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## Analysis of the antimicrobial and anti-biofilm activity of human milk lactoferrin compared to bovine lactoferrin against multi-drug resistant and susceptible *Acinetobacter baumannii* clinical isolates

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Ethics Approval and Consent to Participate

Ethics approval to carry out this study was provided by the Meharry Medical College Institutional Review Board (IRB 081204AAH23119) and by Vanderbilt University Medical Center (IRB 100897). The secondary use of de-identified or coded samples is not considered research involving human subjects under 45 CFR 46. Biospecimens used in this study were de-identified and need for consent was waived by the IRB in accordance with federal regulation (45 CFR 46, Department of Health and Human Services, Authority: 5 U.S.C. 301; 42 U.S.C. 289(a); 42 U.S.C. 300v-1(b)).

Supporting Information Available

Bacterial growth and biofilm assays performed in cultures supplemented with exogenous nutrient iron are available as supplemental files in "Supporting Information". This information is available free of charge on the ACS Publications website.

## Abstract

*Acinetobacter baumannii* is an opportunistic bacterial pathogen that causes severe infections in immunocompromised patients. The emergence of multi- and pan-drug resistant strains of *A. baumannii* from clinical sources has confounded treatment and enhanced morbidity and mortality associated with these infections. One way that *A. baumannii* circumnavigates environmental and antimicrobial challenge is by forming tertiary architectural structures of cells known as biofilms. Biofilm-inhibiting molecules could be deployed as a potential chemotherapeutic strategy to inhibit or disrupt *A. baumannii* biofilms and mitigate adverse outcomes due to infection. Lactoferrin is an innate immune glycoprotein produced in high concentrations in both human and bovine milk which has previously been shown to have antibacterial and anti-biofilm activities. We sought to test lactoferrin against a bank of clinical isolates of *A. baumannii* to determine changes in bacterial growth or biofilm formation. Our results indicate that human lactoferrin has slightly more potent antibacterial activities than bovine lactoferrin against certain strains of *A. baumannii* and that these effects are associated with anatomical site of isolation. Additionally, we have shown that both bovine and human lactoferrin can inhibit *A. baumannii* biofilm formation and that these effects are associated with anatomical site of isolation and whether the strain forms robust or weak biofilms.

## Keywords

*Acinetobacter baumannii* ; biofilm; antimicrobial peptides; glycoproteins; innate immunity

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*Acinetobacter baumannii* is a gram-negative bacterium which causes opportunistic and nosocomial infections in immunocompromised hosts and military personnel with battlefield wounds<sup>1</sup>. *A. baumannii* causes a wide variety of diseases including sepsis, meningitis, skin and soft-tissue infections, and pneumonia<sup>2</sup>. Specifically, hospital-acquired pneumonia is one of the most common clinical manifestations of *A. baumannii* infections. It occurs commonly in patients who are mechanically ventilated in intensive care unit facilities and is, therefore, frequently referred to as “ventilator-associated pneumonia” (VAP<sup>3</sup>). A recent meta-analysis of 126 studies across 29 countries determined that *A. baumannii* is associated with 80% of all hospital-acquired pneumonia and VAP cases worldwide, indicating that *A. baumannii* is a huge global burden on hospitalized and ventilated patients<sup>4</sup>. The emergence of SARS-CoV-2 and the resultant CoVID-19 pandemic has increased the risk of hospitalized patients developing VAP caused by opportunistic bacterial pathogens including *A. baumannii*<sup>5-9</sup>. The absence of a commercially available vaccine combined with emerging multi- and pan-drug resistant strains are contributing factors which hamper effective control of this infectious pathogen<sup>10</sup>. The limited options of antibiotic chemotherapeutics for these infections, coupled with the shortage of antibiotics in the development pipeline, have prompted the Centers for Disease Control and Prevention to label *A. baumannii* an urgent threat; the highest threat designation for bacterial pathogens<sup>11</sup>. Thus, new antimicrobial strategies are needed against *A. baumannii* and targeting pathways essential for bacterial virulence or survival in the host or hospital environments could prove useful for the chemotherapeutic strategies of the future.

Almost all bacterial pathogens require nutrient iron to survive and proliferate. As such, approaches to sequester or limit iron from entering the bacterial cell are promising

therapeutic strategies. We recently reported that lactoferrin, an iron-binding glycoprotein that is highly abundant in innate immune cells such as neutrophils and human milk, has antimicrobial and anti-biofilm activities against the gram-positive pathogen, *Streptococcus agalactiae* or Group B *Streptococcus* (GBS)<sup>12</sup>. Lactoferrin is a 76 to 80 kDa glycosylated protein comprised of two lobes designated the N-lobe (amino acids 1–333) and the C-lobe (amino acids 345–692)<sup>13</sup>. Each lobe consists of two subdomains, (designated N1 and N2 in the N-lobe and C1, C2 in the C-lobe), harboring one iron binding site, and one glycosylation site within each lobe. The degree of glycosylation of the protein varies and therefore the molecular weight of lactoferrin varies between 76 and 80 kDa. Additionally, the stability of lactoferrin varies with glycosylation state. Each lactoferrin molecule can reversibly bind one iron molecule in each of two discrete binding sites. The binding sites are comprised of two tyrosine residues, one histidine residue and one aspartic acid residue coordinated with two carbonate or bicarbonate ions. Lactoferrin's binding affinity for iron is 300 times higher than that of transferrin, making it one of the innate immune system's most effective iron chelators<sup>14</sup>. Because of this, we postulated that lactoferrin could be an effective antimicrobial strategy against *A. baumannii*, including strains which are multi-drug resistant.

## Results

### Analyses of bacterial growth in increasing concentrations of bovine or human lactoferrin.

Lactoferrin has antimicrobial activity against a variety of microbial pathogens, thus its potential as a chemotherapeutic has been proposed, especially against bacteria which are resistant to multiple classes of antibiotics<sup>15–17</sup>. Bovine lactoferrin has been extensively studied due to the abundance of bovine milk as a purification source, however, bovine lactoferrin and human lactoferrin have structural and functional differences which could contribute to variations in antimicrobial activity. To this end, we sought to test the antimicrobial activity of freshly purified lactoferrin from human milk and compare this to commercially available lactoferrin purified from bovine milk against a bank of strains of *A. baumannii* from diverse anatomical sources with varying antibiotic susceptibility profiles (Table 1). Analyses of *A. baumannii* growth in response to exposure to increasing concentrations of bovine or human lactoferrin revealed a dose-dependent antimicrobial effect that varies across strains and lactoferrin isoforms (Figure 1). In *A. baumannii* strains isolated from wounds, human lactoferrin significantly inhibited bacterial growth at concentrations of 250 µg/mL and above (\*\*P<0.001 and \*\*\*\*P<0.0001, respectively, one-way ANOVA, Figure 1A). Similarly, bovine lactoferrin inhibited the growth of wound-isolated *A. baumannii* at concentrations of 250 µg/mL and above (\*P<0.05 and \*\*\*\*P<0.0001, respectively, one-way ANOVA, Figure 1B). In *A. baumannii* strains isolated from sputum collected from patients with respiratory infections, human lactoferrin significantly inhibited bacterial growth at concentrations of 250 µg/mL and above (P<0.001 and P<0.0001, respectively, one-way ANOVA, Figure 1C). Conversely, these strains were less susceptible to bovine lactoferrin's antimicrobial activity, which inhibited growth at higher concentrations than the human isoform, 500 µg/mL and above (\*\*\*\*P<0.0001, one-way ANOVA, Figure 1D). *A. baumannii* isolates derived from blood were highly susceptible to the antimicrobial activity of human lactoferrin, with concentrations as low

as 62.5 µg/mL inhibiting bacterial growth significantly ( $P < 0.05$ , one-way ANOVA, Figure 1E). Conversely, inhibition of blood isolates of *A. baumannii* by bovine lactoferrin required concentrations of 250 µg/mL and above ( $P < 0.01$  and  $P < 0.0001$ , respectively, one-way ANOVA, Figure 1F). Isolates of *A. baumannii* derived from urinary tract or abdominal cavity infections were highly susceptible to human and bovine lactoferrin, with growth inhibition observed at 62.5 µg/mL ( $P < 0.05$  and  $P < 0.01$ , respectively, one-way ANOVA) 125 µg/mL ( $P < 0.01$  and  $P < 0.05$ , respectively, one-way ANOVA) and above ( $P < 0.0001$ , one-way ANOVA, Figure 1G–H). Interestingly, supplementation with 250 µM ferric chloride in the media or saturation of the lactoferrin preparations to the holo-isoform abrogated the antimicrobial activity of both human and bovine lactoferrin against *A. baumannii* (Figure S1 and Figure S2), underscoring the important role of lactoferrin-dependent iron chelation in this phenotype.

### **Analysis of the effect of bovine or human lactoferrin on biofilm formation by clinical strains of *A. baumannii***

Previous work from our lab and others has shown that in addition to antimicrobial activity, lactoferrin also has anti-biofilm activity against a wide range of pathogens and commensals<sup>18–21</sup>. Additionally, our previous work demonstrated that clinical *A. baumannii* strains isolated from different anatomical sites exhibited significant differences in biofilm formation<sup>22</sup>. We hypothesized that these differences could contribute to differential responses in lactoferrin's ability to alter *A. baumannii* biofilm formation. To test this, we grew *A. baumannii* strains from the aforementioned bank of clinical isolates statically to promote bacterial biofilm formation. Strains were grown overnight in medium alone or medium supplemented with 125 µg/mL of lactoferrin isolated from either human or bovine milk. This concentration was selected because it is within the physiological range encountered *in vivo*. Our growth data suggest this concentration is sufficient to perturb bacterial growth for many strains tested (11/17) but the inhibition of growth was less than 50% (and often less than 25%). The following day, biofilms were quantified by colorimetric assay. Results indicate that 13 out of 17 strains exhibited significant reduction in biofilms when exposed to either human or bovine lactoferrin, and the level of this inhibition varied across strain types (Table 1).

Among *A. baumannii* isolates derived from wound infections, 75% of strains tested showed significant inhibition of biofilm formation by either human or bovine lactoferrin (Figure 2). Strains 19, 37, and 101 exhibited 41%, 68%, and 70% decrease in biofilm formation, respectively, when exposed to human lactoferrin, results that were statistically significant ( $*P < 0.05$ , one-way ANOVA). Only one isolate, strain 101, exhibited a significant decrease (42%) in biofilm formation when exposed to bovine lactoferrin ( $*P < 0.05$ , one-way ANOVA). Interestingly, only one strain isolated from wounds (strain 81, one of the weakest biofilm-forming strains) did not exhibit significant changes in biofilm formation in response to lactoferrin.

The results revealed that in *A. baumannii* isolates derived from sputum collected from patients suffering from respiratory tract infections, 86% of strains tested (6/7 strains) showed significant inhibition of biofilm formation by either human or bovine lactoferrin

(Figure 3). Strains 16, 31, 35, and 112 exhibited 32%, 53%, 67%, and 56% decrease in biofilm formation, respectively, when exposed to bovine lactoferrin; results that were statistically significant (\* $P < 0.05$ , one-way ANOVA). Strains 35, 38, 79, and 112 exhibited 76%, 34%, 38%, and 84% decrease in biofilm formation, respectively, when exposed to human lactoferrin; results that were statistically significant (\* $P < 0.05$ , one-way ANOVA). Interestingly, only one strain isolated from sputum (strain 4, one of the weaker biofilm-forming strains) did not exhibit significant changes in biofilm formation in response to lactoferrin.

Among *A. baumannii* isolates derived from patient blood samples, 100% of strains tested (3/3 strains) showed significant inhibition of biofilm formation by both human and bovine lactoferrin (Figure 4). Strains 13, 15, and 17 exhibited 75%, 72%, and 50% decrease in biofilm formation, respectively, when exposed to bovine lactoferrin and 77%, 68%, and 70% decrease in biofilm formation, respectively, when exposed to human lactoferrin; results which were statistically significant (\* $P < 0.05$ , one-way ANOVA). It is pertinent to note that all three strains isolated from patient blood are strong biofilm formers *in vitro*, a phenotype consistent with our previously published results<sup>22</sup>.

Among *A. baumannii* isolates derived from urinary tract or abdominal cavity infections, 33% of strains tested (1/3 strains) showed significant inhibition of biofilm formation by lactoferrin (Figure 5). Strain 5 exhibited 41% and 62% decrease in biofilm formation when exposed to bovine or human lactoferrin, respectively; results which were statistically significant (\* $P < 0.05$ , one-way ANOVA). It is noteworthy that the two strains in this cohort which were not affected by lactoferrin exposure were observed to be weak biofilm formers *in vitro*, a phenotype consistent with our previously published results<sup>22</sup>.

In order to determine if the iron-chelation activity of lactoferrin was also critical for the observed perturbations of *A. baumannii* biofilm formation, strains were grown in medium supplemented with 250  $\mu\text{M}$  ferric chloride, an excess concentration that was sufficient to overcome the chelation activity. Interestingly, the addition of 250  $\mu\text{M}$  ferric chloride to the media or saturation of the lactoferrin molecule with iron to create holo-isoforms abrogated the anti-biofilm activity of both human and bovine lactoferrin (Figure S2 and Figure S3), again, highlighting the important role of lactoferrin-dependent iron chelation in lactoferrin's anti-biofilm activity.

### **Analysis of the effect of bovine or human lactoferrin on *A. baumannii* strains with respect to anatomical isolation site.**

Because the incidence of susceptibility to lactoferrin-dependent inhibition of biofilm formation across *A. baumannii* clinical isolates varied across strain types, we hypothesized that isolation source could be a potential predictor for susceptibility to lactoferrin-association biofilm inhibition. To test this, we analyzed the effect of lactoferrin (either human or bovine) on biofilm formation based on anatomical site of isolation of the strain of *A. baumannii*. Results indicated that blood isolates exhibit the largest decrease in biofilm formation when exposed to bovine or human lactoferrin (66% and 81%, respectively), followed by sputum isolates which exhibit the second largest decrease in biofilm formation when exposed to bovine or human lactoferrin (42% and 52%, respectively) (Figure

6). Among urine and abdominal infection isolates, only human lactoferrin significantly inhibited biofilm formation by 46%, a result that was not observed with bovine lactoferrin. Collectively, biofilm formation by wound isolates was not significantly impacted by either human or bovine lactoferrin.

### **Analysis of the effect of bovine or human lactoferrin on high vs. low biofilm forming strains of *A. baumannii***

As we observed that strains which formed more biofilm were more likely to experience biofilm inhibition by lactoferrin, we hypothesized that strong biofilm formation could be a potential predictor for susceptibility to lactoferrin-associated inhibition of biofilm. To test this, we calculated the geometric mean of biofilm formed by all clinical isolates in medium alone and imposed this as a cutoff (geometric mean across all strains ratio of biofilm  $OD_{560}$ /biomass  $OD_{600} = 1.0$ ) to delineate between high and low biofilm formers. Upon binning the strain specific biofilm values into these two designations, we applied a one-way ANOVA to ensure that the phenotypes observed across these designations were significantly different in medium alone ( $P < 0.0001$ ). We then analyzed the effect of either human or bovine lactoferrin on clinical isolates of *A. baumannii* based on these criteria. Results in Figure 7 indicate that high biofilm forming strains of *A. baumannii* exhibit a 55% decrease in biofilm formation in response to exposure to bovine lactoferrin ( $P < 0.0001$ , one-way ANOVA) and a 74% decrease in biofilm formation in response to exposure to human lactoferrin ( $P < 0.0001$ , one-way ANOVA). Low biofilm forming strains of *A. baumannii* do not exhibit significant changes in biofilm formation in response to either human or bovine lactoferrin.

### **Analysis of iron associated with human and bovine lactoferrin samples**

Because our previous work has demonstrated that the antimicrobial activity of lactoferrin is associated with its capacity to bind and chelate iron, we hypothesized that differences in lactoferrin-associated iron might contribute to differential antimicrobial activities between human and bovine lactoferrin in our studies. To test this, purified lactoferrin from human breast milk, as well as commercially-available bovine milk lactoferrin were subjected to ICP-MS analysis to enumerate the iron ions associated with 100  $\mu\text{g}$  of sample (Figure 8). Freshly purified fractions of lactoferrin from expressed human breast milk (Human Lf) exhibited a mean value of 12 parts per billion (PPB) of iron (Fe) ions, while commercially-sourced bovine lactoferrin (Bovine Lf) preparations exhibited a mean value of 88 PPB of Fe ions, results that were statistically significant ( $P < 0.05$ , Student's *t* test with Welch's correction). Additionally, iron-saturation to yield holo-isoforms resulted in enhanced iron associated with human lactoferrin ( $P < 0.0001$ , One Way ANOVA) and bovine lactoferrin ( $P = 0.046$ , unpaired Student's *t* test). Together, these results indicate that commercially-sourced bovine lactoferrin is significantly more iron-saturated than the freshly purified human breast milk isoform which is largely in the apo- isoform.

## **Discussion**

A survey of clinical isolates of *A. baumannii* from Nashville, Tennessee revealed that antibiotic resistance to the following drugs was prevalent in most strains: ampicillin-sulbactam, amikacin, ceftriaxone, ceftazidime, cefotaxime, ciprofloxacin, cefepime,

gentamicin, levofloxacin, meropenem, piperacillin, trimethoprim-sulfamethoxazole, ticarcillin- K clavulanate, tetracycline, and tobramycin<sup>22</sup>. *A. baumannii* isolated from wounds exhibited higher motility than strains isolated from blood, urine or Foley catheter, or sputum/bronchial wash. Blood-isolated *A. baumannii* strains formed significantly more biofilm than isolates from wounds, sputum or bronchial wash samples<sup>22</sup>. An inverse relationship between motility and biofilm formation was observed in the cohort of 17 clinical isolates of *A. baumannii* tested in this study<sup>22</sup>. Motility was also inversely correlated with induction of hemolysis<sup>22</sup>. An inverse correlation was observed between hemolysis and resistance to ticarcillin-k clavulanate, meropenem, and piperacillin<sup>22</sup>. An inverse correlation was also observed between motility and resistance to ampicillin-sulbactam, ceftriaxone, cefotaxime, ceftazidime, ciprofloxacin, or levofloxacin<sup>22</sup>. Together, this work demonstrated that strain-dependent variations in biofilm and motility are associated with anatomical site of isolation, and that there are cross-relationships between the regulation of virulence and antimicrobial resistance pathways<sup>22</sup>. To build on this study, we sought to determine if the antimicrobial glycoprotein lactoferrin could have utility in altering *A. baumannii* growth or biofilm formation. Specifically, the purpose of the current study was to analyze the utility of either bovine or human lactoferrin against a diverse collection of clinical strains of *A. baumannii* from varying isolation sources. We hypothesized that individual strain virulence phenotypes (such as biofilm formation) or anatomical site of isolation might contribute to the efficacy of lactoferrin as an antimicrobial strategy.

The emergence of multi- and pan-drug resistant *A. baumannii* strains, in particular strains resistant to carbapenems, pose a serious threat to hospitalized patients<sup>23</sup>. The need for novel treatment options is urgently required, and antimicrobial peptides and glycoproteins with selective toxicity against antibiotic-resistant bacteria are promising candidates. Lactoferrin, an antimicrobial glycoprotein that is abundant in human and bovine milk, innate immune cell granules, and mucus, has been shown to have wide antimicrobial activity against a variety of bacteria, fungi, parasites, and viruses, including the novel coronavirus SARS-CoV-2<sup>24</sup>. Seminal work by Dijkshoorn *et al.*, has shown that the lactoferrin-derived peptide hLF(1–11) was highly bactericidal against five multi-drug resistant strains of *A. baumannii* *in vitro*, leading to a 3–4 log reduction in bacterial viability<sup>25</sup>. Similarly, results published by de Léséleuc and colleagues has revealed that bovine and murine sera have antimicrobial effects which are bypassed by some *A. baumannii* strains via a heme oxygenase gene which facilitates heme consumption as an iron source to circumnavigate the potential iron restriction within sera (likely contributed by transferrin and lactoferrin)<sup>26</sup>. Mahdi and colleagues further refined these studies by purifying lactoferrin and lactoperoxidase from camel colostrum and applying this to multi-drug resistant *A. baumannii*. Their results indicate that camel colostrum lactoferrin can exert an antimicrobial effect against *A. baumannii* in a dose-dependent fashion, and that the combination of lactoferrin and lactoperoxidase can ameliorate *A. baumannii* infections *in vivo*<sup>27</sup>. Additional work by Kamoshido *et al.* revealed that susceptibility to colistin was inversely related to resistance to lactoferrin in *A. baumannii*. Specifically, loss of lipopolysaccharide (LPS) production due to colistin resistance selection enhanced the susceptibility of the type strain *A. baumannii* 19606<sup>T</sup> to human lactoferrin, implicating LPS as a potential barrier against lactoferrin challenge in this specific strain which survived 1000 µg/mL lactoferrin challenge<sup>28</sup>. Our

work expands on these studies, leveraging lactoferrin from milk sources such as human milk and the more abundant bovine milk and a larger bank of *A. baumannii* isolates from diverse anatomical isolates and antimicrobial susceptibility profiles. Our work indicates that 15 out of 17 strains were susceptible to growth inhibition by either bovine or human lactoferrin, highlighting the broad antimicrobial activity that these molecules exert against clinical strains of *A. baumannii*. Human lactoferrin had a lower MIC than bovine lactoferrin for 8 strains of *A. baumannii*, 6 of which were MDR, in our study. Conversely, bovine lactoferrin had a lower MIC for 2 strains (one MDR and one susceptible strain) compared to human lactoferrin, underscoring strain variability with respect to susceptibility to these isoforms of lactoferrin.

In addition to its ability to inhibit growth, lactoferrin has been demonstrated to possess anti-biofilm activities against a wide range of commensal and pathogenic microorganisms. Lactoferrin has been shown to inhibit bacterial biofilms in the opportunistic pathogen *Pseudomonas aeruginosa* at concentrations well beneath those that inhibit bacterial growth<sup>18</sup>. Human lactoferrin has potent anti-biofilm and anti-adhesion properties against intestinal symbionts such as *Bacteriodes fragilis* and *Bacteriodes thetaiotamicron*<sup>19</sup>. Additionally, bovine lactoferrin, and its cognate peptides which can be generated by hydrolysis, have been established as an effective anti-biofilm molecule against several bacteria including *Actinobacillus pleuropneumoniae*, *Listeria monocytogenes*, *Escherichia coli*, *P. aeruginosa*, *Pseudomonas fluorescens*, and *Streptococcus mutans*<sup>20,21,29–31</sup>. We are the first to demonstrate the anti-biofilm activity of human and bovine lactoferrins against clinical *A. baumannii* strains, and the first to report strain heterogeneity in this pathogen's response to both human and bovine isoforms of this important antimicrobial glycoprotein.

Human and bovine lactoferrin possess very high sequence similarity, however, differences between these isoforms within *in vitro* and *in vivo* studies have been observed. A recent study by Rosa and colleagues has attributed differences in function and efficacy of human and bovine lactoferrin to physico-chemical properties such as glycosylation state, thermostability, resistance to proteolysis, iron-saturation of the molecule, and cross-metalation by aluminum, copper, magnesium, manganese, and zinc, which are chelated by lactoferrin at lower affinities<sup>32</sup>. Indeed, our results mirror those previously described as we observed that the commercially available bovine lactoferrin we utilized had overall lower antimicrobial and antibiofilm activity against the bank of 17 strains of *A. baumannii* we tested, compared to the freshly purified human milk lactoferrin samples. Elemental analyses revealed that the bovine isoform was partially saturated with iron; which could ultimately hamper iron-chelation-dependent antimicrobial activity. Conversely, our fresh preparations of human milk lactoferrin were comprised of apo-isoforms of the protein which likely contributed to the heightened antimicrobial activity this glycoprotein has against *A. baumannii*.

The utility of antimicrobial peptides as an alternative class of antibiotics is an area of increasing interest. Unlike antibiotics currently in use, bacteria are less effective at developing resistance to antimicrobial peptides; a result that is likely due to co-evolutionary forces at play during the host-pathogen interaction<sup>33,34</sup>. To that end, lactoferrin and its cognate peptides such as lactoferricin have been used as effective therapies to treat urinary



tract infections, as an oral treatment for irritable bowel syndrome due to its normalizing effects on the gut microbiome<sup>35–39</sup>. Additionally, lactoferrin antimicrobial peptides have been shown to have utility against four strains of MDR *A. baumannii* in an experimental infection in mice, with treatment leading to a 2–3 log reduction in bacterial burden *in vivo*<sup>25</sup>. Our work refines the existing literature to provide a clear rationale for the potential utility of apo-lactoferrin against a broad bank of *A. baumannii* strains from diverse anatomical isolation sources.

## Conclusions

In conclusion, we report that physiologically relevant concentrations of either human or bovine lactoferrin have the capacity to inhibit the growth of most clinical isolates of *A. baumannii* tested. Additionally, both human and bovine lactoferrin can inhibit biofilm formation by a variety of *A. baumannii* strains at concentrations well beneath MIC<sub>50</sub> for growth. While human lactoferrin tended to show greater statistically significant differences from control than bovine, there were no statistically significant differences between results obtained from the two forms. Furthermore, we report that freshly isolated human milk lactoferrin exhibits antimicrobial and anti-biofilm effects and that these effects vary across *A. baumannii* isolates from diverse anatomical origin and varying phenotypes with respect to antibiotic susceptibilities. Specifically, human lactoferrin significantly inhibited biofilm formation from strains isolated from sputum, blood, urinary tract or abdominal infections, whereas bovine lactoferrin was only able to significantly inhibit biofilm formation in strains isolated from sputum or blood. Interestingly, wound-isolated *A. baumannii* strains' biofilm was not significantly impacted by human or bovine lactoferrin and were previously identified as being weak biofilm formers<sup>22</sup>. Analysis of low versus high biofilm forming phenotypes revealed that this was the strongest predictor for the efficacy of lactoferrin in inhibiting biofilms, indicating cellular processes required for development of robust biofilm formation are likely the target of the antimicrobial activity of lactoferrin. Our work underscores the potential utility of the antimicrobial glycoprotein lactoferrin against *A. baumannii* as the age of antibiotics continues to wane.

## Methods

### Bacterial strains and culture conditions

Seventeen clinical isolates of *A. baumannii* were utilized in this study (Table 1). These strains were selected from diverse anatomical origin and from a wide range of disease presentations including urinary tract, respiratory, wound, intra-abdominal infections, and bacteremia. They represented a wide range of quantitative biofilm formation phenotypes as well as susceptibility or resistance to antibiotics. Antimicrobial susceptibility to ampicillin-sulbactam (A/S), amikacin (AK), ceftriaxone (CAX), ceftazidime (CAZ), cefotaxime (CFT), ciprofloxacin (CP), cefepime (CPE), gentamicin (GM), levofloxacin (LVX), meropenem (MER), piperacillin (PI), trimethoprim-sulfamethoxazole (T/S), tetracycline (TE), ticarcillin-K clavulanate (TIM), and tobramycin (TO) was determined as previously described<sup>22</sup>. All isolates were grown overnight in Luria-Bertani (LB) broth (ThermoFisher) at 37 °C in room

air under shaking conditions 180 rpm to an optical density of 600 nm (OD<sub>600</sub>) between 0.8–1.0.

### Purification of lactoferrin from human milk

Expressed human breast milk was gathered 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) from healthy donors between 3 days and 3 months post-partum and purified as previously described<sup>40</sup>. Lactoferrin was purified from human milk as previously described<sup>12</sup>. Briefly, de-identified human milk samples were centrifuged at 8000 g for 45 minutes to separate milk fats from the soluble fraction. Following centrifugation, the upper fat layer was removed, and proteins were precipitated from the soluble fraction by the addition of ammonium sulfate and incubation at 4°C overnight. Precipitated proteins were fractionated by ion-exchange chromatography. The purity and identity of the fractions were determined by high resolution mass spectrometry analysis. Fractions containing greater than 99% lactoferrin were combined and used in the subsequent assays. Holo-isoforms of both bovine and human lactoferrin were prepared by saturating lactoferrin samples with ferric chloride and dialyzing to remove unbound iron as previously described<sup>24</sup>.

### Bacterial growth curve assays

*A. baumannii* biofilms were cultured and analyzed as previously described<sup>41,42</sup> with some modifications. Briefly, overnight cultures were diluted ten-fold in fresh media comprised of 50% LB broth and 50% calprotectin buffer with increasing concentrations (0, 62.5 μM, 125 μM, 250 μM, 500 μM, 750 μM, 1000 μM) of human or commercially available bovine lactoferrin (Friesland Campina) alone or in medium supplemented with 250 μM ferric chloride. Cultures were incubated at 37 °C for 24 h in shaking conditions (180 rpm). The OD<sub>600</sub> was measured to quantify the density of the bacterial cells in each culture. Minimum inhibitory concentration (MIC) was determined as the concentration at which statistically significant inhibition of growth was observed.

### Bacterial biofilm quantification

*A. baumannii* biofilms were cultured and analyzed as previously described<sup>22,43</sup> with some modifications. Briefly, overnight cultures were diluted ten-fold in fresh media comprised of 50% LB broth and 50% calprotectin buffer alone or supplemented with 250 μM ferric chloride, then treated with 125 μg/mL of either bovine or human lactoferrin (concentrations that were subinhibitory to growth for most strains). Samples were incubated at 37 °C for 24 h in dark static conditions. The OD<sub>600</sub> was measured to quantify the density of the bacterial cells in each culture. Biofilm formation was determined by crystal violet staining. A 1% solution (w/v) of crystal violet (Sigma-Aldrich) was used to stain bacterial cells, decanted, and then washed twice with distilled water. 200 μL of 80%/20% ethanol/acetone solution (Sigma-Aldrich) was added to each well to solubilize the crystal violet and the absorbance at 560 nm was recorded. Total biofilm (as determined by OD<sub>560</sub> of crystal violet stain) for each isolate was normalized to its respective total mass by calculating the ratio of absorbance at 560 nm to 600 nm (biomass). Each biofilm assay consisted of 2–3 technical replicates and the assay was repeated at least three times using fresh overnight cultures. All OD<sub>560</sub>/OD<sub>600</sub>

ratios from strains grown in medium alone above 1.0, the geometric mean, were considered strong biofilm formers, while strains exhibiting values below this cutoff were considered weak biofilm formers. To validate this cutoff value, absorbance values were subjected to the D'Agostino and Pearson test of normality as previously described<sup>44</sup> and after segregation into weak vs. strong cohorts, a one-way ANOVA was applied ( $P < 0.0001$ ).

### Inductively coupled plasma mass spectrometry analyses

To enumerate the iron molecules associated with lactoferrin isoforms, Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was performed as previously described<sup>40</sup>. Briefly, 100  $\mu\text{g}$  of each lactoferrin isoform were digested in 0.9 mL 50% nitric acid (Thermo Fisher) overnight at 37°C in sealed Teflon sample containers (VWR). The following day, samples were diluted in 9 mL of deionized water in metal-free tubes (VWR). Elemental quantification was performed using a Thermo-Element 2 HR- ICP-MS instrument.

### Statistical analyses

Statistical analyses of growth were performed by Student's *t* test comparing to medium alone control within discrete strain experiments and comparing between bovine and human lactoferrin. Analyses of biofilm formation were performed using 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 or 8 software (GraphPad Prism Software Inc., La Jolla, California) or Microsoft Excel.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

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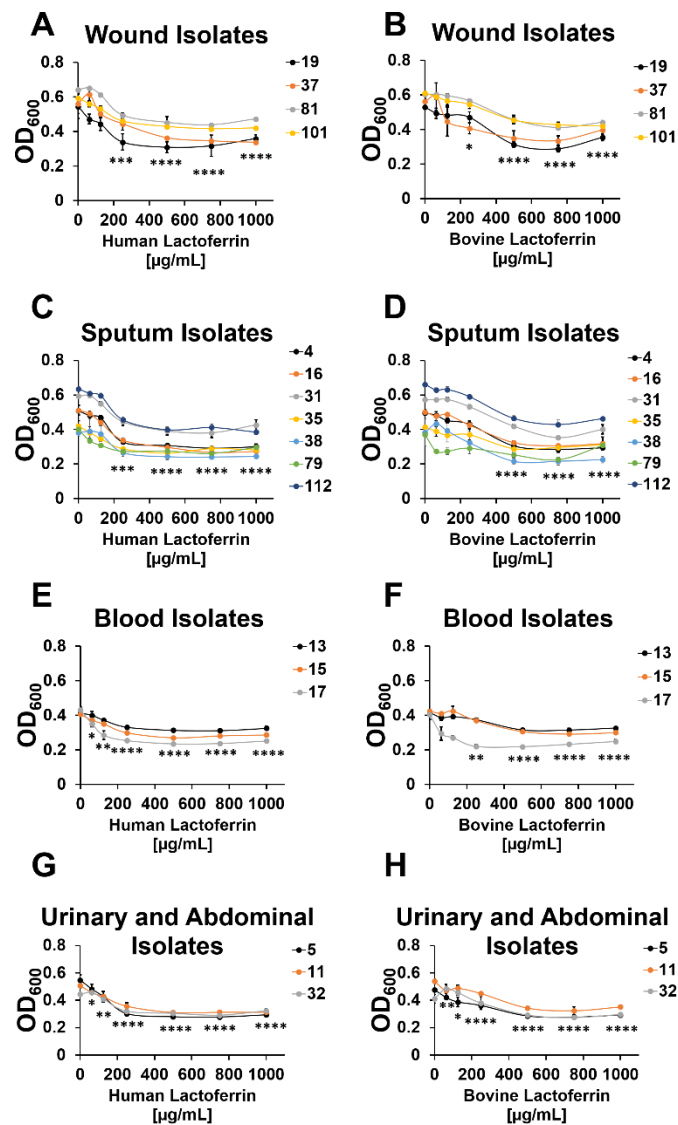
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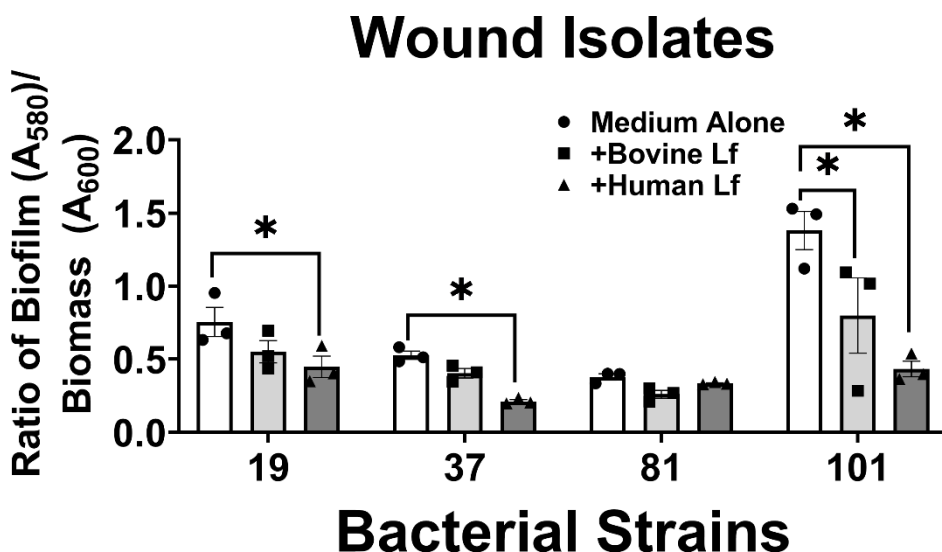
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**Figure 1.**

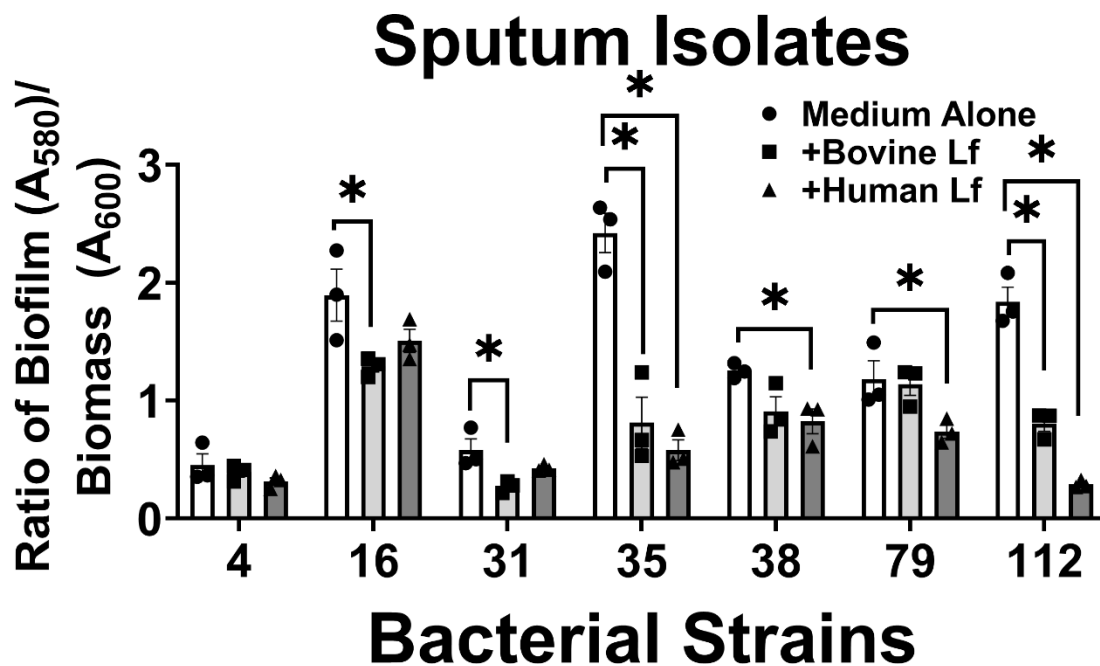
Analyses of bacterial growth in increasing concentrations of bovine or human lactoferrin. Bacterial growth was assessed at 24 hours post-inoculation by spectrophotometric measurement of optical density at 600 nm (OD<sub>600</sub>). Cultures were grown in increasing concentrations of human (A, C, G, E) or bovine lactoferrin (B, D, F, H). *A. baumannii* strains tested were isolated from wounds (A-B), sputum (C-D), blood (E-F), or urinary tract or abdominal cavity infections (G-H). Significant inhibition of bacterial growth compared to medium alone negative control was determined by one-way ANOVA with Tukey's *post-hoc* test, \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001, n=3.



**Figure 2.**

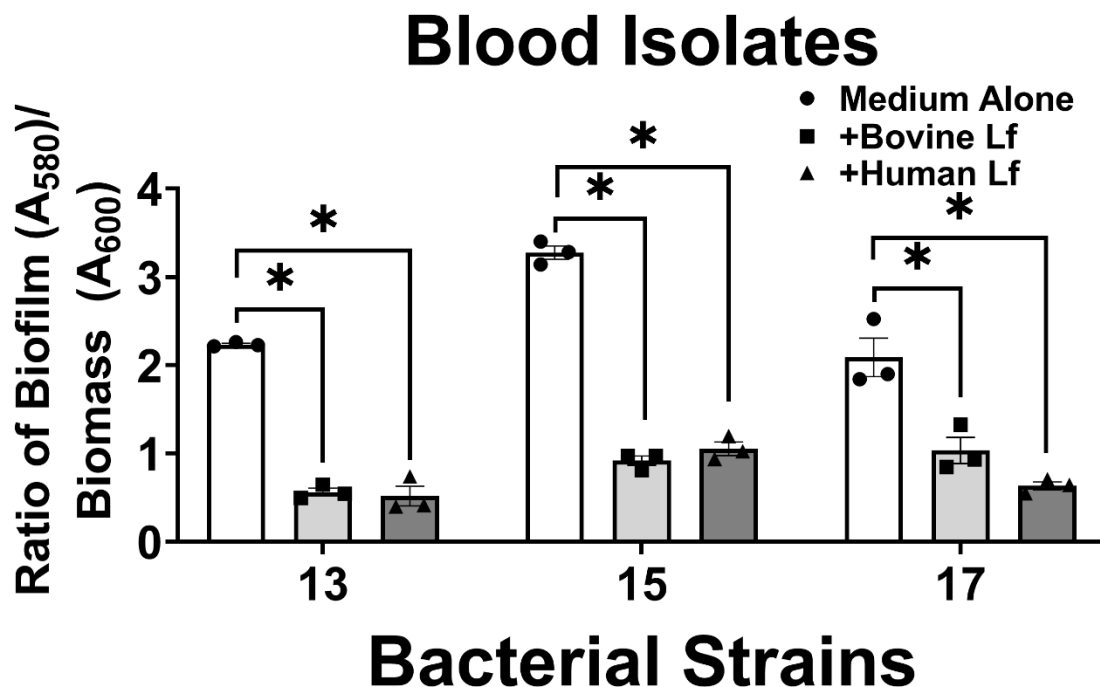
Analysis of the effect of 125  $\mu\text{g}/\text{mL}$  bovine or human lactoferrin on biofilm formation by clinical strains of *A. baumannii* isolated from wounds (strains 19, 37, 81, and 101). Bacterial biomass was measured at 24 hours post-inoculation by spectrophotometric measurement of optical density at 600 nm ( $\text{OD}_{600}$ ). Cultures were decanted, washed, and adherent biofilms were stained with crystal violet. Biofilm was quantified by solubilizing crystal violet in an 80%/20% ethanol: acetone solution and evaluation at 560 nm ( $\text{OD}_{560}$ ). Cultures were grown in medium alone (Medium Alone, designated by white bars and circle points) or medium supplemented with 125  $\mu\text{g}/\text{mL}$  of either bovine lactoferrin (Bovine Lf, designated by light gray bars and square points) or human lactoferrin (Human Lf, designated by dark gray bars and triangle points). Points indicate individual experiments, bars indicate mean  $\pm$  SEM. Significant inhibition of bacterial biofilm compared to medium alone negative control was determined by one-way ANOVA with Tukey's *post-hoc* test,  $*P < 0.05$ ,  $n = 3$ .





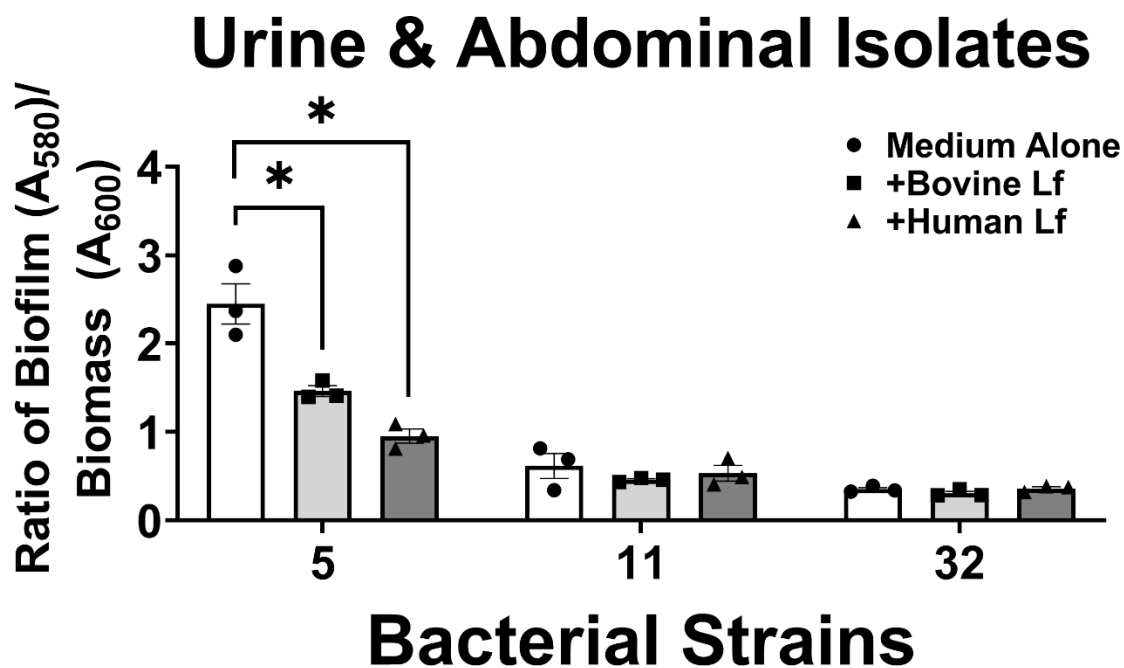
**Figure 3.**

Analysis of the effect of bovine or human lactoferrin on biofilm formation by clinical strains of *A. baumannii* isolated from sputum (strains 4, 16, 31, 35, 38, 79, and 112). Bacterial biomass was measured at 24 hours post-inoculation by spectrophotometric measurement of optical density at 600 nm (OD<sub>600</sub>). Cultures were decanted, washed, and adherent biofilms were stained with crystal violet. Biofilm was quantified by solubilizing crystal violet in an 80%/20% ethanol: acetone solution and evaluation at 560 nm (OD<sub>560</sub>). Cultures were grown in medium alone (Medium Alone, designated by white bars and circle points) or medium supplemented with 125 µg/mL of either bovine lactoferrin (Bovine Lf, designated by light gray bars and square points) or human lactoferrin (Human Lf, designated by dark gray bars and triangle points). Points indicate individual experiments, bars indicate mean  $\pm$  SEM. Significant inhibition of bacterial biofilm compared to medium alone negative control was determined by one-way ANOVA with Tukey's *post-hoc* test, \*P<0.05, n=3.



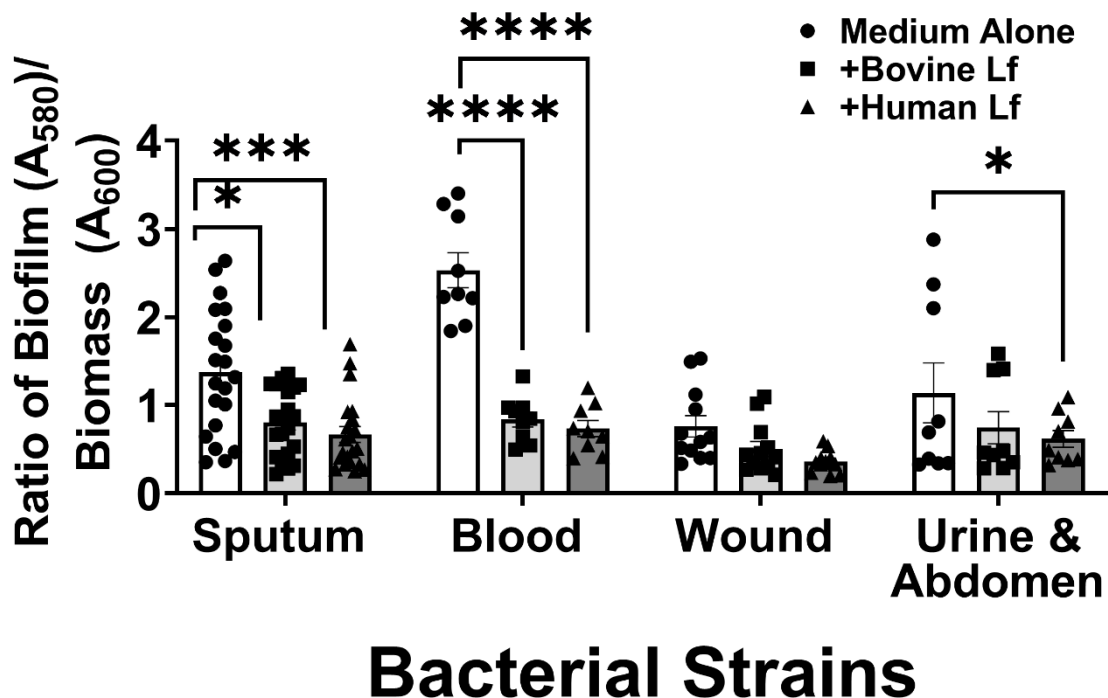
**Figure 4.**

Analysis of the effect of bovine or human lactoferrin on biofilm formation by clinical strains of *A. baumannii* isolated from blood (strains 13, 15, and 17). Bacterial biomass was measured at 24 hours post-inoculation by spectrophotometric measurement of optical density at 600 nm ( $OD_{600}$ ). Cultures were decanted, washed, and adherent biofilms were stained with crystal violet. Biofilm was quantified by solubilizing crystal violet in an 80%/20% ethanol: acetone solution and evaluation at 560 nm ( $OD_{560}$ ). Cultures were grown in medium alone (Medium Alone, designated by white bars and circle points) or medium supplemented with 125  $\mu\text{g}/\text{mL}$  of either bovine lactoferrin (Bovine Lf, designated by light gray bars and square points) or human lactoferrin (Human Lf, designated by dark gray bars and triangle points). Points indicate individual experiments, bars indicate mean  $\pm$  SEM. Significant inhibition of bacterial biofilm compared to medium alone negative control was determined by one-way ANOVA with Tukey's *post-hoc* test, \* $P < 0.05$ ,  $n = 3$ .



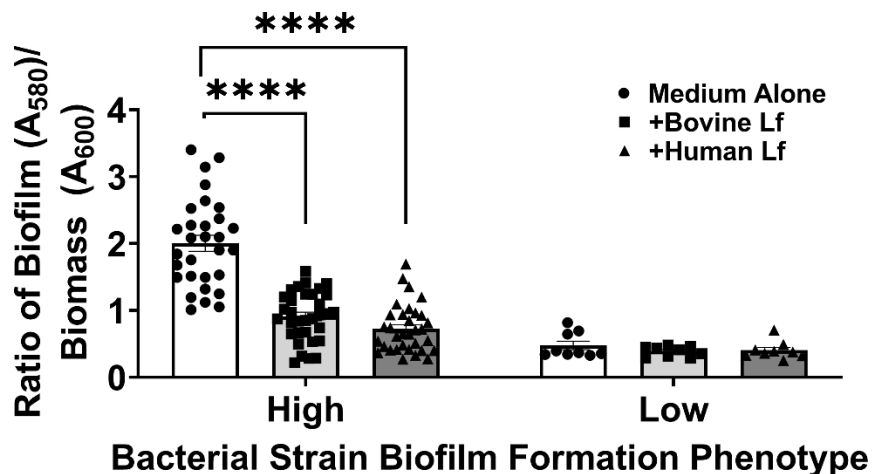
**Figure 5.**

Analysis of the effect of bovine or human lactoferrin on biofilm formation by clinical strains of *A. baumannii* isolated from urinary tract or abdominal cavity infections (strains 5, 11, and 32). Bacterial biomass was measured at 24 hours post-inoculation by spectrophotometric measurement of optical density at 600 nm ( $OD_{600}$ ). Cultures were decanted, washed, and adherent biofilms were stained with crystal violet. Biofilm was quantified by solubilizing crystal violet in an 80%/20% ethanol: acetone solution and evaluation at 560 nm ( $OD_{560}$ ). Cultures were grown in medium alone (Medium Alone, designated by white bars and circle points) or medium supplemented with 125  $\mu\text{g}/\text{mL}$  of either bovine lactoferrin (Bovine Lf, designated by light gray bars and square points) or human lactoferrin (Human Lf, designated by dark gray bars and triangle points). Points indicate individual experiments, bars indicate mean  $\pm$  SEM. Significant inhibition of bacterial biofilm compared to medium alone negative control was determined by one-way ANOVA with Tukey's *post-hoc* test,  $*P < 0.05$ ,  $n=3$ .



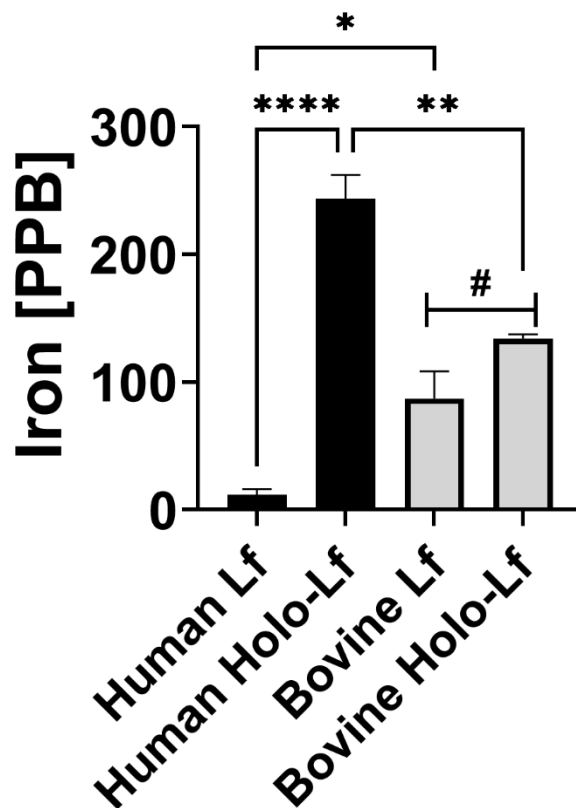
**Figure 6.**

Analysis of the effect of bovine or human lactoferrin on biofilm formation by clinical strains of *A. baumannii* based on isolation source (sputum, blood, wound, urinary tract & abdominal infections). Bacterial biomass was measured at 24 hours post-inoculation by spectrophotometric measurement of optical density at 600 nm ( $OD_{600}$ ). Cultures were decanted, washed, and adherent biofilms were stained with crystal violet. Biofilm was quantified by solubilizing crystal violet in an 80%/20% ethanol: acetone solution and evaluation at 560 nm ( $OD_{560}$ ). Cultures were grown in medium alone (Medium Alone, designated by white bars and circle points) or medium supplemented with 125  $\mu\text{g}/\text{mL}$  of either bovine lactoferrin (Bovine Lf, designated by light gray bars and square points) or human lactoferrin (Human Lf, designated by dark gray bars and triangle points). Points indicate individual experiments, bars indicate mean  $\pm$  SEM. Significant inhibition of bacterial biofilm compared to medium alone negative control was determined by one-way ANOVA with Tukey's *post-hoc* test, \* $P < 0.05$ ,  $n = 3$ .



**Figure 7.**

Analysis of the effect of bovine or human lactoferrin on high vs. low biofilm forming strains of *A. baumannii*. Strains with biofilm quantification values above the geometric mean (1.0) were designated “High” biofilm formers. Strains with biofilm quantification values below the geometric mean (1.0) were designated “Low” biofilm formers. Bacterial biomass was measured at 24 hours post-inoculation by spectrophotometric measurement of optical density at 600 nm ( $OD_{600}$ ). Cultures were decanted, washed, and adherent biofilms were stained with crystal violet. Biofilm was quantified by solubilizing crystal violet in an 80%/20% ethanol: acetone solution and evaluation at 560 nm ( $OD_{560}$ ). Cultures were grown in medium alone (Medium Alone, designated by white bars and circle points) or medium supplemented with 125  $\mu\text{g}/\text{mL}$  of either bovine lactoferrin (Bovine Lf, designated by light gray bars and square points) or human lactoferrin (Human Lf, designated by dark gray bars and triangle points). Points indicate individual experiments, bars indicate mean  $\pm$  SEM. Significant inhibition of bacterial biofilm compared to medium alone negative control was determined by one-way ANOVA with Tukey’s *post-hoc* test, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ ,  $n = 3$ .



**Figure 8. ICP-MS elemental analysis of lactoferrin preparations.**

Freshly purified fractions of lactoferrin from expressed human breast milk (Human Lf, black bars), or commercially-sourced bovine lactoferrin preparations (Bovine Lf, grey bars), as well as iron-saturated isoforms of each respective preparation (holo-Lf) were subjected to ICP-MS elemental analysis to enumerate the iron (Fe) ions associated with 100  $\mu$ g of sample. Bars indicate mean values of at least three biological replicates  $\pm$  SEM.

\* $P < 0.0001$ , \*\* $P < 0.001$ , \* $P < 0.05$ , one-way ANOVA with Tukey's post-hoc test, # $P < 0.05$ , unpaired Student's  $t$  test.

**Table 1.**

*A. baumannii* clinical strains, isolation source, antibiotic resistance, and effect of bovine or human lactoferrin on growth or biofilm

Strain ID	Source	Antibiotic <sup>R</sup> profile	Growth MIC (Bovine Lf)	Growth MIC (Human Lf)	Biofilm Reduction (Bovine Lf)	Biofilm Reduction (Human Lf)
4	bronchial wash	A/S <sup>I</sup> , AK, CAX, CAZ, CFT, CP, CPE, GM, LVX, MER, PI, T/S, TE <sup>I</sup> , TIM	62.5 µg/mL *	62.5 µg/mL *	—	—
5	abdominal cavity	—	62.5 µg/mL **	62.5 µg/mL ****	41% *	62% *
11	urine	A/S <sup>I</sup> , CAX, CAZ, CFT, CP, LVX, MER <sup>I</sup> , PI, T/S, TE, TIM	250 µg/mL *	125 µg/mL **	—	—
13	blood	A/S <sup>I</sup> , AK, CAX, CAZ, CFT, CP, CPE, GM, LVX, MER, PI, T/S, TE <sup>I</sup> , TIM, TO	125 µg/mL *	125 µg/mL ***	77% *	75% *
15	blood	A/S, AK <sup>I</sup> , CAX, CAZ, CFT, CP, CPE, GM, LVX, MER, PI, T/S, TIM, TO	250 µg/mL ****	62.5 µg/mL ****	72% *	68% *
16	sputum	CAX, CAZ, CFT, CP, CPE <sup>I</sup> , GM, LVX, PI, T/S, TE <sup>I</sup> , TIM <sup>I</sup> , TO <sup>I</sup>	250 µg/mL *	125 µg/mL *	32% *	—
17	blood	AK <sup>I</sup> , CAX, CAZ, CFT, CP, CPE, GM, LVX, MER, PI, T/S, TE <sup>I</sup> , TIM, TO	62.5 µg/mL *	62.5 µg/mL **	50% *	70% *
19	wound	CAX <sup>I</sup> , CFT <sup>I</sup>	<1000 µg/mL	<1000 µg/mL	—	41% *
31	sputum	A/S, AK, CAX, CAZ, CFT, CP, CPE, GM, LVX, MER, PI, T/S, TE, TIM, TO	250 µg/mL *	125 µg/mL **	53% *	—
32	Foley catheter	A/S <sup>I</sup> , CAX, CAZ, CFT, CP, CPE, GM, LVX, MER, PI, T/S, TE <sup>I</sup> , TIM, TO	750 µg/mL ****	250 µg/mL **	—	—
35	sputum	—	750 µg/mL **	125 µg/mL *	67% *	76% *
37	wound	A/S <sup>I</sup> , AK, CAX, CAZ, CFT, CP, CPE, GM, LVX <sup>I</sup> , MER, PI, T/S, TE <sup>I</sup> , TIM, TO	250 µg/mL *	750 µg/mL **	—	68% *
38	sputum	A/S <sup>I</sup> , CAX, CAZ, CFT, CP, CPE, GM, LVX <sup>I</sup> , MER, PI, T/S, TIM, TO	750 µg/mL ****	750 µg/mL ****	—	34% *
79	sputum	—	<1000 µg/mL	<1000 µg/mL	—	38% *
81	wound	—	62.5 µg/mL **	125 µg/mL *	—	—
101	wound	—	250 µg/mL *	125 µg/mL *	42% *	70% *
112	sputum	A/S, CAX, CAZ, CFT, CP, CPE, GM, LVX, T/S, TE, TO	750 µg/mL ****	250 µg/mL ****	56% *	84% *

**Key terms and symbols.** *A. baumannii* strain identification (Strain ID). Source of isolation from clinical patient (Source). Antibiotic abbreviations are as follows: Ampicillin-Sulbactam (A/S), amikacin (AK), ceftriaxone (CAX), ceftazidime (CAZ), cefotaxime (CFT), ciprofloxacin (CP), cefepime (CPE), gentamicin (GM), levofloxacin (LVX), meropenem (MER), piperacillin (PI), trimethoprim-sulfamethoxazole (T/S), tetracycline (TE), ticarcillin-K clavulanate (TIM), and tobramycin (TO). Intermediate resistance is designated with a superscript (I). Minimum inhibitory concentration (MIC) was defined as the lowest concentration at which statistically significant inhibition of growth was observed, and biofilm reduction was defined as statistically significant inhibition of bacterial biofilm, determined by one-way one-way ANOVA compared to bacterial grown in medium alone (\*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001). For MIC analyses, bacteria were grown in increasing concentrations of either bovine lactoferrin (Bovine Lf) or human lactoferrin (Human Lf). For biofilm assays, bacteria were grown in medium alone or medium

supplemented with 125 µg/mL Bovine Lf or Human Lf and percent calculated is the mean reduction in biofilm formation compared to cultures grown in medium alone.

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