also in lower concentrations in tears, neutrophil granules, and blood (Chen et al. 2013). Its concentrations are highest in colostrum (5 g/L) and decline gradually the further into lactation to ~1 g/L in mature milk (Harada et al. 1999). As previously discussed, lactoferrin can bind and sequester free iron, and its ability to bind iron and sequester it from pathogens contributes to its bacteriostatic properties (Farnaud & Evans 2003; Chen et al. 2013; Arnold et al. 1980). When administered orally, both bovine and human lactoferrin have been shown to be bactericidal, not just bacteriostatic (Chen et al. 2013). Consequently, this makes administration of lactoferrin to treat bacterial infections of potential clinical significance. The bacteriostatic properties of lactoferrin and sensitivity ranges of various bacterial pathogens, including *S. aureus*, have been studied and demonstrated *in vitro* (Farnaud & Evans 2003; Arnold et al. 1980). The bactericidal properties of lactoferrin pertain to direct interactions between the bacterial cell wall and the protein (Farnaud & Evans 2003). Of main significance to this study are the bacteriostatic properties of lactoferrin against *S. aureus* under *in vitro* conditions. Concentrations of lactoferrin in serum were calculated based on a study measuring lactoferrin serum concentration of piglets fed bovine lactoferrin orally (Harada et al. 1999).

METHODOLOGY

Reagents: Staphylococcus aureus, isolate no. S54F9 was obtained from a chronic embolic pulmonary abscess in a Danish slaughter pig (Department of Veterinary Pathobiology journal no. 36444) and was generously provided by Dr. Bent Aalbaek at the University of Copenhagen, Denmark (Nielsen et al. 2009). Bovine lactoferrin (Bioferrin 2000) was purchased from Glanbia Nutritionals (Overland Park, KS) and bovine hemin from Sigma-Aldrich Corporation (St. Louis, MO). Serum was obtained from a late gestation sow purchased from the Swine Research Center at the University of Illinois, Urbana.

Culturing of S. aureus S54F9: Sterile glass test tubes containing 10 mL of sow serum (Ctrl) or serum + hemin (HM), lactoferrin (LF) or a combination of LF and HM (Cmb) were inoculated with 6.25 x 10^3 CFU/mL *S. aureus* S54F9 from frozen stock. The *S. aureus* S54F9 dose was based on the concentration of *S. aureus* present in the blood in a 7-day-old piglet infected with *S. aureus* (Nielsen et al. 2009). Serum has been previously used to grow *S. aureus* to study virulence factors and provides results that are translatable to the *in vivo* condition (Oogai et al. 2011). The cells were exposed to LF at 1, 3 and 6 μM to represent concentrations that were sub-physiological, physiological and supra-physiological, respectively. The physiological LF dose (3 μmol LF/L) was based on the concentration observed in the serum concentration of piglets 2-hr. following consumption of formula containing the colostral concentration of lactoferrin (1 g/L) (Harada et al. 1999). Bovine HM was tested at these same concentrations to assess how a heme source of iron affects cell growth and gene expression relative to Ctrl and LF. Lastly, the Cmb treatment was tested to determine whether LF interferes with HM uptake by *S. aureus* S54F9. The same total concentrations were tested (1, 3 and 6 μM), with LF and HM in a 1:1 ratio.

Staphylococcus aureus S54F9 Growth: Cells were cultured at 37° C in shaking water bath within an anaerobic chamber in the laboratory of Dr. Michael Miller in the Department of Food Science and Human Nutrition at the University of Illinois. Bacterial growth was quantified by measuring the optical density at 600 nm every 12 hr. for 96 hr. Data were compared over time as well as between treatment groups.

Isolation of mRNA and RT-qPCR: After 96 hr., tubes were centrifuged at 4° C at 4000 RPM for 10 min to obtain a bacterial pellet. Bacterial mRNA was extracted from the pellets using a FastRNA Pro Blue kit (MP Biomedicals, Salon, OH) with bead beating at 6 m/sec for 40 sec. The mRNA was purified using the RNeasy Plus Mini Kit (Qiagen, Valencia CA). Abundance of mRNA was determined fluorometrically using a Qubit (Life Technologies, Grand Island, NY), and mRNA quality was assessed using a 2100 Bioanalyzer instrument (Agilent Technologies, Inc., Santa Clara, CA). The mRNA was then reverse transcribed to cDNA for SYBR Green-based quantitative PCR. Expression of I*sdC, IsdG*, and *gyrA* was measured using the 7900HT Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA). The V6 region of the *S. aureus* 16S rRNA (Atshan et al. 2013) was used as an internal control. Primers were designed using Primer Express (Thermo Fischer Scientific Inc., 2014) and are shown in **Table 1**. RT-qPCR data were analyzed using the SDS v2.3 software (Life Technologies 2013).

Table 1. Primer Sequences used for qPCR.

Gene	Custom Assay Sequence
IsdG	TGA CGT TAA CAA AAG GAA CAG CAA
	CAT GCC ATC AA ACC TTC TAA TGT
IsdC	TCA TCA TCG CGA CAT TCA GTA AT
	CTC ATT GGT ATT GTA TTT GTA AAC CTC
gyrA	TCA AAT TGA CCG ACA AAG CTT GTA
	TCC GTA GCC CAG CAA TGG
16s RNA	GGG ACC CGC ACA AGC GGT GG
	GGG TTG CGC TCG TTG CGG GA

Statistical Analyses: Data were statistically analyzed within SAS (Version 6.09, SAS Institute, Cary, NC). Bacterial growth over time was analyzed using a repeated measures analysis of variance (ANOVA). Factors within the model included time, treatment, concentration and their interactions. The effect of treatments on bacterial growth was further analyzed at 36 and 72 hr. using 2-way ANOVA with treatment, concentration and their interaction. Gene expression at 96 hr. was also assessed by 2-way ANOVA with treatment, concentration and their interaction. A post-hoc analysis identified significant differences between groups for models displaying overall statistical significance of time, treatment concentration or their interactions, as appropriate. Statistical significance was defined as $p<0.05$. Data are expressed as mean ± SD.

RESULTS AND DISCUSSION

Optical Density: After 24 hr., a clump of cells was observed at the bottom of each experimental treatment tube (excepting control), suggesting the formation of a biofilm (Donlan & Costerton 2002; Kaplan 2010). A cell clump had not been observed when *S. aureus* was grown in sow serum alone as a control. In terms of OD readings, there were overall effects of time, treatment, and concentration in the model ($P<0.0001$). In addition, interactions between treatment*concentration ($P<0.0001$), treatment*time ($P=0.009$) were observed. There was a trend for an interaction between concentration*time ($p=0.08$). No statistically significant 3-way interactions were observed. The results for the HM, LF and Cmb treatments are shown in **Fig 1a, 1b and 1c**, respectively.

Focusing on treatment*concentration for HM-treated cells, there were only differences between Ctrl and HM at 3 µM and between HM at 1 µM and HM at 3 µM ($p<0.0001$). For the interaction of treatment*time for HM, significant differences between Ctrl and HM were noted at all of the time points except 12

and 24 hr. At 48 and 60 hr., the OD readings for all HM groups were greater than Ctrl (**Fig 1a**).

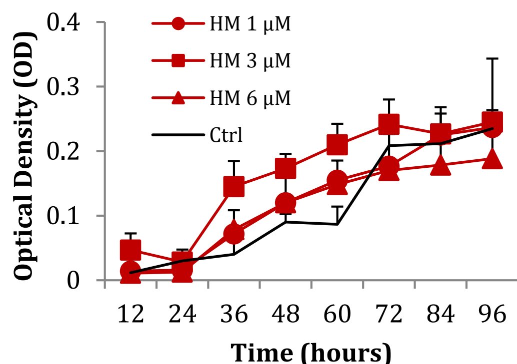

Figure 1a. Optical density over time of *S. aureus* S54F9 grown in sow serum (Crtl) alone or with 1µM, 3 µM, or 6 µM bovine hemin (HM). Data are expressed as mean ± SD. There were statistical effects of treatment*concentration (P<0.0001) and treatment*time (P=0.009).

A treatment*concentration interaction for the LF-treated cells was observed with differences between Ctrl and LF at 3 µM or 5 µM (p=0.0001) and a trend from a difference between Ctrl and LF at 1 µM (p=0.09). For the interaction of treatment*time for LF, significant differences between Ctrl and LF were only observed at the later time points (72, 84 and 96 hr.) with the OD readings for the LF-treated cultures being lower than Ctrl (**Fig 1b**).

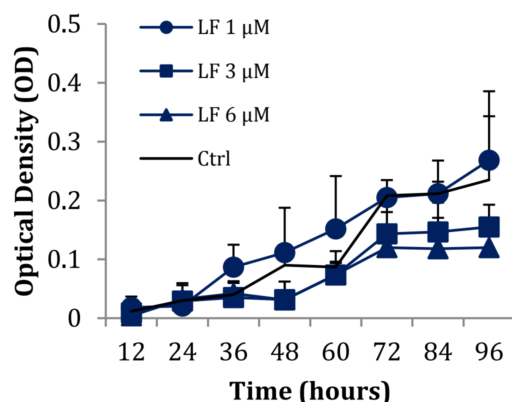

Figure 1b. Optical density over time of *S. aureus* S54F9 grown in sow serum (Crtl) alone or with 1 µM, 3 µM, or 6 µM bovine lactoferrin (LF). Data are expressed as mean ± SD. There were statistical effects of treatment*concentration (P<0.0001) and treatment*time (P=0.009).

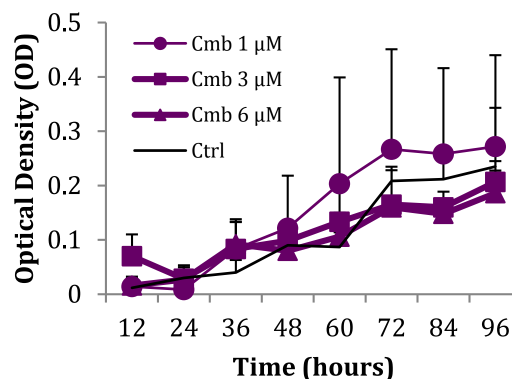

Fig 1c. Optical density over time of *S. aureus* S54F9 grown in sow serum (Crtl) alone or with 1 µM, 3 µM, or 6 µM bovine lactoferrin mixed 1:1 with bovine hemin (Cmb). Data are expressed as mean ± SD. There were statistical effects of treatment* concentration (P<0.0001). Cmb at 1 µM differed from 3 µM (p=0.003), and 6 µM (p<0.0001), while 3 and 6 µM did not differ from each other (p=0.13).

For the Cmb treatment, there were differences between Ctrl and Cmb at 1, 3 and 6 µM (p<0.0001). Cmb at 1 µM differed from 3 µM (p=0.003), and 6 µM (p<0.0001), while 3 and 6 µM did not differ from each other (p=0.13). For the interaction of treatment*time for Cmb, significant differences between Ctrl and Cmb were only observed at 36 and 60 hr. (**Fig 1c**). Thus, combining HM and LF somewhat ameliorated the inhibitory effect of LF alone on *S. aureus* growth that was observed at the later time points (**Fig 1b**).

To further examine the effect of the treatments on cell growth, the mean OD readings at two time points (36 and 72 hr.) were compared. These two points were selected to represent phases of rapid (36 hr.) vs. slower (72 hr.) *S. aureus* growth. At 36 hr., there were significant effects of treatment (p<0.0001) and treatment*concentration (p<0.003), but not concentration alone (p=0.20). At 36 hr., OD readings at all three HM doses, LF at 1 µM and Cmb at 1 µM and 3 µM were greater than Ctrl (**Fig 2a**). In contrast the two higher doses of LF did not allow cells to grow faster than Ctrl.

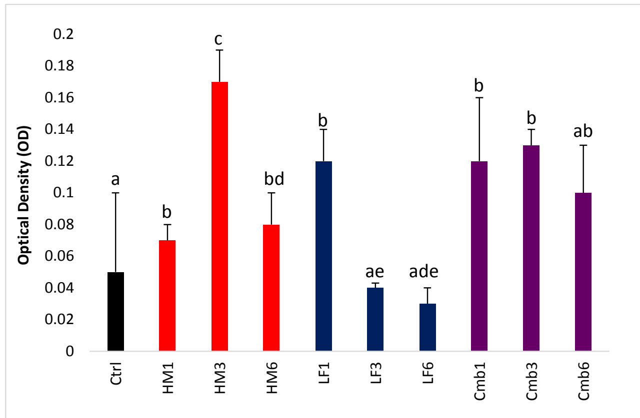

Figure 2a. Effect of treatments on optical density measurements of *S. aureus* S54F9 grown in sow serum (Crtl) alone or 1 μM, 3 μM, or 6 μM hemin (HM1, HM3, HM6), lactoferrin (LF1, LF3, LF6) or combined (1:1) hemin and lactoferrin (CB1, CB3, CB6) for 36 hr. Data are expressed as mean ± SD. Significant effects of treatment (p<0.0001) and treatment* concentration were observed (p<0.003). Different letter superscripts denote significant differences between experimental

At 72 hr., the overall model was significant at p<0.033, but there was only a trend (P<0.08) for an interaction between treatment*concentration (**Fig 2b**).

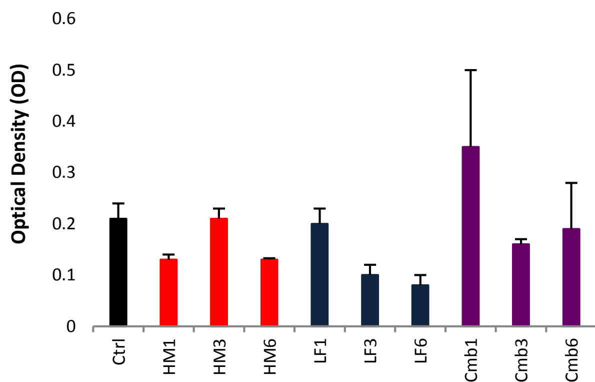

Figure 2b. Effect of treatments on optical density measurements of *S. aureus* S54F9 grown in sow serum (Crtl) alone or 1 μM, 3 μM, or 6 μM hemin *H1, H3, H6), lactoferrin (L1, L3, L6) or combined (1:1) hemin and lactoferrin (Cmb1, Cmb3, Cmb6) for 72 hr. Data are expressed as mean ± SD. A trend for an interaction between treatment*concentration was observed (p<0.08).

IsdC, IsdG and gryA Expression: The results of RT-qPCR of gene expression in *S. aureus* S54F9 exposed to the treatments for 96 hr. indicated overall statistical significance for *IsdG* (p<0.03) and *gryA* (p<0.007) gene expression. No significant differences among the treatment groups were found for *IsdC* expression (p=0.30) (**Fig 3**).

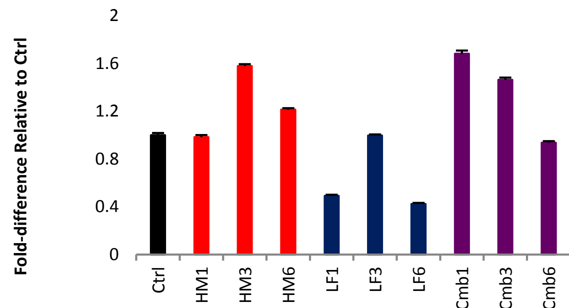

Figure 3: *IsdC* mRNA expression in *S. aureus* S54F9 grown in sow serum (Crtl) alone or 1 μM, 3 μM, or 6 μM hemin (HM1, HM3, HM6), lactoferrin (LF1, LF3, LF6) or combined (1:1) hemin and lactoferrin (CB1, CB3, CB6) for 96 hr. Data are expressed as mean ± SD. The overall model was not statistically significant (p=0.30).

Statistical differences were observed for *IsdG* mRNA expression with an interaction between treatment*concentration (**Fig 4**). The overall pattern was for increasing concentrations of HM to increase *S. aureus* growth, whereas LF and Cmb decreased cell growth at concentrations increased. No treatment differed significantly from Ctrl, however, significant differences were observed between: HM 1 μM and HM 6 μM and LF 1 μM; Cmb 1 μM and Cmb 3 μM; and Cmb 6 μM with LF 1 μM, Cmb 1 μM and Cmb 3 μM.

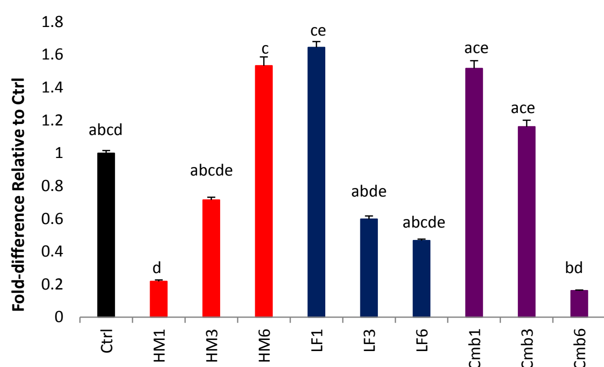

Figure 4: *IsdG* mRNA expression in *S. aureus* S54F9 grown in sow serum (Crtl) alone or 1 μM, 3 μM, or 6 μM hemin (HM1, HM3, HM6), lactoferrin (LF1, LF3, LF6) or combined (1:1) hemin and lactoferrin (Cmb1, Cmb3, Cmb6) for 96 hr. The overall model was statistically significant (p=0.03), but the only the interaction of treatment*concentration was a significant main effect (p<0.003) Different letter superscripts denote significant differences between experimental treatments at p<0.05.

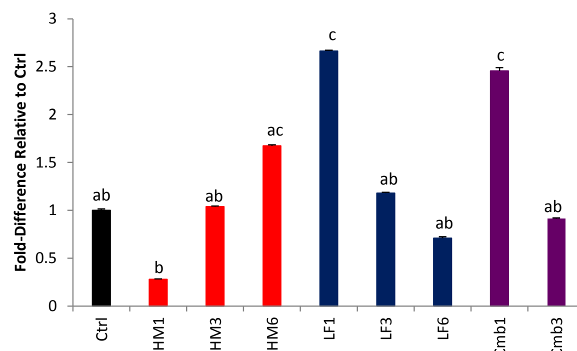

Figure 5: *GyrA* mRNA expression in *S. aureus* S54F9 grown in sow serum (Crtl) alone or 1 μM, 3 μM, or 6 μM hemin (HM1, HM3, HM6), lactoferrin (LF1, LF3, LF6) or combined (1:1) hemin and lactoferrin (Cmb1, Cmb3) for 96 hr. There was insufficient mRNA from the Cmb6 group. The overall model was statistically significant (p=0.007), but the only the interaction of treatment*concentration was a significant main effect (p<0.003) Different letter superscripts denote significant differences between experimental treatments at p<0.05.

Lastly, *gryA* mRNA expression was significantly affected by an interaction of treatment*concentration (**Fig 5**). The LF1 and CB1 treatments increased *gyrA* relative to all other treatments except HM6, including Crtl. Expression was lowest in the HM1 treatment, but it was only significantly different from LF1 and CB1. The overall pattern of *gryA* expression was similar to that of *IsdG*, with HM dose-dependently increasing *gryA* expression and LF and Cmb decreasing *gryA* expression.

CONCLUSIONS

Staphylococcus aureus is responsible for many cases of septicemia and death, particularly in hospitalized and immunocompromised patients (Stoll et al. 2002; Ağca et al. 2014). The overall goal of this study was to investigate potential mechanisms whereby lactoferrin prevents *S. aureus* septicemia in VLBW neonates (Pammi & Abrams 2015). *S. aureus* was cultured in sow serum as the media with or without hemin and lactoferrin, which were proposed to either donate or sequester iron from the *S. aureus*, affecting its growth and gene expression. After 24 hr. of incubation, biofilm formation was observed in all treatment tubes except in the Ctrl tubes. Formation of a biofilm at 24 hr. suggests a clinically relevant survival mechanism for *S. aureus* (Donlan & Costerton 2002; Kaplan 2010).

The OD readings, an indicator of cell growth, decreased at 36 hr. of incubation for treatments of 3 μM and 6 μM LF in comparison to HM, Cmb, and Ctrl treatments. Importantly, the OD readings for higher treatment concentrations over time were lower than OD readings for 1-μM treatments and Ctrl treatments. In other words, 6 μM was the most effective concentration for decreasing *S. aureus* S54F9 growth, followed by 3 μM and 1 μM, suggesting a dose-dependent reduction.

The goal was to reach a logarithmic growth phase (OD readings of 0.4-0.6) for optimal mRNA extraction, however, OD readings stabilized in the last 24-36 hr. (60 to 96 hr. post inoculum). Stabilization of OD readings may have resulted from *S. aureus* having inadequate

nutrients to grow, in addition to the bacteriostatic effects of lactoferrin. We observed a treatment trend, and a statistically significant effect of HM and LF concentration on OD over time, suggesting that the treatments impacted viability and may potentially regulate iron concentration in the serum.

Gene expression of *gyrA* and *IsdG* exhibited significance as overall models and at concentration as a factor of treatment. Treatment groups with increased *IsdG* expression also had increased *gyrA* expression. Increased *IsdG* expression in 1 μM LF and in 1 and 3 μM Cmb treatments in contrast to 1 μM HM and Ctrl treatments may suggest that *S. aureus* cells were particularly starved for iron in LF and Cmb treatments. The increased expression of *IsdG* leads to relaxation of the bacterial chromosome and an increase in *gyrA* expression in response. Elevated *gyrA* expression has also been shown to occur in stressed bacteria; suggesting *S. aureus* in the 1 μM LF and 1 μM Cmb were particularly stressed for nutrients. Expression of both genes was greatly reduced in 3 μM and 6 μM LF treatments (Fig 4, 5), which may be due to the low viability suggested by OD readings of the treatment group as observed in Fig 1b. This may also be the case for the reduced *IsdG* expression in 6 μM of Cmb treatment (Fig 4).

In summary, reduced S. aureus growth in the presence of LF, suggest that it is bacteriostatic at serum concentrations of 3 μM and highly bacteriostatic at a supra-physiologic serum concentration of 6 μM. The results for *IsdG* and *gyrA* mRNA expression provide evidence of nutrient stress affecting bacteria grown in all LF and Cmb treatments. Together, the cell growth and gene expression results suggest that the viability of *S. aureus* S54F9 was reduced by the 6-μM LF and Cmb treatments, leading to low gene expression of both target genes. The results obtained herein provide evidence to support clinical applications of physiological and supra-physiological concentrations of lactoferrin in the treatment and prevention of *S. aureus* infection.

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