LUNG SURFACTANT PROTECTS HOST AGAINST STAPHYLOCOCCUS AUREUS PATHOGENESIS

by

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DEDICATION

Dedicated to all those affected by staphylococcal diseases.

Separately dedicated to my father, Vsevolod Predtechenskiy, who argued with doctors trying to prove his Staphylococcus epidermidis infection was not caused by a 'commensal' organism and who passed away during my PhD: with this work, I have contributed to the collective understanding of staphylococcal research. Thank you for directing me to science and instilling in me the belief that I can achieve anything I desire.

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ABSTRACT

Lungs are the first organ to encounter aspirated pathogens and have many lines of defense against these diverse invaders. For the most part, these immunological barriers to infection comprised of resident epithelial cells, mucus clearance, and patrolling immune cells are successful in defending the host from infection. Only in cases of viral infections where such a barrier is breached, is an infectious agent more likely to establish an infection. *Staphylococcus aureus* (*S. aureus*), found in the anterior nares of more than 20% of the population, is known for causing bacterial lung pneumonia after viral infections like influenza. Previous studies have observed a different pattern of virulence factor gene expression in *S. aureus* following influenza A virus compared to *S. aureus* infection only. We hypothesized that either a diseased lung environment triggers *S. aureus* to be more virulent or the healthy lung naturally suppresses *S. aureus* colonization. Previous studies have shown that influenza A targets cells producing lung surfactant. In the current study we investigate the influence of surfactant on *S. aureus* virulence. By assessing *S. aureus* cytotoxicity against immune cells in the presence of lung surfactant, we discovered that lung surfactant protects host immune cells from *S. aureus* toxins. To uncover the mechanism behind this protection, we demonstrated that surfactant down-regulated virulence genes regulated by the SaeR/S two-component gene regulatory system. We also investigated one of the more common fatty acids found in lung surfactant, palmitate, for how it impacted *S. aureus* cytotoxicity and virulence transcription. Palmitate recapitulated the protective phenotype of whole surfactant and modulated *S. aureus* virulence as seen by cytotoxicity and transcriptional assays. Palmitate was also found to directly act on *S. aureus* toxins, although the precise mechanism behind this needs to be elucidated. This work provides a rationale for why healthy *S. aureus* carriers are able to aspirate the bacterium into the lungs and not get primary *S. aureus* pneumonia. Furthermore, these studies reinforce the potential of surfactant replacement therapy as a treatment or prevention strategy for secondary bacterial pneumonia.

CHAPTER ONE

INTRODUCTION TO *STAPHYLOCOCCUS AUREUS* AND LUNG PATHOGENESIS

Staphylococcus aureus

Staphylococcus aureus, also known as *S. aureus*, is a bacterium that colonizes between 20-50% of the human population, and staphylococcal diseases affect around 500,000 individuals every year in the US (Gorwitz *et* al. 2008; Wertheim *et al.* 2005; Mashruwala *et al.* 2017; King *et al*. 2016). Historically, this pathogen was first isolated in 1884 by Friedrich Rosenbach and was named due to its golden color attributed to carotenoid pigments (Adhikari 2021). One hundred-fifty years ago, this skin-inhabiting pathogen was overshadowed by more lethal infective agents behind cholera, smallpox, typhus, yellow fever, and scarlet fever. Yet today, *S. aureus* is on the forefront of global healthcare concern as the organism can infect nearly all tissues ranging from bone, joints, muscle, heart and lung and can effectively spread through the bloodstream leading to morbid clinical consequences. The recognition that *S. aureus* was not only a commensal organism but also a pathogen started in mid-1900s when antibiotics were beginning to be used in hospitals. Throughout the 1960s, methicillin-resistant strains of this bacteria emerged, albeit their sparce incidences were localized to hospitals. Methicillin resistant *S. aureus*, also known as MRSA, spread rapidly in the early 2000s as community-acquired MRSA strains passed between family members and close connections dominated the healthcare scene. One of these more common strains in North America known as USA300 or LAC originating from Los Angeles is a hypervirulent and cytotoxic strain that causes severe skin and soft tissue infections (King *et al*.

2016). Furthermore, resistance also expanded to other antibiotics like lincosamides, macrolides, aminoglycosides, fluoroquinolones, and their various combinations by horizontal gene transfer (von Eiff *et al*. 2001). Now, there are even VRSA (Vancomycin-resistant *S. aureus*) strains of *S. aureus* which are resistant to the last-line antibiotic, vancomycin, although they are not as common due to growth cost. These multi-drug resistant strains have especially been taxing in the context of hospital acquired infections, with MRSA being one of the leading causes of death (Harper *et al.* 2018). Globally, other strains like USA100, USA200, USA400, and USA600 continue to cause many lethal conditions like bacteremia, endocarditis, pneumonia and toxic shock syndrome (King *et al.* 2016). Worldwide, *S. aureus* causes 940,000 deaths annually in individuals older than fifteen (GBD 2022). It is estimated that by 2050, antibiotic resistant bacteria may claim 10 million lives annually (O'Neil 2016), and *S. aureus* will be on the forefront of this global catastrophe.

S. aureus is a pathogen that is very accustomed to inhabiting the human body, surviving in many different environments in both healthy and compromised individuals. It can be found on skin, mucosa, perineum, pharynx, gastrointestinal tract, vagina, axillae, and other diverse bodily systems (Wertheim *et al.* 2005). Most commonly, *S. aureus* is found in the anterior nares. About 20% of the human population are persistent carriers of this bacterium (Chmielowiec-Korzeniowska *et al*. 2020). This subpopulation not only carries higher loads of *S. aureus* but is also more prone to *S. aureus* infections, especially since the endogenous *S. aureus* strain found on the skin or the nares is usually the strain that causes the infection (Wertheim *et al*. 2005; Harper *et al.* 2018). Furthermore, nasal carriage increases the chances of infection post-surgery or in patients receiving dialysis (von Eiff *et al*. 2001). For the 60% of the human population that are intermittent carriers, this bacterium is found in the nares transiently and for various durations. Finally, 20% of

the population are noncarriers who never get colonized by *S. aureus*. Although the exact reasons behind noncarriage are unknown, there are suggestions about incumbent bacterial flora and genetic determinants (Sollid *et al.* 2014).

This pathogen can cause a variety of disease manifestations such as bloodstream infections, pneumonia, bone and joint infections, endocarditis, necrotizing fasciitis, and skin and soft tissue infections due to its wide array of virulence factors. Unlike other pathogens which rely on only a few toxins, this pathogen is able to colonize a variety of host niches due to its virulence arsenal composed of adhesion factors, cytotoxins (hemolysins, cytolytic peptides, and leucocidins), immunomodulatory proteins (superantigens, superantigen-like proteins, and complement-inhibitory proteins), proteases, and factors that interfere with immune cell recognition all of which donate to morbidity and mortality (Spaulding *et al*. 2014; Cheung *et al*. 2021; Alonzo *et al*. 2014). Exotoxins like the cytolytic biocomponent pore-forming leukocidins target a whole range of immune cells like macrophages, monocytes, neutrophils, dendritic cells, B and T cells, and NK cells (Alonzo *et al*. 2014). Because *S. aureus* can avoid both the innate and adaptive immune systems, many therapeutic and vaccine strategies against this bacterium have been rendered ineffective (Alonzo *et al*. 2014).

Because these diverse virulence factors are so potent, *S. aureus* tightly regulates their expression under specific contexts through the use of two component systems. The bacterium is able to make the switch between persisting in its current niche or switching to a more virulent phenotype by measuring bacterial density through quorum sensing two component system Agr and reading environmental stimuli by other systems. While there is low bacterial density, *S. aureus* is able to survive asymptomatically for long periods of time and cause chronic infection in the host

by down-regulating virulence factors until there is a change in the local environment. This chronic phenotype is characterized by a biofilm lifestyle, surviving in various host cells, and developing a silent small-colony variant phenotype with inactive metabolism (Cheung *et al*. 2021). Once there are enough bacteria, *S. aureus* switches to a hypervirulent pathogenic profile with heavy production of toxins and colonization factors. One of the more prominent virulence two component systems important for successful infections is SaeRS, responsible for regulating major virulence factors such as hemolysins, leucocidins, superantigens, surface proteins, and proteases (Voyich *et* al. 2009; Nygaard *et al*. 2010; Liu *et al*. 2014; Montgomery *et al.* 2010). These factors help establish a severe infection in the current location and escape to other niches. Not only can *S. aureus* survive and travel in the blood by utilizing host nutrients by itself, but also the organism has a Trojan-like ability to live in neutrophils allowing it to travel undetected and spread to other locations (Gresham *et al*. 2000). The contexts under which *S. aureus* uses its potent toxins and various virulence factors should be studied in order to thwart the infections process early on.

S. aureus Pneumonia

Pneumonia is a bacterial or viral infection of the lungs where air sacs fill up with pus and are inflamed. There are 24 bacterial pneumonia cases per 10,000 patients every year in the US (Regunath *et al*. 2024). The usual pneumonia bacterial agents are *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus* (Kosai *et al*. 2004). Historically, staphylococcal pneumonia has been common for infants (Prince 2013). Pneumonia caused by USA300 strains of *S. aureus* has been on a steady rise since 2000s, and staphylococcal pneumonia is associated with increased morbidity and mortality in up to 37% of cases (Parker *et al*. 2012; Prince 2013).

A secondary pneumonia (i.e. a bacterial pneumonia following a primary viral infection) or mixed bacterial pneumonia (i.e. bacterial pneumonia that develops simultaneously with a viral infection) is more common than a primary pneumonia (i.e. bacterial or viral pneumonia that develops by itself) and usually is observed in the later course of disease (Kosai *et al*. 2004). Influenza is one of the viral infections where secondary pneumonias are common. About 65% of influenza cases are followed by a bacterial co-infection (Klein *et al*. 2016), and it is the co-infection that increases mortality by 10-15 times compared to just an influenza infection (Xu *et al*. 2016; Heron 2016; Kochanek *et al*. 2019). Hence, in the 1918 Spanish flu which caused 40 million deaths worldwide (Weinheimer *et al*. 2012), up to 95% of the mortalities were linked to the bacterial pneumonia that emerged and not actually due to the initial viral infection (Borgogna *et al*. 2022). Even now, there are between 3,000-49,000 annual deaths from influenza A infections (Weinheimer *et al*. 2012). Of these, many deaths are caused by bacterial pneumonia complications. The health burden of bacterial pneumonia is not only limited to influenza. Bacterial pneumonias are common in cases of other respiratory viral infections like human parainfluenza virus, adenovirus, human metapneumovirus, measles, respiratory syncytial virus, human rhinovirus, and coronavirus (Sharov 2020; Prasso *et al*. 2017).

It is important to understand the disease progression of bacterial lung infections. *S. aureus*, using clumping factor B to attach to nasal epithelial cells, inhabits the anterior nares (Parker *et al*. 2012). The pathogen is inhaled and transported through the trachea, bronchus, and tiny airways into the alveolar lumens. In this space, the bacteria are exposed to pulmonary surfactant which coats alveolar surfaces (Ishii *et al*. 2014). Here, *S. aureus* modifies its virulence factors as it senses that the lung environment is different from that of the nose. For example, the lung environment is

low in nutrients such as iron and is coated in mucus composed of mucin (Pivard *et al*. 2021). Once it's in the lungs, *S. aureus* coordinates expression of surface proteins like microbial surface components recognizing adhesive matrix molecules for colonization, iron acquisition systems, and factors to evade the immune system (Parker *et al*. 2012). *S. aureus* uses fibronectin binding proteins to adhere to airway epithelial cells and get internalized (Parker *et al*. 2012). In airway epithelial cells, Hla toxin is known to affect calcium fluxes, pro-inflammatory signaling, change of ciliary beat frequency, increased vascular leakage, and permeabilization of epithelial cells (Parker *et al*. 2012; Bubeck Wardenburg *et al*. 2008). After adhering and impairing epithelial and mucosal lining, *S. aureus* evades the complement system with Protein A, Sbi, SCIN, and CHIPS (Prince 2013). It is also in this space that *S. aureus* confronts or evades neutrophil and alveolar macrophages and shifts the cytokine responses (Prasso *et al*. 2017). *S. aureus* two component system Agr up-regulates prominent damaging virulence factors such as Hla, phenol-soluble modulins, and LukAB (Kitur *et al*. 2015). Hla toxin targets ADAM10 in the lungs, enabling systemic dissemination and increased pathology, as well as stimulates production of highly inflammatory cytokines in the lung (Kitur *et al*. 2015). *S. aureus* uses PVL toxin to target alveolar macrophages, and PVL aids in increased colonization of alveolar airspaces (Prince *et al*. 2017). *S. aureus* also uses its wide array of cytolytic toxins to combat neutrophils, as neutrophils are critical for *S. aureus* clearance from the lung (Prince 2013). *S. aureus* can persist intracellularly in epithelial cells, macrophages, and neutrophils, and with the help of Agr, the organism can escape and continue to colonize (Parker *et al.* 2012). Other than Agr system, the SaeRS two-component system has also been found particularly important for a severe lung infection, and its responsible for regulating many of the virulence factors mentioned above. Influenza has been shown to

modulate the lung environment to then up-regulate *S. aureus* virulence through the SaeRS system which controls Hla, HlgABC, LukAB, and PVL (Borgogna *et al*. 2018; Montgomery *et al*. 2010). Once *S. aureus* determines there is enough bacteria with Agr quorum sensing, it then proceeds further with dissemination and colonization of other niches (Pivard *et al*. 2021). Due to the numerous virulence factors detailed above, USA300 strains of *S. aureus* are known to cause highly inflammatory necrotizing pneumonia, with fatal MRSA pneumonia cases characterized by lack of alveolar architecture, extensive fluid accumulation, hemorrhage, and lung parenchyma consolidation (Kitur *et al*. 2015; Parker *et al*. 2012; Prince 2013).

Lung Surfactant in the Context of Infections

To successfully launch a bacterial lung infection, bacteria must first reach the lungs and dominate the primary interactions that happen between the bacteria and the host. In the case of *S. aureus*, it has an ease of passage since it already inhabits the anterior nares. Although both intermittent carriers and persistent carriers of nasal *S. aureus* breathe the bacteria into their lungs daily, we do not see many primary *S. aureus* pneumonias because the host defenses successfully clear the pathogens. However, by studying when this defense is breached (i.e. bacterial pneumonia after a primary viral infection), we can elucidate the critical defenses of the host. In order to do this, we must investigate the changes in the lung environment that are moderated by the viral infection. For example, influenza is known to infect and replicate in type II pneumocytes to then release virions (Weinheimer *et al.* 2012). The primary role of type II pneumocytes in the lungs is to produce lung surfactant. Without these cells, less surfactant is made, and it is not regulated properly. Abnormal levels of lung surfactant are observed in lung diseases like ARDS, pulmonary fibrosis, emphysema, cystic fibrosis, COPD, and RDS in neonates (Chroneos *et al*. 2010).

Furthermore, drastic changes in pulmonary surfactant are related to inflammation in RSV pneumonias (Shan *et al*. 2018). SARS-CoV-2 infections are similarly characterized by damage to type II pneumocytes and loss of surfactant followed by deadly bacterial pneumonia (Mirastschijski *et al*. 2020; Ji *et al*. 2021).

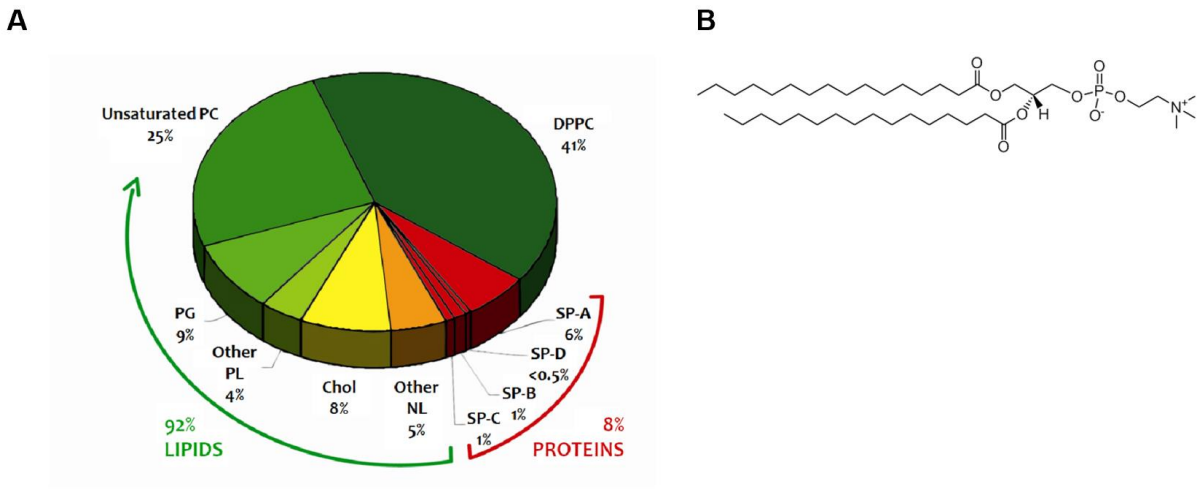

Figure 1. Composition of lung surfactant. A) Obtained from bronchoalveolar lavages of mammalian lungs. PL: phospholipids; PC: phosphatidylcholine; DPPC: dipalmitoyl phosphatidylcholine; PG: phosphatidylglycerol; Chol: cholesterol; NL: neutral lipids; SP-A/B/C/D: surfactant protein A/B/C/D. Taken from Parra Ortiz 2013. B) Structure of DPPC.

This aforementioned lung surfactant is a lipid-protein complex composed of 90% lipids and 10% proteins (Huck *et al*. 2021). Most lipids are phospholipids, of which dipalmitoylphosphatidylcholine or DPPC is the most abundant, as seen in Figure 1A. There are four surfactant proteins, SP-A, SP-B, SP-C, SP-D, that are responsible for the structure and functionality of the surfactant. DPPC enriched with bilayer lipids and proteins forms a monolayer surface film which reduces surface tension and prevents collapse of the lungs during exhalation. This is due to DPPC supporting high order states during compression and surfactant proteins

facilitating interfacial spreading and absorption (Huck *et al*. 2021). Although surfactant is produced and maintained by type II pneumocytes, it is also taken up again, processed, and re-secreted as recycled material by the same cells (Andreeva *et al*. 2007). Otherwise, surfactant is cleared by internalization by epithelial cells or alveolar macrophages (Wright 1990). Hence, there is constant maintenance and screening of the surfactant for pathogens. A lack of lung surfactant during a primary viral infection may be the reason behind the establishment of a secondary bacterial infection. It has also been suggested that bacteria damage the lung surfactant by producing free radicals that cause lipid peroxidation, and this furthers the progression of pneumonia (Bouhafs *et al*. 1999). Given the involvement of this liquid during infections, this work aimed to see if lung surfactant itself has a protective role against lung pathogens.

In lung surfactant, there are several lipid groups, with DPPC being the most abundant at about 40% of the surfactant composition (Figure 1A). DPPC itself is composed of two groups of palmitic acid attached to phosphatidylcholine headgroup (Figure 1B). Thus, this palmitic acid, also known as palmitate, is the most abundant free fatty acid in pulmonary surfactant, making up about 80% of total fatty acid in lung surfactant (Ishii *et al*. 2014; Schmidt *et al*. 2002). This is one of the reasons we chose to investigate this compound, with the other reason being a plethora of research into how fatty acids have antibacterial effects. This is unsurprising, as *S. aureus* often comes in contact with fatty acids in the host, not only in the lungs, but also in the blood and plasma, nasal mucosa, and other tissues (Kengmo Tchoupa *et al*. 2020; Kuiack *et al*. 2023). Skin specifically secretes fatty acids in a lipid mixture for their antimicrobial activity, and there is enough research about how *S. aureus*, a commensal skin organism, develops resistance to one of the more potent unsaturated fatty acids, sapienic acid (Arsic *et* al. 2012; Neumann *et al*. 2015). In the anterior nares,

S. aureus comes in contact with sapienic and linoleic unsaturated fatty acids in nasal secretions (Arsic *et al*. 2012). Monoglycerides and fatty acids have been regarded as antibacterial lipids due to their ability to destabilize bacterial cell membranes, induce cellular component leakage, disrupt respiration, and inactivate membrane-bound proteins (Yoon *et al*. 2018; Kuiack *et al*. 2023; DeMars *et al*. 2021). Although, not all fatty acids have the same effectiveness: with unsaturated fatty acids being more potent than saturated acids and medium chain sized fatty acids being able to impact the fluidity and compactness of the bacterial membrane the most (DeMars *et al*. 2021). Particularly, unsaturated fatty acids like linoleic acid, palmitoleic, and sapienic acid have been reported to have the greatest antibacterial effects (Kengmo Tchoupa *et al*. 2020; Arsic *et al*. 2012). Palmitate, a saturated fatty acid, inhibits many different bacterial species like *Staphylococcus* sp., Pneumococci, and *Streptococcus* group A and D among many others (Yoon *et al*. 2018). To combat toxic effects of fatty acids, *S. aureus* can incorporate fatty acids from the host into phospholipids via fatty acid phosphorylation with the help of fatty acid kinase (Kengmo Tchoupa *et al*. 2020; Ridder *et al*. 2020; DeMars *et al*. 2020). Fatty acids have also been involved with SaeRS two component system. In particular, an overabundance of fatty acids shuts down the SaeRS system through an interplay between respiratory activity, redox balance, and SaeRS activity during growth in fatty acids (DeMars *et al*. 2021). Hence, fatty acid kinase maintains a low fatty acid pool by incorporating the inhibitory molecules into phospholipids to be inserted in its own membrane, which indirectly activates the production of virulence factors by SaeRS (Ericson *et al*. 2017). Thus, the presence of host fatty acids like palmitate stimulates *S. aureus* pathogenesis.

Hypothesis

Staphylococcus aureus (*S. aureus*) is a common pathogen living in the anterior nares that gets aspirated into the lungs daily. Although *S. aureus* is a frequent cause of secondary bacterial infections after a primary viral infection, primary bacterial pneumonia is uncommon in healthy hosts. It remains a question whether aspirated *S. aureus* is repressed by a healthy lung environment. Nitric oxide has been postulated to inhibit *S. aureus* virulence in the nasal space (Urbano *et al*. 2018) so repression of virulence in the lung is a possibility. Alternatively, a diseased lung environment may trigger *S. aureus* virulence. To begin to answer this question, the key is to examine existing lung defenses and the changes that convert a normal healthy lung into a diseased one. Previous research heavily focused on the host response to infections, such as influenza A and the followings immune response including type I interferon induction (Lee *et al*. 2010; Parker *et al*. 2012). However, the bacterial pathogen that is capable of sensing and responding to changes in environments also dictates the outcome under superinfection conditions (e.g. influenza A followed by *S. aureus*) (Guerra *et al*. 2017). Currently, there is a gap in understanding of how *S. aureus* senses and responds to a diseased lung state. Several prior observations led to investigation into this area. Earlier studies by our group observed that an influenza A virus lung infection prior to a *S. aureus* lung infection changed the virulence profile of *S. aureus* (Borgogna *et al*. 2018). There are reports on how a primary viral infection (including those caused by influenza A virus) destabilizes the lung by affecting lung surfactant production (Mirastschijski *et al*. 2020; Ji *et al*. 2021), specifically influenza A virus targets type II pneumocytes that produce lung surfactant (Weinheimer *et al.* 2012). Hence, we thought to investigate the role of surfactant in modulating *S. aureus* virulence. The overarching hypothesis of this work is that **pulmonary surfactant protects**

the host from *S. aureus* lung infection by modulating virulence and interaction with immune cells.

Chapter Two directly tests this hypothesis by assessing how lung surfactants from mouse, rat and a commercially available surfactant (of bovine origin) impact *S. aureus* virulence and cytotoxicity against immune cells. Data from this work confirm that lung surfactants have a protective role against *S. aureus* by down-regulating SaeRS-dependent virulence factors. Lung surfactant interferes with the cytotoxicity of these factors against neutrophils and other immune cells. To determine the mechanism, Chapter Three of this work investigates a major component found in lung surfactant, palmitate, and whether the effects observed in Chapter Two are due to this component. Palmitate was able to recapitulate a lot of the phenotype observed in whole surfactant, establishing that lipid components in lung surfactant suppress *S. aureus* virulence. Ongoing work, delineated in Chapter Four, assesses the bacterial response to palmitate. Of the virulence factors secreted by *S. aureus* in response to palmitate, cysteine protease SspB was identified by SDS-PAGE experiments and evaluated for its capacity to modulate *S. aureus* virulence following exposure to palmitate. Collectively, this work underscores the importance of lung surfactant in protecting the host from *S. aureus* pathogenesis by affecting the toxins regulated by the SaeRS system and provides rationale for lung surfactant therapy for treating secondary bacterial infections.

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CHAPTER TWO

LUNG SURFACTANT REDUCES *STAPHYLOCOCCUS AUREUS* TOXIN PRODUCTION AND PROTECTS HOST IMMUNE CELLS FROM MEMBRANE DAMAGE

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Lung Surfactant Reduces *Staphylococcus aureus* Toxin Production and Protects Host Immune Cells from Membrane Damage

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ABSTRACT

In this study, we identify that lung surfactant significantly reduces production of *Staphylococcus aureus* (*S. aureus*) membrane damaging toxins. Data demonstrate that natural surfactant from mice and rats and commercially available surfactant Infasurf® protect human primary cells (neutrophils and peripheral blood mononuclear cells) from *S. aureus* cytolytic activity. Supernatants from *S. aureus* grown in surfactant showed significant reduction in plasma membrane damage against primary human cells as compared to supernatants grown without surfactant. This reduction was not due to a direct bactericidal effect of the surfactants on *S. aureus* growth. Rat and mouse surfactants downregulated the gene expression of the *S. aureus* two-component system *saeR/S* that is responsible for production of virulence factors which are important during lung infection and cause membrane damage in host cells. Rat and lung surfactants also reduced transcript abundance of SaeR/S-regulated genes *lukF*-PV, *hla*, and *hlgA*. Interestingly, commercially available surfactant Infasurf® did not recapitulate the effect of natural surfactants and did not decrease gene transcription of the virulence genes tested. These data suggest that components of surfactant protect lungs from *S. aureus* by suppressing *S. aureus* virulence factors and have implications for the role of surfactant in host defense against *S. aureus*.

IMPORTANCE

This study explored the influence of lung surfactant on *Staphylococcus aureus* (*S. aureus*) toxin production. We demonstrate that natural and commercially available lung surfactants minimize the cytolytic capacity of *S. aureus* supernatants against primary human cells. Data indicate that reduction was partially due to surfactants reducing transcript abundance of virulence factors. This work identifies a novel role for surfactant and suggests its importance in modulating severity of *S. aureus* lung infections.

INTRODUCTION

Pulmonary surfactant is a lipid-rich complex within the alveoli that prevents atelectasis during respiration by maintaining surface tension at the air-liquid interface (1, 2). Lung surfactant predominantly produced

by alveolar type II epithelial cells and is composed of 90% lipids, of which dipalmitoylphosphatidylcholine (DPPC) is the most abundant (3), and 10% proteins (1-4). The four lung surfactant proteins important for alveolar integrity and surfactant homeostasis (5) are SP-A, SP-B, SP-C, and SP-D, of which SP-B and SP-C are responsible for the structural stability of the surfactant (2, 6). Historically, lung surfactant has primarily been described as an interface for the oxygenation of our bodies; however, more recently data has emerged showing that lung surfactant has immunological properties (7, 8). Components of lung surfactant play a role in anti-angiogenesis (9) and can inhibit the generation of reactive oxygen intermediates in neutrophils and monocytes (10). Vesicles containing lung surfactant components, like DPPC, have been shown to decrease macrophage inflammation by inducing expression of innate immune receptors, including Fc receptors, CD11b, scavenger and mannose receptors, and complement receptor CR1 or interfering with toll-like receptor mediated inflammatory responses (3).

In addition, abnormal levels of lung surfactant have been associated with many respiratory problems, among which are pulmonary fibrosis, cystic fibrosis, and COPD, (3) as well as during infection e.g. influenza A virus (11). During lower respiratory infections with influenza A virus, alveolar type II cells are preferentially targeted (12). Infection of these cells leads to measurable disruptions in surfactant production (13). In a previous study, we identified that antecedent influenza A virus infection in mice increased virulence gene expression in *S. aureus* compared to virulence gene expression during *S. aureus* lung infection only (14). Taken together, these observations provided the basis for the current study to investigate whether surfactant may directly impact virulence gene expression and toxin production in *S. aureus*. Our results demonstrate that the presence of natural as well as commercially available lung surfactants prevent membrane damage in neutrophils and peripheral blood mononuclear cells by *S. aureus* toxins. Murine and rat lung surfactants repressed transcription of *S. aureus* secreted toxins, whereas commercially available surfactant did not decrease transcripts of virulence genes tested. This work adds to our knowledge of the role of surfactant in healthy lungs and implies that lung surfactant has a role in reducing the pathogenicity of bacterial infections.

MATERIALS AND METHODS

Bacteria Strains and Culture Conditions

Staphylococcus aureus (*S. aureus*) PFGE-type USA300 strain LAC (15) was used in all experiments. Unless noted otherwise, overnight and sub-cultured bacteria (1:100 dilution of overnight) were grown in 20 mL of tryptic soy broth (TSB; EMD Millipore) supplemented with 0.5% glucose with shaking (250 rpm) at 37°C. Optical density at 600 nm (OD_{600}) was measured using a NanoDrop 2000c Spectrophotometer (ThermoFisher Scientific) and colony forming units (CFUs) were determined by plating diluted samples on tryptic soy agar (TSA; EMD Millipore) and enumerated the following day.

Lung Surfactant Extraction and Growth Assays

Pulmonary surfactant isolation was performed using an adaptation of the method described in Inselman *et al.* (16). Briefly, murine (C57BL/6) or rat (F344BN) lung tissue (0.2 g) was homogenized in 3 mL of ice-cold Dulbecco's phosphate buffered saline (DPBS) using a tissue grinder. Homogenate slurry was passed through a 70 µm cell strainer followed by centrifugation at 300 x g for 10 minutes at 4°C. The supernatant was collected and placed in microcentrifuge tubes for centrifugation at 18,000 x g for 30 minutes at 4°C. The resulting supernatant was aspirated and discarded. The remaining surfactant pellet was resuspended in 1 mL of DPBS at 60°C. For experiments, this was considered 100% surfactant. Research grade Infasurf® was purchased from Onybiotech. For experiments investigating growth in surfactant, 1 mL of surfactant (at varied concentrations) was added to 4 mL of TSB pre-warmed to 37°C and inoculated with 50 µL of overnight culture. Samples were incubated for five hours at 37°C with shaking (250 rpm).

Relative Quantitative Real Time RT-PCR

Transcription of *S. aureus* genes was assessed using TaqMan® real time reverse transcriptase-PCR (RT-PCR) as previously described (17-19). Briefly, sub-cultured strains were harvested at mid-exponential (ME; OD_{600} = 1.5) or early stationary (ES; OD_{600} = 3.0) phase of growth, mechanically disrupted using a

FastPrep FP120 cell disrupter (ThermoFisher Scientific), and RNA purified using RNeasy Kit (Qiagen) as described in (17). TaqMan® real-time RT-PCR was performed using primer and probe sets as published previously (14).

Human PMN or PBMC Plasma Membrane Integrity Assays

Heparinized venous blood from healthy donors was collected in accordance with a protocol approved by the Institutional Review Board for Human Subjects at Montana State University. All donors provided written consent to participate in the study. Human polymorphonuclear leukocytes (neutrophils or PMNs) and/or peripheral blood mononuclear cells (PBMCs) were isolated under endotoxin-free conditions (<25.0 pg/ml) and cell viability and purity of preparations were assessed using a FACSCalibur Flow cytometer (BD Biosciences) as described in (17) and (20). Assays intoxicating PMNs with extracellular *S. aureus* proteins were performed as previously described (21, 22). Briefly, supernatants from *S. aureus* sub-cultured for 5 h in TSB with glucose were sterile-filtered (0.22 µm, Avantor) and diluted in DPBS. PMNs or PBMCs ($1x10^6$) were exposed to varied dilutions of *S. aureus* supernatant. Samples were incubated at 37°C for 60 min then stained with propidium iodide (PI; ThermoFisher Scientific) following the manufacturer's protocol and then analyzed by FACS as in (22).

RESULTS

Lung surfactant protects immune cells from *S. aureus* toxin-mediated membrane damage.

To test the hypothesis that surfactant influences *S. aureus* virulence, we investigated the role of surfactant on *S. aureus* toxin production. In these assays, we compared plasma membrane damage in human neutrophils and peripheral blood mononuclear cells (PBMCs) following exposure to *S. aureus* supernatants grown in the presence or absence of varied concentrations of mouse or rat lung surfactant (Fig 1A-D). Neutrophils exposed to supernatants grown in 10% mouse surfactant demonstrated a significant reduction in plasma membrane damage and had an average of 2.39±0.39% propidium iodide

(PI)-positive cells compared to 47.39±8.63% PI-positive cells from supernatants grown in TSB only (Fig 1B). *S. aureus* grown in rat surfactant also demonstrated significantly reduced plasma membrane damage e.g. 3.17±0.33% PI positive in 10% rat surfactant compared to neutrophils exposed to supernatants from *S. aureus* grown in TSB only at 60.67±8.44% PI-positive (Fig 1C). However, unlike mouse surfactant, rat surfactant maintained its ability to significantly reduce plasma membrane damage at a concentration as low as 0.2%. Similarly, growth in mouse or rat surfactant significantly reduced PBMC plasma membrane damage from *S. aureus* supernatants. PBMCs exposed to supernatants harvested from *S. aureus* grown in 10% mouse surfactant or 2% rat surfactant were 8.26±0.72% and 13.70±2.68% PI-positive, respectively, compared to control at 38.90±8.50% PI-positive (Fig 1D). The reduction of membrane damage in cells was not due to decreased bacterial growth in the presence of surfactants and no significant differences in bacterial growth was seen in cultures grown with or without surfactant (Figure 1E and 1F).

We next assessed whether this same protective effect could be observed with commercially available surfactant. For these experiments, *S. aureus* was grown in varied concentrations of Infasurf®. Similar to above, supernatants were harvested and plasma membrane damage in neutrophils and PBMCs was assessed. Growth in 1% Infasurf® provided significantly reduced PI uptake in PMNs. An average of 12.31±2.79% propidium iodide (PI)-positive cells compared to 38.53±6.05% PI-positive cells from supernatants grown in TSB only was observed (Fig 2A). This concentration of Infasurf® also reduced death of PBMCs. Without surfactant, PBMCs were 33.21±3.31% PI-positive when exposed to supernatants versus 23.80±2.75% PI-positive with 1% Infasurf® (Fig 2B). As with mouse and rat surfactants, growth in Infasurf® did not impact bacterial viability (Fig 2C). Interestingly, higher Infasurf® concentrations no longer protected neutrophil cells from membrane damage from *S. aureus* supernatants (Figure 2A). It has been shown with another commercially available artificial surfactant, Surfactant TA®, that neutrophils treated with this surfactant had changes consistent with apoptosis (23). We tested if plasma membrane damage was occurring at higher concentrations of Infasurf®. However,

we found that higher concentrations of Infasurf® without any *S. aureus* supernatants did not yield a higher amount of propidium iodide (PI)-positive cells (Supplemental Figure 1).

Lung surfactant modulates *S. aureus* gene expression.

To investigate if the reduction of *S. aureus* toxin production in supernatants was due to an effect of surfactant on transcription of genes, we first investigated transcript abundance of *saeR*, the response regulator of the *S. aureus* SaeR/S system. The SaeR/S system is a two-component system of *S. aureus* responsible for controlling virulence gene expression (24-26). Additionally, we and others have previously identified a role for SaeR/S in *S. aureus* lung infections (14, 25). In the current study, *S. aureus* was grown to mid-logarithmic and early stationary phases of growth with or without surfactant. The relative fold decrease of *saeR* compared to control was: -0.29±0.04 for mouse surfactant and 0.30±0.11 for rat surfactant at mid-logarithmic phase (Figure 3A). Transcript was also reduced at early stationary phase (-0.40±0.01 for mouse surfactant and -0.48±0.22 for rat surfactant) (Fig 3A). For growth in Infasurf®, the relative fold change of *saeR* compared to control was: +0.14±0.05 in mid-logarithmic phase and -0.04±0.30 in early stationary phase (Fig 3B).

We next assessed *S. aureus* virulence genes, *lukF*-PV, *hla*, and *hlgA* that contain the SaeR-binding domain (20) and that have been shown to be differentially regulated during murine lung infection (14). Compared to control treatment with DPBS, we observed decreases in the abundance of transcripts encoding various toxins when *S. aureus* was grown to either mid-logarithmic or early stationary phase with mouse and rat surfactants (Fig 4). At mid-log phase, the relative fold decrease of *lukF*-PV, *hla*, and *hlgA* compared to control for mouse surfactant was: -0.68±0.33, -1.14±0.23, -0.18±0.01, respectively (Fig 4A). Similarly, the relative fold decrease of *lukF*-PV, *hla*, and *hlgA* compared to control for rat surfactant was: -0.43±0.26, -0.61±0.12, -0.12±0.04, respectively (Fig 4C). At early stationary phase, the relative fold decrease of *lukF*-PV, *hla*, and *hlgA* compared to control for mouse surfactant was: -0.80±0.15, -0.57±0.33, -0.23±0.05, respectively (Fig 4B). For rat surfactant, the relative fold decrease of *lukF*-PV, *hla*, and *hlgA* compared to control was: -0.83±0.22, -0.57±0.30, -0.37±0.18, respectively (Fig 4D).

In contrast to the results seen with natural surfactant, presence of Infasurf® upregulated *S. aureus* virulence transcripts by mid-log phase. When grown in Infasurf® to mid-log phase, *lukF*-PV, *hla*, and *hlgA* expression was upregulated compared to control by: +0.56±0.28, +0.12±0.09, +0.04±0.17, respectively (Fig 5A). When grown in Infasurf® to early stationary phase, there was a reduction in *lukF*-PV, and *hla* compared to control: -0.03±0.06, -0.24±0.20, respectively (Fig 5B). However, *hlgA* was increased in the presence of surfactant as seen in mid-exponential (+0.19±0.07) (Fig 5B).

DISCUSSION

In this study we demonstrated that when *S. aureus* is grown in the presence of mouse, rat and commercially available lung surfactant Infasurf®, there was a significant decrease in the membrane damaging ability of secreted *S. aureus* toxins against human neutrophils and PBMCs (Figures 1 and 2). With concentrations of Infasurf® over 1%, we noticed increased membrane damage in cells. However, this was not due to a direct effect of Infasurf® on neutrophil or PBMC membrane integrity (Supplemental Figure 1). We also confirm that decreased cytotoxicity against these immune cells was not due to surfactant directly impacting *S. aureus* growth. This is an important observation since others have shown that surfactant components, including SP-A, SP-D (27) and free fatty acids (28) can be bactericidal.

To investigate if the decrease in cytotoxicity in neutrophils and PBMCs was regulated at the level of transcription, we first investigated gene expression of *saeR*, the response regulator of the two-component gene regulatory system SaeR/S. SaeR/S regulates numerous adhesins, toxins, and immunomodulatory proteins important in neutrophil evasion (29) and is important in mouse models of *S. aureus* lung infections (14, 25). Specifically, we demonstrated that *S. aureus* pathogenesis following antecedent influenza A infection was SaeR/S dependent (14). Growth of *S. aureus* to mid-log and early stationary phases in the presence of mouse and rat surfactants decreased *saeR* transcript abundance (Figure 3). Genes regulated by SaeR/S that encode proteins associated with plasma membrane damage (30) and associated with *S. aureus* lung infections (14, 25, 30) were also downregulated, suggesting that surfactants regulate toxin production at the level of gene transcription.

Our observations with Infasurf® were inconclusive. This surfactant slightly increased the transcription of virulence genes and upregulated *saeR* at mid-logarithmic phase and had a varied response at early stationary phase. Similar to our results, a report published by Ishii *et al*. tested another commercially available surfactant, Surfacten®, that like Infasurf® is derived from bovine lungs (31). Although there were differences in experimental conditions, including strain used, growth conditions and assay used for transcript analysis, this study demonstrated that *lukF*-PV, *hla*, and *hlgA* were not significantly influenced by the surfactant at late logarithmic phase (31). We conclude that Infasurf® may be protecting cells through another mechanism or perhaps the timing of when it impacts virulence gene expression is different than that observed with the mouse and rat surfactants.

The observed differences in the influence of surfactants on *S. aureus* virulence gene expression and toxin production may be due to differences in mammalian surfactant composition. It is known that rat surfactant has a higher phosphatidylcholine concentration than mouse surfactant which is different in composition from bovine surfactant (32). Specific surfactant lipids which have been shown to attenuate inflammation and alter the host response (33) may be at different concentrations in these surfactants. There are reports that lung surfactant proteins SP-A and SP-D are involved in pathogen opsonization (3, 5), puncturing microbial membranes (3, 27), suppressing microbial growth, aiding in detoxifying bacterial LPS, and modulating phagocytosis and inflammatory responses by alveolar macrophages (3, 4). SP-D has also been shown to be able to bind to immune cell receptors, modulate complement activation, and regulate activity of phagocytic cells (5). Ishii *et al*. suggested lipid components in surfactant like palmitate can increase virulence expression of *S. aureus* (31). The authors suggest surfactant components may cause membrane stress, triggering *S. aureus* virulence gene expression through stress response regulator SigB (31). There are other reports of free fatty acids which induce membrane stress and impair growth of *S. aureus* (28, 34). However, our concentration of surfactant did not impair *S. aureus* growth. Future studies will investigate the differences in composition of surfactants of both lipids and proteins to determine the effectors responsible for our observations of reduced virulence gene expression and secreted toxin

production following growth or exposure to surfactant. Future studies will also investigate additional commercially available surfactants. Of note, at the time of this study we were unable to obtain other commercially available surfactants for research purposes.

In summary, this study identified a putative role for surfactant in protection of the lung from *S. aureus* infection. Our data suggest that surfactant may provide a first line of defense in healthy lungs against *S. aureus* infection. This would provide a logical explanation for the observation that despite the high number of individuals who are colonized with *S. aureus* in the nares (29, 35), providing frequent opportunity for exposure to the lungs due to natural aspiration, primary *S. aureus* lung infections are not common. We hypothesize that surfactant suppresses *S. aureus* virulence so the pathogen can be cleared with innate immune defenses. It follows that disruption of surfactant abundance or composition may predispose individuals to *S. aureus* lung infection as occurs following influenza A virus infections (14).

AUTHOR CONTRIBUTIONS

JV, TB, and MP contributed to the conception and design of this study. MP, CA, SS, and TB performed experiments and data analysis. JV and MP wrote and prepared the manuscript for submission. All authors read and approved this manuscript.

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FIGURE LEGENDS

Figure 1. Surfactant from mouse and rat lungs protect human primary cells from *S. aureus*-mediated cytotoxicity. A) Representative flow cytometry plots of data displayed in B) and C). *S. aureus* was grown to early stationary phase in TSB with varied dilutions of B) mouse or C) rat lung surfactant. *S. aureus* supernatants were harvested, diluted to 1:50 final concentration, and incubated with human PMNs for one hour. Plasma membrane damage was assessed using propidium iodide (PI) uptake and analyzed by flow cytometry. D) Human peripheral blood mononuclear cells (PBMCs) exposed to *S. aureus* supernatants diluted to 1:5 final concentration following growth in 10% mouse or 2% rat surfactant (concentration determined in B and C), and plasma membrane damage assessed with PI. *S. aureus* CFUs collected after growth in mouse (E) or rat surfactant (F). Dash (-) represents cells without exposure to supernatants or surfactant while 0% represents cells with exposure to only supernatants. Data are from 3 biological replicates for B), D), E) and F), 6 biological replicates for C) and F). *P <0.05, **P < 0.005 and ****P < 0.0001 one-way ANOVA followed by Dunnett's multiple comparison test. NS = not significant.

Figure 2. Commercially available surfactant Infasurf® protects human primary cells from *S. aureus*-mediated cytotoxicity. A) *S. aureus* was grown to early stationary phase in TSB with varied dilutions of Infasurf® and *S. aureus* supernatants were harvested, diluted to 1:50 final dilution, and incubated with human PMNs for one hour. Plasma membrane damage was assessed by PI staining and flow cytometry. B) Human peripheral blood mononuclear cells were exposed to *S. aureus* supernatants harvested as in (A) following growth in 1% Infasurf® (determined in A), and plasma membrane damage assessed. C) *S. aureus* CFUs collected after growth in Infasurf®. Dash (-) represents cells without exposure to supernatants or surfactant while 0% represents cells with exposure to only supernatants. Data are from 5 biological replicates for A) and C), and 8 biological replicates for B). *P <0.05 and **P <

0.005 one-way ANOVA followed by Dunnett's multiple comparison test for A), B), and C). NS = not significant.

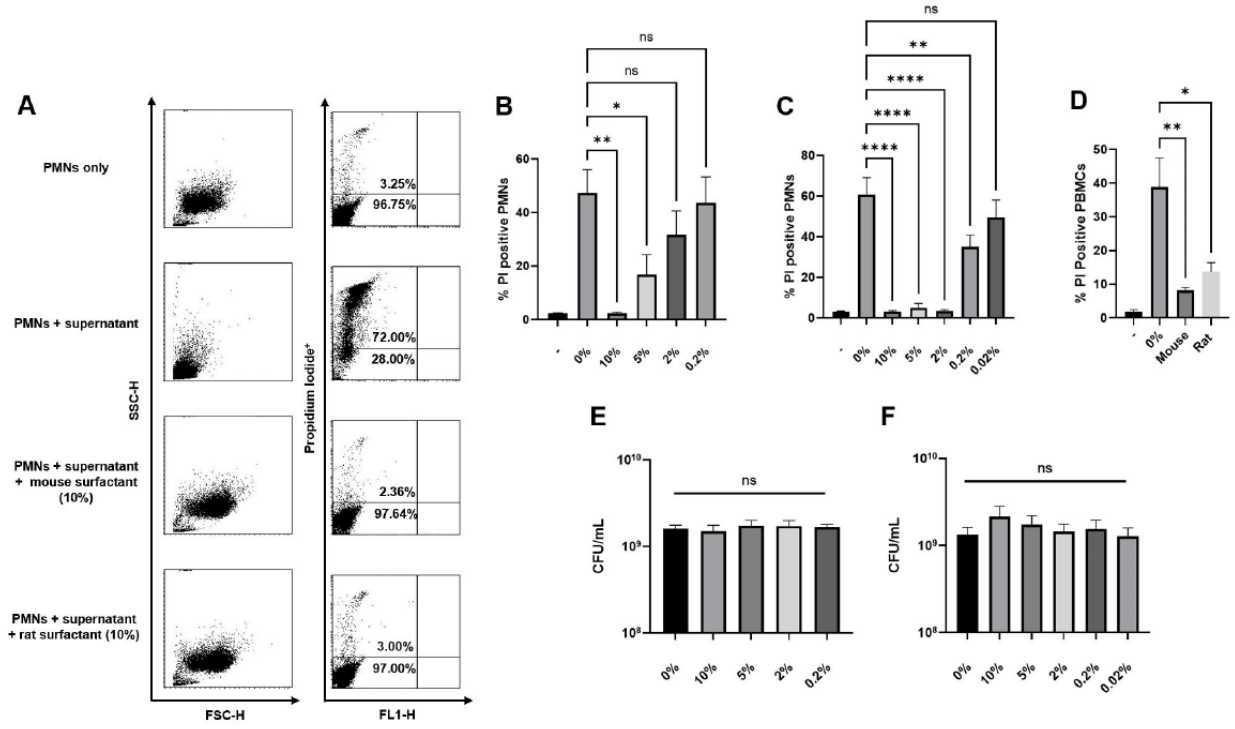
Figure 3. Lung surfactants impact transcription of *S. aureus saeR*. *S. aureus* was grown in mouse (black) or rat (gray) surfactant (A) or Infasurf® (B) to mid-logarithmic or early stationary phase. RNA was harvested and subjected to TaqMan® RT-PCR. Gene transcripts were normalized to *gyrB*. Data shown are the mean fold-change of *S. aureus saeR* relative to treatment with *S. aureus* only. Error bars indicate mean ± SEM of 3 biological replicates for each surfactant tested.

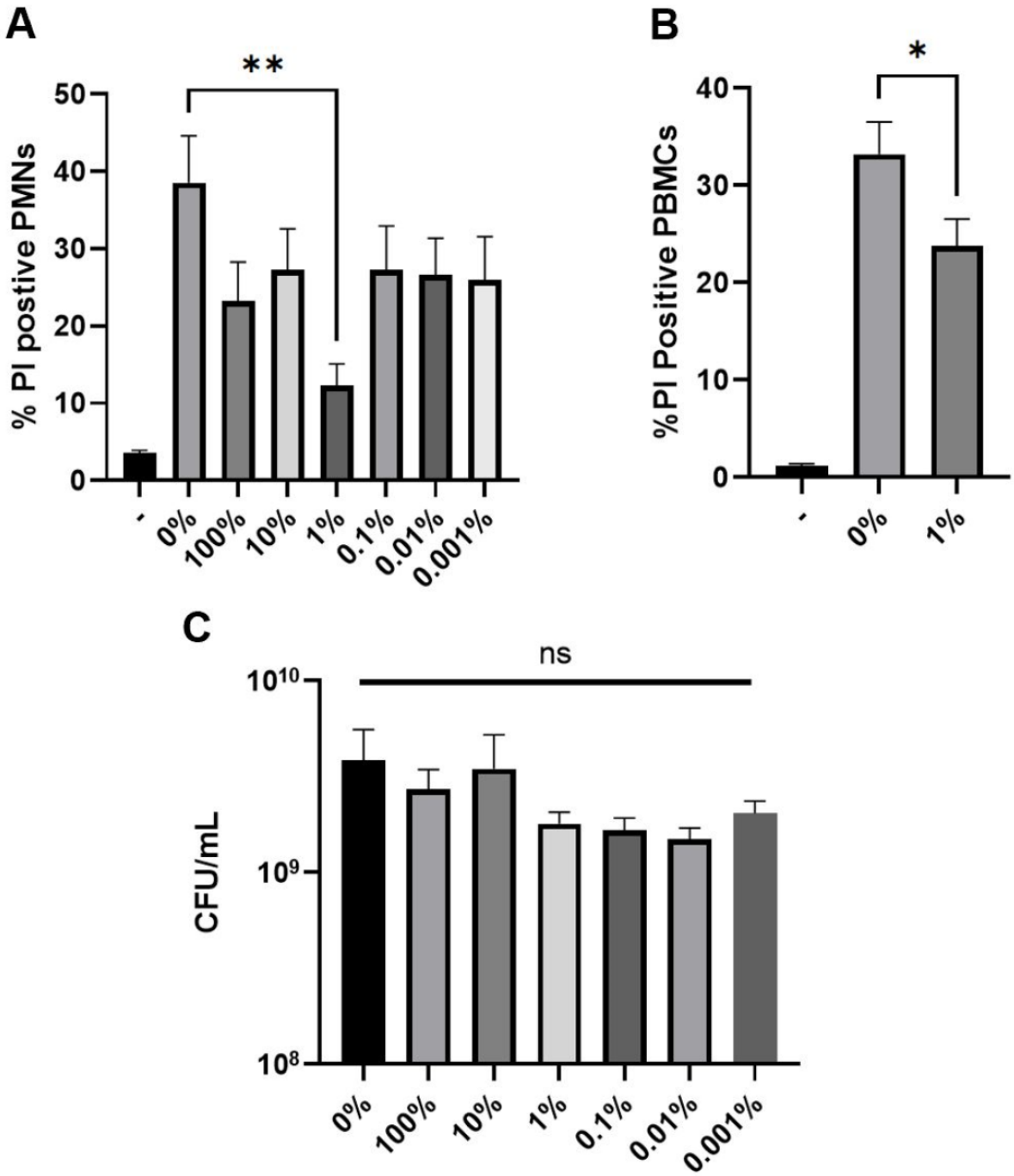
Figure 4. Mouse and rat lung surfactants decrease transcription of *S. aureus* virulence genes. *S. aureus* was grown in mouse (A and B black bars) or rat surfactant (C and D gray bars) to mid-logarithmic phase (A and C) or early stationary phase (B and D) of growth. RNA was harvested and subjected to TaqMan® RT-PCR. Gene transcripts were normalized to *gyrB*. Data shown are the mean fold-change of indicated gene relative to treatment with *S. aureus* only. Error bars indicate mean ± SEM of 3 biological replicates for each surfactant tested.

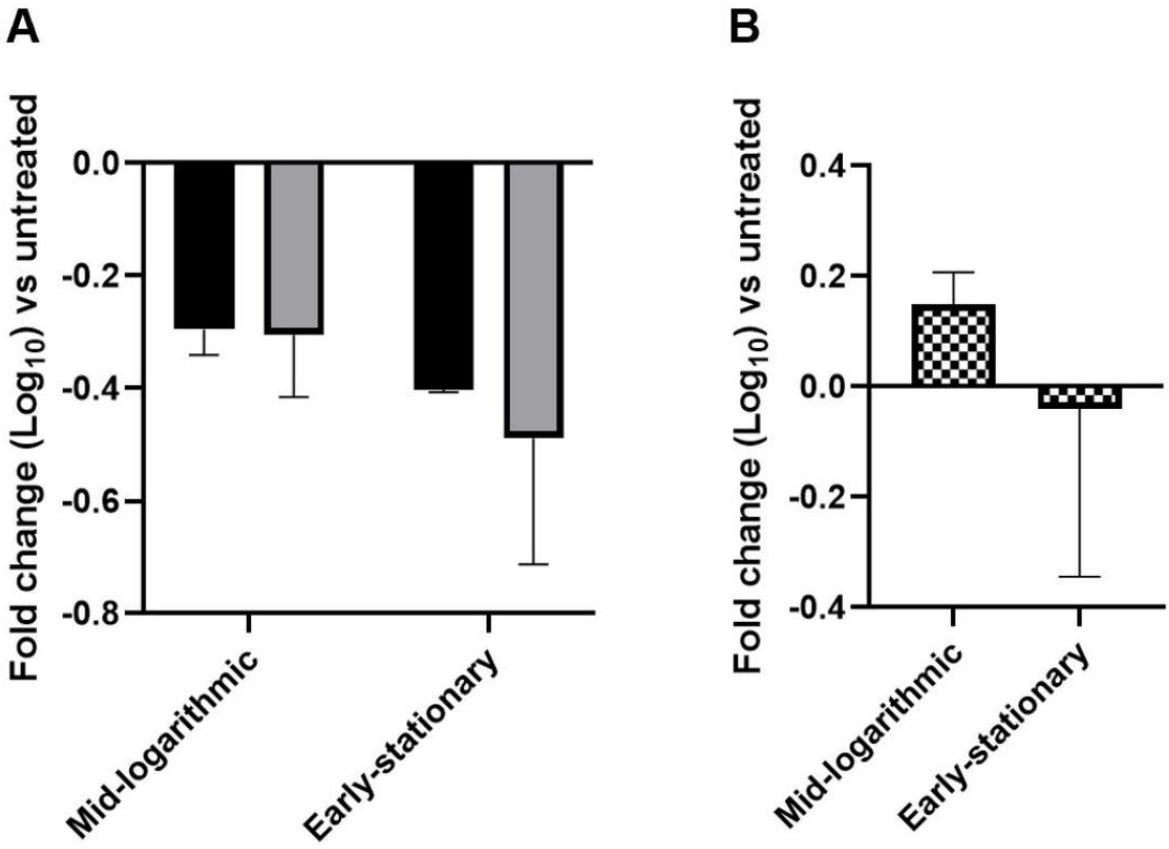
Figure 5. Commercially available surfactant Infasurf® modulates transcription of *S. aureus* virulence genes. *S. aureus* was grown to mid-logarithmic (A) or early stationary phase (B) of growth in TSB with 1% Infasurf®. RNA was harvested and subjected to TaqMan® RT-PCR. Gene transcripts were normalized to *gyrB*. Data shown are the mean fold-change of indicated gene relative to treatment with *S. aureus* only. Error bars indicate mean ± SEM of 3 biological replicates.

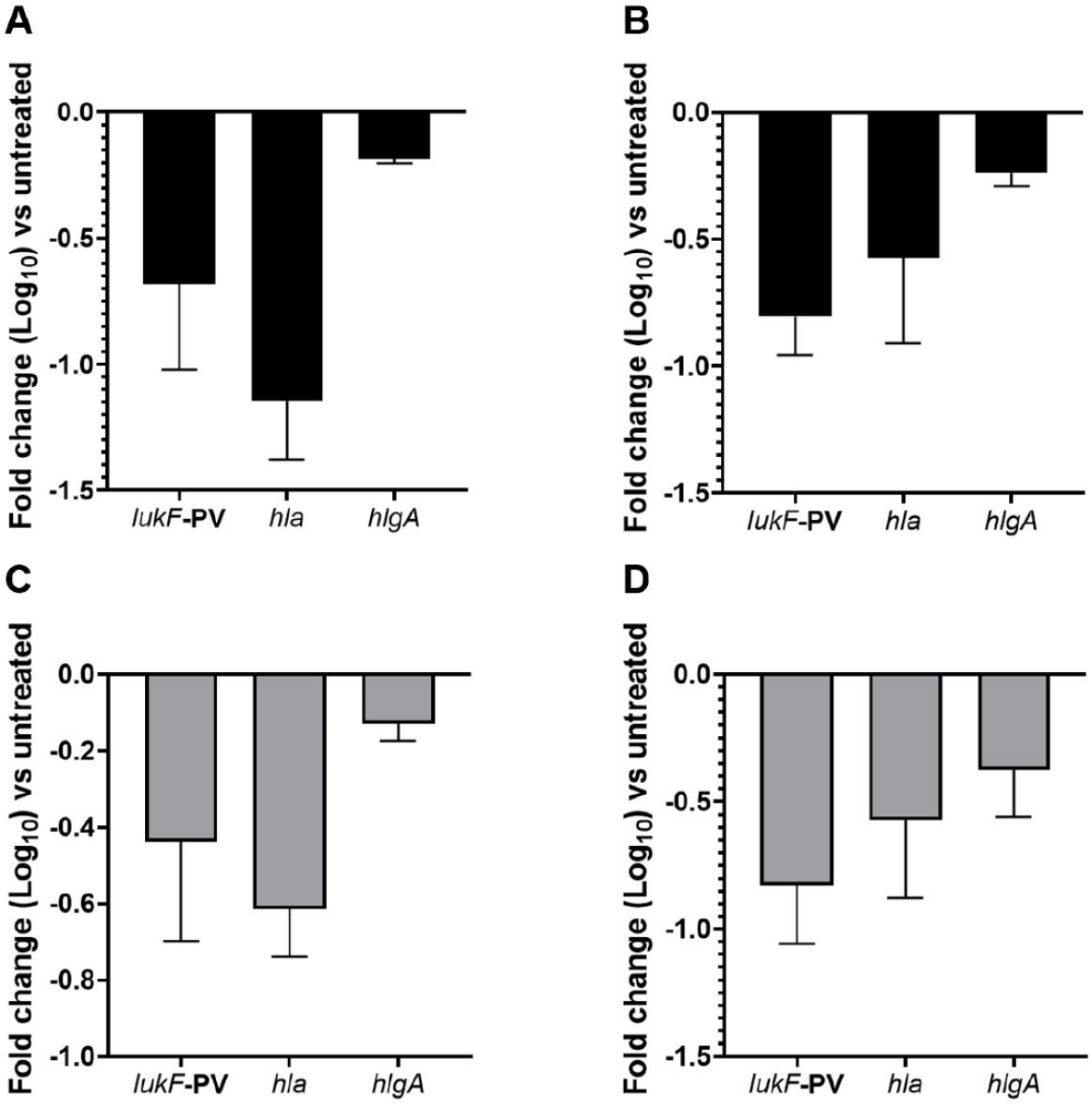
Supplemental Figure 1. Commercially available surfactant Infasurf® does not cause plasma membrane damage in human PMNs. TSB with varied dilutions of Infasurf® was incubated for five

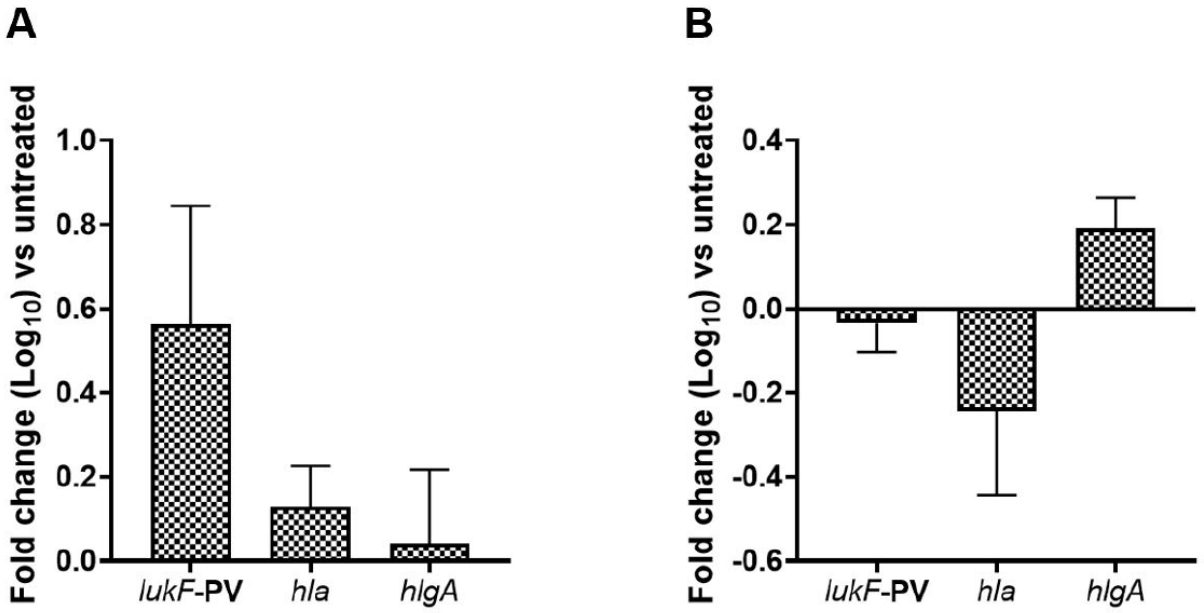
hours with no bacteria and samples were centrifuged, diluted to 1:50 final dilution, and incubated with human PMNs for one hour. Plasma membrane damage was assessed by PI staining and flow cytometry. Dash (-) represents PMN control without treatment. Data are from 3 biological replicates. NS = not significant by one-way ANOVA followed by Dunnett's multiple comparison test.


A
PMNs only
PMNs + supernatant
PMNs + supernatant + mouse surfactant (10%)
PMNs + supernatant + rat surfactant (10%)
SSC-H
FSC-H
Propidium Iodide+
FL1-H
3.25%
96.75%
72.00%
28.00%
2.36%
97.64%
3.00%
97.00%
B
% PI positive PMNs
ns
ns
*
**
C
% PI positive PMNs
ns
**
****
****
D
% PI Positive PBMCs
*
**
Mouse
Rat
E
CFU/mL
ns
F
CFU/mL
ns


A
**
% PI postive PMNs
50
40
30
20
10
0
-
0%
100%
10%
1%
0.1%
0.01%
0.001%
B
*
%PI Positive PBMCs
40
30
20
10
0
-
0%
1%
C
ns
CFU/mL
10^10
10^9
10^8
0%
100%
10%
1%
0.1%
0.01%
0.001%


A
Fold change (Log10) vs untreated
0.0
-0.2
-0.4
-0.6
-0.8
Mid-logarithmic
Early-stationary
B
Fold change (Log10) vs untreated
0.4
0.2
0.0
-0.2
-0.4
Mid-logarithmic
Early-stationary


A
B
C
D
Fold change (Log10) vs untreated
0.0
-0.5
-1.0
-1.5
-0.2
-0.4
-0.6
-0.8
lukF-PV
hla
hlgA


A
Fold change (Log10) vs untreated
1.0
0.8
0.6
0.4
0.2
0.0
lukF-PV
hla
hlgA
B
Fold change (Log10) vs untreated
0.4
0.2
0.0
-0.2
-0.4
-0.6
lukF-PV
hla
hlgA

CHAPTER THREE

PALMITATE SUPPRESSES STAPHYLOCOCCUS AUREUS VIRULENCE TRANSCRIPTION AND PROTECTS INNATE LEUKOCYTES FROM TOXIN MEDIATED DEATH

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

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Contributions: Project design, experimental design and execution, and wrote manuscript.

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Contributions: Reagent preparation.

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Contributions: Reagent preparation.

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Abstract

Staphylococcus aureus (*S. aureus*) is an opportunistic pathogen commonly found in anterior nares of more than 20% of the population. The bacterium gets aspired daily into lungs, however, healthy nasal carriers of *S. aureus* do not typically manifest lung disease. In a previous study we identified that lung surfactant repressed *S. aureus* toxin production and reduced transcript abundance of virulence genes. This study chose to investigate the role of palmitate, the most predominant free fatty acid in lung surfactant and a component of one of the most common lipids in lung surfactant, DPPC. Herein, we tested the hypothesis that palmitate reduces *S. aureus* virulence during interaction with human neutrophils. We found that palmitate down-regulated the *S. aureus* two-component system SaeRS responsible for production of virulence factors important in neutrophil evasion and lung infection. Cytotoxicity assays also revealed that palmitate protected human neutrophils by reducing the membrane damage to PMNs caused by *S. aureus* secreted factors. Palmitate also exhibited a direct, inhibitory effect on *S. aureus* secreted toxins themselves. Moreover, in the presence of both palmitate and neutrophils, *S. aureus* virulence gene expression was further repressed by palmitate. Collectively, this work identifies palmitate as a fatty acid of surfactant that can directly modulate *S. aureus* virulence gene expression and toxin production.

Introduction

Staphylococcus aureus (*S. aureus*) is a pathogen that can colonize a variety of host tissues, ranging from skin, bones, joints, muscles, heart, and lungs. It is also commonly found in the anterior nares of 20-50% of the human population (Mashruwala *et al.* 2017). Although nasal carriers of *S. aureus* breathe in the pathogen daily, primary bacterial pneumonia is uncommon in healthy carriers. However, *S. aureus* is a prevalent cause of devastating secondary bacterial pneumonia after a primary influenza A infection (Borgogna *et al.* 2022). The isolate from the nares is usually identical to the one at the diseased site (von Eiff *et al.* 2001). To understand the reason behind the higher incidence of *S. aureus* secondary bacterial pneumonia, it is worthwhile to investigate the lung environment and the changes that influenza A virus inflicts to make the lung space susceptible to bacterial colonization. There are several known protective barriers in the lungs that bacteria must overcome to cause an infection such as frequent mucus clearance (Quie 1986), opsonizing SP-A and SP-D lung surfactant proteins, and patrolling alveolar macrophages and neutrophils (Wu *et al.* 2003). Other than these more well-known protective barriers, it is also known that defect or deficiency of lung surfactant is associated with respiratory dysfunction (Tonks *et al.* 2005; Chroneos *et al.* 2010). Moreover, there are several reports linking secondary bacterial infections after respiratory illnesses like influenza A and SARS-CoV-2 with a decrease in the amount of lung surfactant present (Mirastschijski *et al.* 2020; Ji *et al.* 2021; Woods *et al.* 2016). Therefore, pulmonary surfactant is of key importance as *S. aureus* encounters it first upon aspiration into the lungs.

Lung surfactant is a lipid-protein complex that lowers surface tension to facilitate breathing. It is made up of 90% lipid and 10% protein. The majority of the lipid is phospholipid,

and in this group DPPC or dipalmitoylphosphatidylcholine is the most abundant (Huck *et al.* 2021). DPPC is made up of two groups of palmitic acid attached to a phosphatidylcholine head group. Palmitic acid, also known as palmitate, is the most abundant free fatty acid in lung surfactant, making up 80% of total fatty acids (Ishii *et al.* 2014; Ding *et al*. 2001; Schmidt *et al*. 2002). Palmitate has been shown to interact with both host cells as well as with bacteria. In the host, palmitate has anti-angiogenic effects (Zhang *et al.* 2017) and can induce reactive oxygen production in neutrophils (Wanten *et al.* 2002). There are a variety of reports showing free fatty acids like palmitate having bactericidal effects through destabilization of the bacterial membrane (Arsic *et al*. 2012; Bouhafs *et al*. 1999; Yoon *et al.* 2018). Palmitate at high doses inhibits growth of *Bacillus* sp., *Staphylococcus* sp., *Micrococcus* sp., *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus bovis*, *Listeria monocytogenes*, and *Helicobacter pylori* among others (Yoon *et al.* 2018).

Given this evidence, we thought palmitate may play an important, protective role in the context of lung infections. Preliminary studies done by our group demonstrated that palmitate has potential to protect human neutrophils from *S. aureus* cytolytic toxins (Borgogna 2019). We hypothesized that palmitate found in lung surfactant modulates *S. aureus* virulence and protects host immune cells. In this work, we provide evidence that palmitate at sub-bactericidal concentrations down-regulates key *S. aureus* virulence genes by repressing the SaeRS system. This study uncovers that palmitate not only acts transcriptionally but also can interact directly with *S. aureus* toxins. Experiments show that the presence of palmitate reduces cytotoxicity of *S. aureus* supernatants towards human immune cells. Taken together, our data highlight that key components in lung surfactant have a protective role against bacterial pathogens, and this underlies why healthy

carriers of nasal *S. aureus* do not get primary pneumonia. While this work explores palmitate's antibacterial effect and its physiological relevance during lung infections, further work is needed to determine the exact mechanisms of action of lung surfactant components like palmitate or DPPC on bacterial pathogens.

Materials and Methods

Bacteria Strains and Culture Conditions

Staphylococcus aureus PFGE-type USA300 strain LAC (Diep *et al.* 2006) was used in all experiments. Unless noted otherwise, overnight and sub-cultured bacterial cultures were grown in 20 mL of tryptic soy broth (TSB; EMD Millipore) supplemented with 0.5% glucose (1:100 dilution) with shaking (250 rpm) at 37°C. Optical density at 600 nm (OD_{600}) was measured using a NanoDrop 2000c Spectrophotometer (ThermoFisher Scientific) and colony forming units (CFUs) were determined by plating diluted samples on tryptic soy agar (TSA; EMD Millipore) and enumerated the following day.

Reagents and Preparations

Palmitate (sodium palmitate) was purchased from TCI (CAS: 408-35-5). Before each experiment, palmitate was dissolved in 1 mL of 50:50 ethanol and DPBS at 60°C to a final concentration of 0.18 M. Subsequent dilutions were dissolved in DPBS.

For detergent study, palmitate was prepared as described above. For preparation of saponin, sodium dodecyl sulfate (SDS), and Tween 80 dilutions, critical micelle concentration (1 x CMC) was dissolved in DPBS at 60°C and further diluted in DPBS. The critical micelle concentration for

palmitate, saponin, SDS, and Tween 80 are as follows: 0.74 mM, 39 mM, 0.693 mM, and 0.012 mM, respectively.

For gel experiments, samples were resuspended with 30 ml of SDS-Laemmli buffer and boiled at 95°C for 10 min. Proteins were separated using 15% SDS-PAGE gels and stained with Silver stain (BioRad).

Growth Assays

For experiments investigating growth in 180 μM palmitate, 0.5 mL of DPBS or 1,800 μM palmitate was added to 4.5 mLs of TSB pre-warmed to 37°C and inoculated with 50 μL of overnight culture. Samples were incubated for five hours at 37°C with shaking (250 rpm).
For experiments with pre-incubation, supernatants from *S. aureus* sub-cultured for 5 h in TSB with glucose were sterile-filtered (0.22 μm, Avantor) and diluted in DPBS. Then, supernatants were incubated with various compounds for varied time periods at 37°C.

Relative Quantitative Real Time RT-PCR

Transcription of *S. aureus* genes was assessed using TaqMan® real time reverse transcriptase-PCR (RT-PCR) as previously described previously (Nygaard *et al.* 2010; Voyich *et al.* 2005; Voyich *et al.* 2009). Briefly, sub-cultured strains were harvested at mid-exponential (ME; OD_{600} = 1.5) or early stationary (ES; OD_{600} = 3.0) phase of growth, mechanically disrupted using a FastPrep FP120 cell disrupter (ThermoFisher Scientific), and RNA purified using RNeasy Kit (Qiagen). TaqMan® real-time RT-PCR was performed using primer and probe sets listed in Table 1 below and fold-change was analyzed using the nalyzed via the 2^(-ΔΔCt) method as described previously (Nygaard *et al.* 2010; Voyich *et al.* 2005; Voyich *et al.* 2009).

Table 1. qRT-PCR primers and probe sequences from Borgogna *et al.* 2018.

RT-PCR Primers and Probes	Sequence
gyrB fwd	5' - CAAATGATCACAGCTTTGGTACAG - 3'
gyrB rvs	5' - CGGCATCAGTCATAATGACGAT - 3'
gyrB probe	5' - AATCGGTGGCGACTTTGATCTAGCGAAAG - 3'
hla fwd	5' - CAACAACACTATTGCTAGGTTCCATATT - 3'
hla rvs	5' - CCTGTTTTTACTGTAGTATTGCTTCCA - 3'
hla probe	5' - ATGAATCCTGTCGCTAATGCCGCAGA - 3'
hlgA fwd	5' - ACTTATTTGACAAGACCCAACTG - 3'
hlgA rvs	5' - CCACTTTGAATTAAAGGAGGTAATTGAT - 3'
hlgA probe	5' - CAGCAGCAAGAGACTATTTCGTCCCAG - 3'
lukA fwd	5' - GCGTCATCATTATCATGTGCAA - 3'
lukA rvs	5' - TCTTTCTTATTTTGGTVTTGAGAGTCTT - 3'
lukA probe	5' - CAGCAACGACTCAAGCAAATTCAGCTCA - 3'
lukF-PVL fwd	5' - TTGCTTTTGCTATCCAATACAGTTG - 3'
lukF-PVL rvs	5' - TCGGAATCTGATGTTGCAGTTG - 3'
lukF-PVL probe	5' - TGCAGCTCAACATATCACACCTGTAAGT - 3'

Human PMN or PBMC Plasma Membrane Integrity Assays

Heparinized venous blood from healthy donors was collected in accordance with a protocol approved by the Institutional Review Board for Human Subjects at Montana State University. All

donors provided written consent to participate in the study. Human polymorphonuclear leukocytes (neutrophils or PMNs) and/or peripheral blood mononuclear cells (PBMCs) were isolated under endotoxin-free conditions (< 25.0 pg/mL) and cell viability and purity of preparations were assessed using a FACSCalibur Flow cytometer (BD Biosciences) as described previously (Nygaard *et al.* 2010; Voyich *et al.* 2005). Assays intoxicating PMNs with extracellular *S. aureus* proteins were performed as previously described (Nygaad *et al.* 2012; Flack *et al.* 2014). Briefly, supernatants from *S. aureus* sub-cultured for 5 h in TSB with glucose were sterile-filtered (0.22 μm, Avantor) and diluted in DPBS. PMNs or PBMCs ($1x10^6$) were exposed to varied dilutions of *S. aureus* supernatant. Samples were incubated at 37°C for 60 min then stained with propidium iodide (PI; ThermoFisher Scientific) following the manufacturer's protocol and then analyzed with a FACSCalibur Flow cytometer.

Results and Discussion

Palmitate Protects Human PMNs from *S. aureus* Toxin-Mediated Killing

In a previous study (Predtechenskaya *et al.* submitted, Chapter Two) we demonstrated that supernatants harvested from *S. aureus* grown in natural surfactants from mouse and rat lungs had significantly reduced toxicity to human neutrophils and peripheral blood mononuclear cells (PBMCs). In the current study we aimed to determine if palmitate could recapitulate the phenotypes seen with whole surfactants. When grown to early stationary phase in the presence of 180 μM palmitate, there was approximately a two-fold reduction of colony forming units (CFU) (Figure 1A). As such, for all additional assays described below, CFU matching was performed to adjust for the lesser amount of starting *S. aureus* cells when grown in palmitate. This observation

is not novel as fatty acids have been shown to have detrimental effects on bacterial viability (Arsic *et al.* 2012). Supernatants of stationary phase *S. aureus* significantly reduced the membrane damage of human polymorphonuclear neutrophils (PMNs) compared to the control (Figure 1B). Next, we wanted to assess if palmitate had the same effect on PBMCs. Contrary to our expectations, preliminary experiments suggested that the presence of palmitate during *S. aureus* growth did not have a protective effect on PBMCs (Figure 1C). However, more replicates are needed to make the conclusion that PBMCs are less sensitive to *S. aureus* cytotoxins than PMNs.

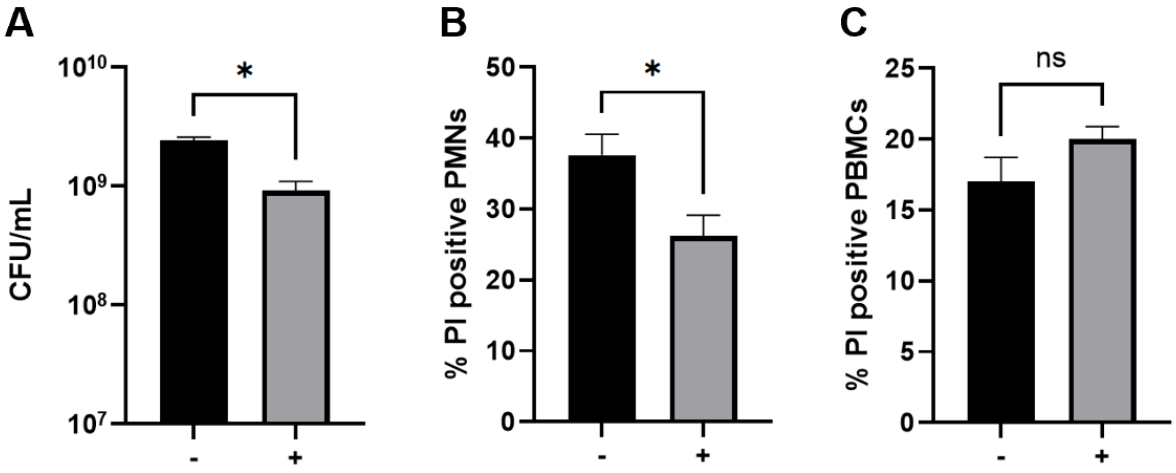


Figure 1. Growth in palmitate influences *S. aureus* cytotoxicity against PMNs. *S. aureus* was grown to early stationary phase in TSB with (+) or without (-) 180 µM palmitate. A) Bacterial concentration of LAC cultured with (+) and without (-) 180 µM palmitate at early stationary growth phase. Supernatants were harvested, diluted to 1:50 final concentration, and incubated with human B) polymorphonuclear neutrophils (PMNs) or C) peripheral blood mononuclear cells (PBMCs) for one hour. Plasma membrane damage was assessed using propidium iodide (PI) uptake and analyzed by flow cytometry. Graphs displayed show mean ± SEM of 3 biological replicates for A), B), and C). *P <0.05 and **P < 0.005 paired t-test for A), B), and C).

Palmitate Modulates *S. aureus* Virulence Gene Expression

To investigate the reason behind palmitate's protective effect on PMNs, we assessed if palmitate is modulating the virulence gene expression of *S. aureus* during growth. Our chosen target, the SaeRS system, is a two-component system of *S. aureus* that senses neutrophils and controls virulence gene expression of toxins that target immune cells. This system is also important for *S. aureus* lung infection (Borgogna *et al*. 2018). We used TaqMan™ relative RT-PCR to determine *saeR* gene regulation at various growth phases. The presence of palmitate during *S. aureus* growth down-regulated expression of *saeR* transcripts in mid-exponential phase-grown cell, but not in early stationary phase-grown cells (Figure 2A), demonstrating that palmitate affects the SaeRS system early on in growth. These results are confirmed by another group which also found that at the concentration of palmitate used in this work, Sae activity is inhibited (DeMars *et al*. 2021). Earlier literature has also noted other fatty acids inhibiting SaeRS system and proposed several mechanisms including: membrane curvature elastic stress altering SaeS conformation, bilayer net negative charge response by SaeS, or direct inactivation of SaeS by fatty acids (Ericson *et al.* 2017). This finding is not exclusive to palmitate, as other fatty acids like sapienic and oleic acid have been found to down-regulate expression of SaeRS transcripts (DeMars *et* al. 2021; Neumann *et al*. 2015). It has been even further suggested that fatty acids may interfere with SaeR and other regulatory proteins from binding to target DNA (DeMars *et al*. 2021). This is further complicated if we consider internalized palmitate versus palmitate in the supernatant (DeMars *et al*. 2020). While we did not attempt to narrow down the exact mechanism of palmitate's effect on transcription with our experiments, we confirm that palmitate down-regulates transcription of *saeR*.

We then wanted to validate this finding by exploring how palmitate impacts a specific set of cytolytic toxins controlled by the SaeRS system (Rogasch *et al.* 2006, Voyich *et al.* 2009). Transcriptional analysis was performed on genes encoding leukocidins LukAB (*lukAB*), HlgAB (*hlgA*), and PVL (*lukF*-PV). We observed that at mid-exponential phase, palmitate significantly down-regulated *lukAB* and *lukF*-PV while *hlgA* was only slightly down-regulated (Figure 2B). Interestingly, by early stationary phase *lukAB* and *lukF*-PV were up-regulated in the presence of palmitate compared to mid-logarithmic control while *hlgA* again showed modest transcriptional responses to the presence of palmitate (Figure 2B). This data recapitulated what we have seen with *saeR*, where palmitate's effect repressed the expression of genes at mid-exponential phase and showed no change by early stationary phase. It makes sense that the expression of *hlgA*, *lukAB*, and *lukF*-PV reflected *saeR* since these toxins are controlled by the SaeRS system (Nygaard *et al.* 2010).

To advance these findings we have started to investigate the influence of palmitate on transcription of *S. aureus* using RNA sequencing (RNAseq). To date we have only done these experiments at early stationary phase. RNAseq showed the following: *saeR*, *hlgA*, *lukB* were not significantly modulated at early stationary, but that *lukF*-PV was significantly up-regulated by early stationary phase. RNAseq needs to be conducted at mid-exponential to confirm our TaqMan™ findings. All these results fall in line with context of lung colonization – at first only a few bacteria reach the lungs and sense the environment. This low bacterial density is read by quorum sensing systems which then repress virulence factors until a larger bacterial density is observed (Pivard *et al*. 2021). So, we may potentially be observing repression at mid-exponential

phase by palmitate which is overpowered by a large bacterial density at early stationary phase when the bacterium is trying to spread and infect other tissues.

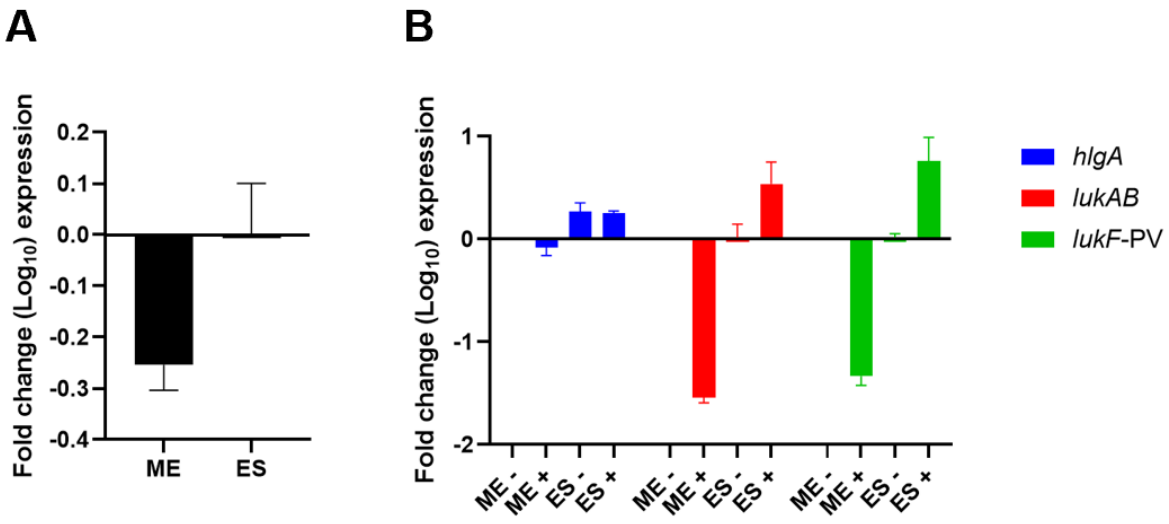
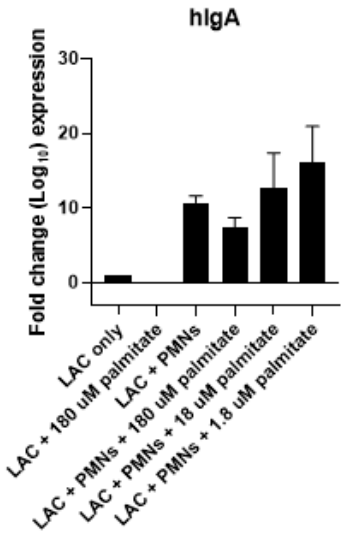


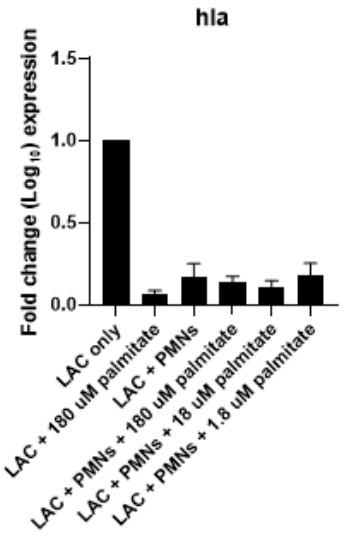
Figure 2. Palmitate down-regulates gene expression of *S. aureus saeR* and select toxins in mid-exponential growth phase. *S. aureus* was grown with (+) or without (-) 180 µM palmitate to mid-exponential (ME) phase and to early stationary (ES) phase, and RNA was harvested and subjected to TaqMan™ real-time RT-PCR. Gene transcripts were normalized to *gyrB*. Data displayed are the mean fold-change (Log_{10}) of indicated gene relative to treatment with *S. aureus* only at respective grow th phase for A) and compared to mid-exponential phase for B). Data displayed show mean ± SEM of 3 biological replicates.

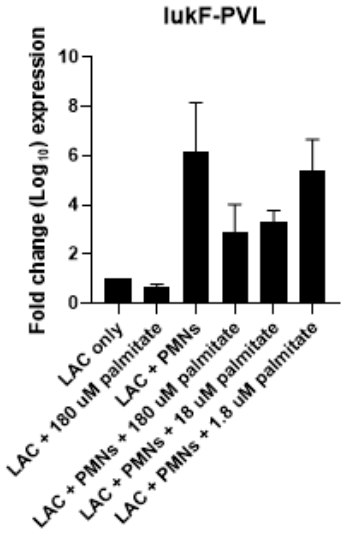
Having demonstrated that palmitate modulates the virulence gene expression of *S. aureus* early on during growth, we wanted to assess how palmitate would impact *S. aureus* and neutrophil interactions that would normally occur early on in lung pathogenesis. So, for the next set of experiments *S. aureus* was grown to mid-exponential phase and then exposed simultaneously to neutrophils and varied concentrations of palmitate. We assayed *hlgA*, *hla*, *lukF*-PV, and *lukAB* virulence genes using real-time RT-PCR as above. We also assessed *essC* which is part of the Type VII Secretion System, a virulence secretion system up-regulated in the presence of human serum

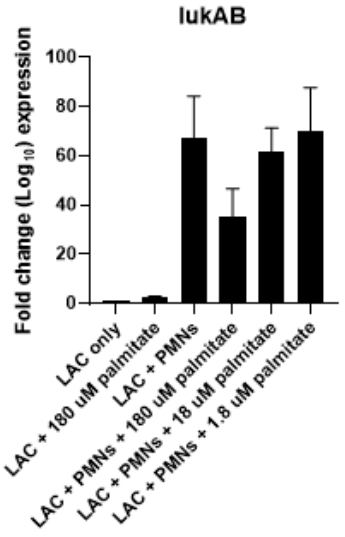
and pulmonary surfactant (Lopez *et al*. 2017). Additionally, we investigated *fakA* which is a fatty acid kinase necessary for incorporation of fatty acids like palmitate into *S. aureus* lipid membranes (Ridder *et al.* 2020). For this set of experiments, *S. aureus* grown to mid-exponential phase was exposed simultaneously to varied concentrations of palmitate and neutrophils. The presence of PMNs up-regulated the expression of this gene as we have shown previously (Flack *et al*. 2014). Despite the strong expression of this gene due to presence of neutrophils, palmitate reduced expression compared to when there was just bacteria and neutrophils present in a dose dependent manner (Figure 3). For *hla*, exposure to palmitate or PMNs immediately down-regulated this gene (Figure 3). Both *lukF*-PV and *lukAB* behaved similarly in this assay. Unlike in palmitate growth experiments where we observed significant down-regulation of both of these toxins at mid-exponential phase (Figure 2B), one hour of incubation with palmitate did not impact expression of these two toxins (Figure 3). Both were potently expressed in the presence of PMNs, but as concentrations of palmitate increased, this signal was repressed. Hence, for *hlgA*, *lukF*-PV, and *lukAB*, but not *hla*, palmitate repressed the strong expression signal induced by presence of PMNs. In context of infection, when bacteria enter the lipid-rich environment of the lungs where palmitate is the most abundant free fatty acid, bacteria may respond distinctly to this specific environment. For the *essC* gene, only the presence of both PMNs and palmitate up-regulated this gene (Figure 3). Finally, for *fakA*, there was up-regulation in both presence of palmitate and PMNs (Figure 3). Fatty acid kinase (FakA) indirectly modulates the signaling of the SaeRS system by keeping a low fatty acid intracellular pool (Ericson *et al.* 2017) due to this relationship FakA has been shown to be important for *S. aureus* virulence (Lopez *et al*. 2017). Overall, this assay displayed that the transcriptional response differs between just palmitate, PMNs, or both. Palmitate has a repressive

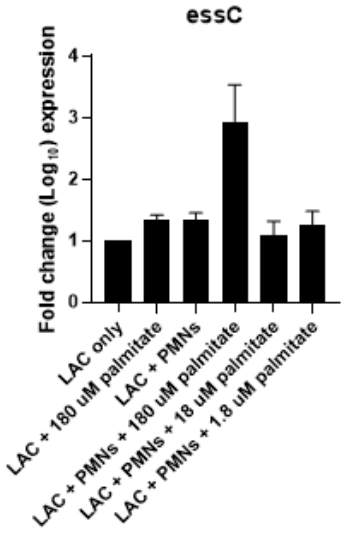
effect on *S. aureus* virulence gene expression which would normally be up-regulated in the presence of PMNs. This is physiologically relevant for the lung environment where *S. aureus* encounters neutrophils but also senses the presence of palmitate in lung surfactant.


hlgA
Fold change (Log10) expression
30
20
10
0
LAC only
LAC + 180 uM palmitate
LAC + PMNs
LAC + PMNs + 180 uM palmitate
LAC + PMNs + 18 uM palmitate
LAC + PMNs + 1.8 uM palmitate


hla
Fold change (Log10) expression
1.5
1.0
0.5
0.0
LAC only
LAC + 180 uM palmitate
LAC + PMNs
LAC + PMNs + 180 uM palmitate
LAC + PMNs + 18 uM palmitate
LAC + PMNs + 1.8 uM palmitate


lukF-PVL
Fold change (Log10) expression
10
8
6
4
2
0
LAC only
LAC + 180 uM palmitate
LAC + PMNs
LAC + PMNs + 180 uM palmitate
LAC + PMNs + 18 uM palmitate
LAC + PMNs + 1.8 uM palmitate


lukAB
Fold change (Log10) expression
100
80
60
40
20
0
LAC only
LAC + 180 uM palmitate
LAC + PMNs
LAC + PMNs + 180 uM palmitate
LAC + PMNs + 18 uM palmitate
LAC + PMNs + 1.8 uM palmitate


essC
Fold change (Log10) expression
4
3
2
1
0
LAC only
LAC + 180 uM palmitate
LAC + PMNs
LAC + PMNs + 180 uM palmitate
LAC + PMNs + 18 uM palmitate
LAC + PMNs + 1.8 uM palmitate

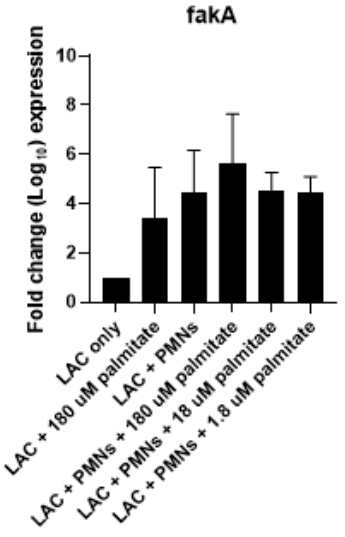

fakA
Fold change (Log10) expression
10
8
6
4
2
0
LAC only
LAC + 180 uM palmitate
LAC + PMNs
LAC + PMNs + 180 uM palmitate
LAC + PMNs + 18 uM palmitate
LAC + PMNs + 1.8 uM palmitate

Figure 3. Palmitate modulates relative gene expression of various *S. aureus* genes of interest in the presence of neutrophils. *S. aureus* was grown to mid-exponential phase and exposed to varied palmitate concentrations and PMNs for 1 hour at 37°C. RNA was harvested and subjected to TaqMan™ real-time RT-PCR. Gene transcripts were normalized to *gyrB*. Data displayed are the mean fold-change (Log_{10}) of indicated gene relative to treatment with *S. aureus* only. Data displayed show mean ± SEM of 3 biological replicates.

Palmitate Interacts Directly with *S. aureus* Toxins

Although we identified that palmitate modulates *S. aureus* transcription and toxin production, providing an explanation for how growth in palmitate can protect PMNs, a question we wanted to tackle was if palmitate could directly interact with the toxins in the supernatant. To investigate this, *S. aureus* was grown to early stationary phase, supernatants extracted and preincubated for varied timepoints with 180 μM palmitate. PMN membrane damage was assessed after one hour of incubation. While palmitate did not have an instant effect, a 30 minute or an hour-long preincubation of toxins with palmitate lowered their cytotoxicity against PMNs (Figure 4A). This indicates that palmitate has a direct effect on toxins but requires some time to act on the toxins. Given that palmitate could interact with toxins, another question of interest to us was whether palmitate could directly protect PMNs. In these assays, PMNs were pre-incubated with palmitate for varied timepoints and then exposed to supernatants. Palmitate had a slight toxic effect on PMNs, but not comparable to PMNs exposed to supernatants (Supplemental Figure 2). Regardless of the preincubation time, palmitate did not seem to have a direct protective effect on PMNs as there were no differences compared to the controls (Supplemental Figure 2).

To understand the direct interaction between palmitate and toxins, we hypothesized that palmitate was degrading toxins during the preincubation. To test this hypothesis early stationary toxins were incubated with palmitate for 60 minutes and used SDS-PAGE to compare profile of toxins only to toxins treated with palmitate. Lighter bands were observed at 35 kDa, 27 kDa, 25

kDa, 15 kDa and 13 kDa (Figure 4B). Although other methods are needed to determine the exact identity of the secreted factors seen on the gel, the 35 kDa band may be *lukH* or *lukB* (consult Supplemental Table 1). Several additional experiments are needed to determine what virulence factors are impacted by palmitate. Ultimately, experiments will include utilizing 2D gel electrophoresis followed by mass spectrometry.

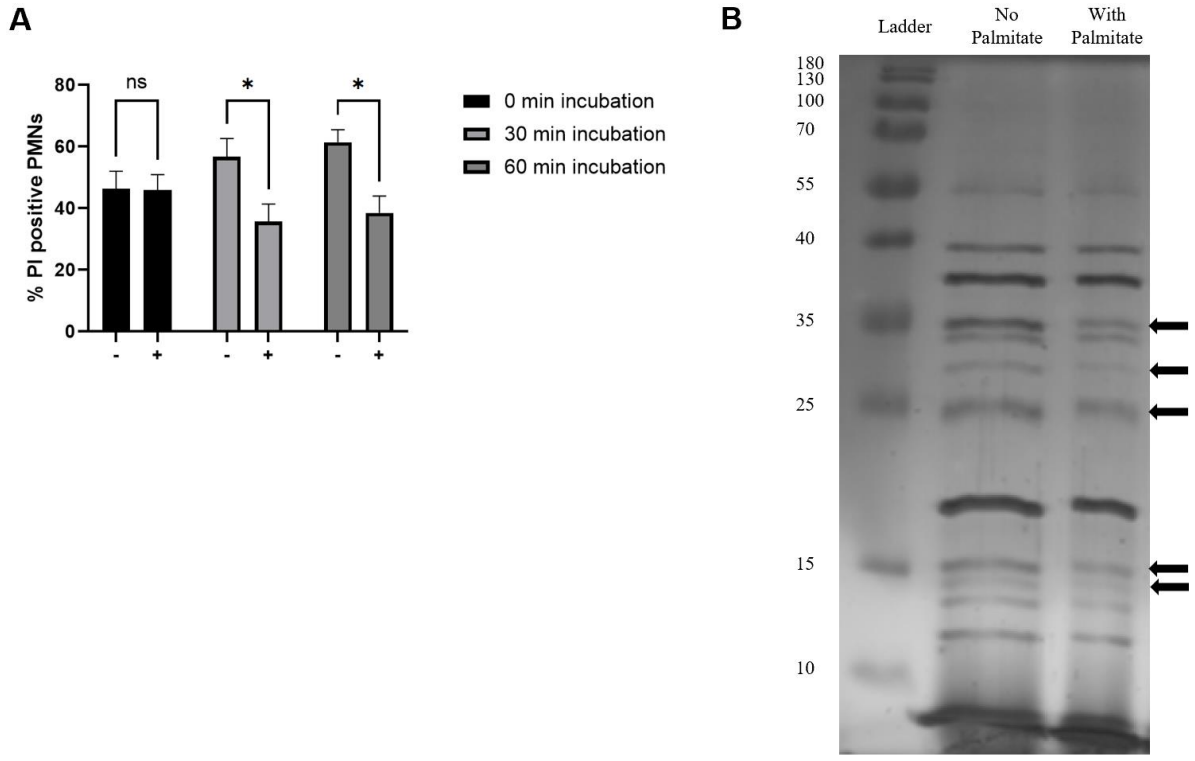


Figure 4. Preincubation of *S. aureus* supernatants with palmitate reduces cytotoxicity against PMNs. A) *S. aureus* was grown to early stationary phase. *S. aureus* supernatants were harvested, diluted to 1:50 final concentration, and preincubated with (+) or without (-) 180 μM palmitate at 37°C for 1 hour, then incubated with human PMNs for one hour. Plasma membrane damage was assessed using propidium iodide (PI) uptake and analyzed by flow cytometry. Data are from 4 biological replicates for A) with error bars showing mean ± SEM. *P <0.05 Two-way ANOVA followed by Tukey's multiple comparison test. B) SDS-PAGE gel of *S. aureus* supernatants pre-incubated with palmitate. LAC was sub-cultured to early stationary phase. Supernatants were preincubated with or without 180 μM palmitate at 37°C for 1 hour, and samples were then silver stained on 15% SDS-PAGE gel. Arrows indicate differences in band intensity.

Supplemental Table 1. Protein sizes of various *S. aureus* toxins.

Gene	Protein Size	Source
hla	33 kDa	Bhakdi et al., 1991
hlgA	32 kDa	Staali *et al.*, 2021
lukED	LukE 32 kDa, LukD 34.3 kDa	Du *et al.*, 2018
lukAB	LukA 37 kDa, LukB 35 kDa	DuMont *et al.*, 2014
lukFS	LukS 33 kDa, LukF 34 kDa	Kaneko *et al.*, 2004

Common Chemical Surfactants do not Reduce Cytotoxicity of *S. aureus* Secreted Toxins

All detergents, including those consisting of fatty acids, have a critical micelle concentration (CMC), which is the concentration at which micelles, or aggregates of surfactant molecules, begin to form. In order to test this, *S. aureus* supernatants were exposed to palmitate and chemical detergents saponin, sodium dodecyl sulfate (SDS), and Tween 80 at varied concentrations. For this assay, we exposed early stationary *S. aureus* supernatants to a detergent of choice and PMNs for 1 hour. Palmitate had a protective effect at $1/10^{th}$ dilution of its CMC (Figure 5). For reference, the 180 μM palmitate concentration used for all previous assays is representative of the $1/4^{th}$ of the CMC. Among the different chemical detergents, no concentrations were able to recapitulate the effect of the biological detergent palmitate implying that the physical formation of micelles may not be the mechanism behind the protective effect of palmitate. Further tests with other biological detergents remain to be done to rule out a micellular effect.

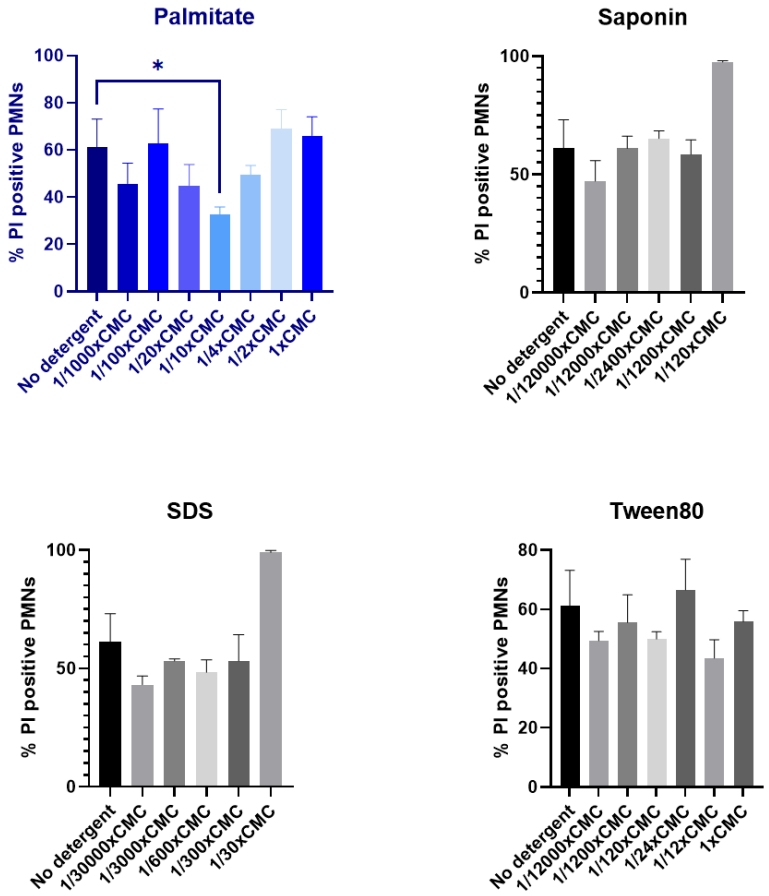


Figure 5. Palmitate's protective effect is not recapitulated by other chemical surfactants. LAC was sub-cultured until early stationary phase, supernatants were harvested, diluted to 1:50 final concentration, and added to detergents at varied concentrations and PMNs at 37°C for 1 hour. The critical micelle concentration (CMC) for the following detergents palmitate, saponin, SDS, Tween80 are as follows: 0.74 mM, 39 mM, 0.693 mM, 0.012 mM, respectively. Detergents were dissolved in DPBS. Plasma membrane damage was assessed using propidium iodide (PI) uptake and analyzed by flow cytometry. Data are from 3 biological replicates for all detergents with error bars showing mean ± SEM. *P <0.05 One-way ANOVA followed by Bonferroni's multiple comparison test.

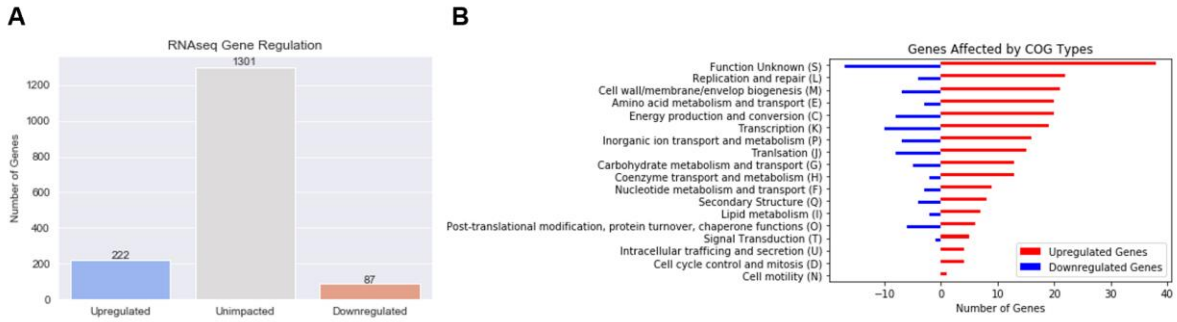
Conclusions

S. aureus is a common pathogen behind bacterial pneumonia that dramatically increases morbidity and mortality. Inasmuch as it is an adept lung colonizer, primary *S. aureus* pneumonia

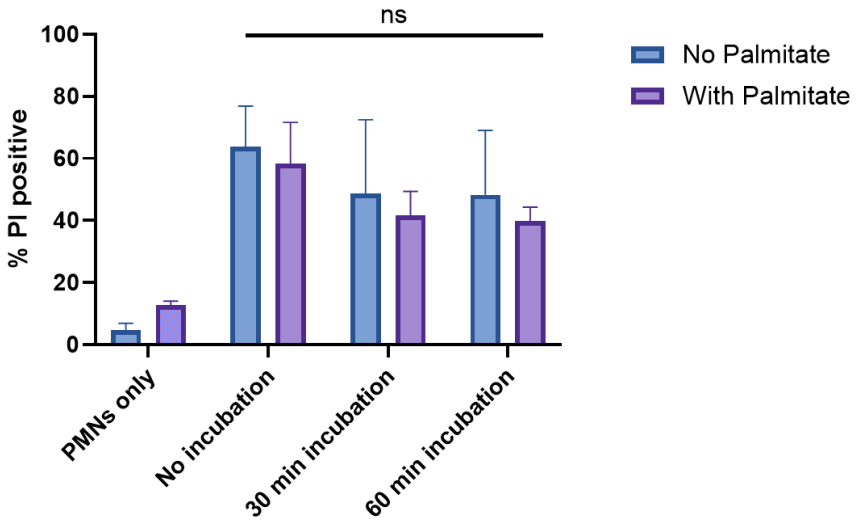
is not common. Previous work highlighted the importance of lung environment in context of S. aureus infection (Borgogna *et al.* 2018, Predtechenskaya *et al.* submitted Chapter Two). To further elucidate the mechanism behind how lung surfactant protects the host from bacterial infection, this study investigated the influence of palmitate, a common lung surfactant fatty acid, on *S. aureus* virulence. We showed that palmitate reduced cytotoxicity of *S. aureus* secreted factors against human neutrophils. Palmitate down-regulated the *S. aureus* two component system SaeRS along with key toxins it regulates. Palmitate further repressed transcript abundance of select *S. aureus* virulence factors when incubated with neutrophils. Palmitate also had a direct effect on *S. aureus* secreted toxins although this observation was not shared by chemical detergents.

Literature has previously noted a potential for interaction between toxins and lipids. It has been suggested before that fatty acids may bind toxins to membranes of cells (Kapral 1976). Similarly, delta toxin of *S. aureus* is known to bind to phospholipids (Alouf *et al.* 1989), while alpha toxin is known to bind to lipids (Füssle *et al.* 1981). This direct binding may be competitively interfering with toxins binding to actual immune membranes or receptors. Further studies need to be done to elucidate the mechanism behind the protective effect of palmitate. In context of lung infections, the effect of palmitate on other common lung pathogens such as *Streptococcus pneumoniae* and *Haemophilus influenzae* remains to be determined. Next steps of this work would be to assess the protective effect of palmitate on PMNs from supernatants of other lung bacterial pathogens. This work underscores the importance of a protective lung environment against bacterial pathogens like *S. aureus* and demonstrates that components of lung surfactants modulate bacterial pathogenesis.

Supplemental Data



Supplemental Figure 1. Analysis of RNAseq data of *S. aureus* grown in palmitate. RNAseq was performed on samples sub-cultured for 5 hours in TSB with glucose with and without 180 µM palmitate, with three samples in each group.



Supplemental Figure 2. Pre-incubation of palmitate with PMNs has no effect on cytotoxicity. Human PMNs preincubated with or without 180 µM palmitate at 37°C for varied incubations. *S. aureus* was grown to early stationary phase, supernatants were harvested, diluted to 1:50 final concentration, and added to samples for incubation at 37°C for 1 hour. Plasma membrane damage was assessed using propidium iodide (PI) uptake and analyzed by flow cytometry. Data are from 3 biological replicates with error bars showing mean ± SEM. *P <0.05 Two-way ANOVA followed by Tukey's multiple comparison test.

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CHAPTER FOUR

ROLE OF S. AUREUS CYSTEINE PROTEASE SSPB IN LUNG PNEUMONIA

Contribution of Authors and Co-Authors

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Manuscript Information

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Abstract

Pneumonia is the sixth leading cause of death in the United States (Pahal *et al.* 2023). Usually, bacterial pneumonia complicates an initial viral infection. Even though bacterial pneumonia occurs as a co-infection or a secondary infection, it is the colonization of the lung space by bacteria which determines the severity of lung infection leading to morbidity and mortality. In the early lung colonization stages, pathogens like *Staphylococcus aureus* (*S. aureus*) secrete virulence factors that initiate an infection and help escape detection by immune cells. Although lung surfactant and specific surfactant components such as palmitate are known to suppress virulence of *S. aureus* and protect host immune cells from bacterial toxins, during infection these natural defenses are weakened. There is a gap in knowledge about how *S. aureus* initially responds to presence of lung surfactant. Herein, we studied the response of *S. aureus* to the surfactant component palmitate to understand early pathogen response to the lung environment. In the current study, *S. aureus* grown in palmitate was shown to secrete SspB, which encodes a cysteine protease shown to cleave fibronectin, fibrinogen, kininogen, and phagocyte surface proteins. An isogenic mutant strain, LACΔ*sspB*, demonstrated reduced cytotoxicity against PMNs and aided in blood lysis. Experiments were done to study *sspB* expression following incubation in palmitate and surfactant. Additional experiments were conducted to determine if SspB digested *S. aureus* toxins, directly interacted with palmitate, or cleaved neutrophil chemokines, but results were inconclusive. Further studies will need to establish the role that SspB has in the context of lung infections and how it interacts with lung surfactant components to promote a successful *S. aureus* infection.

Introduction

Pneumonia is a common cause for hospital admission in developed countries, and one of the main causes of death of children in developing countries (Shann 2001). Furthermore, bacterial pneumonia is a major cause of morbidity and mortality after an initial viral infection. *Staphylococcus aureus* (*S. aureus*) is a concerning antibiotic-resistant pathogen and a common cause of secondary bacterial pneumonia (Borgogna *et al*. 2018). Previous studies (Chapter Two, Chapter Three) demonstrate that lung surfactant and palmitate, a predominant component of lung surfactant, reduce *S. aureus* virulence.

Data collected in Chapter Two identified up-regulation of the gene encoding the cysteine protease SspB following growth in palmitate. This observation was intriguing because *S. aureus* extracellular proteases have been shown to impair immunity of the lung and promote tissue damage (Paharik *et al*. 2016; Kantyka *et al*. 2013). *S. aureus* has fifteen extracellular proteases, of which five are exfoliative toxins responsible for cell-to-cell adhesion and produced only by 5% of *S. aureus* strains (Ramirez *et al*. 2020). The remaining ten proteases, produced by almost every strain (Gimza *et al.* 2019) consist of six serine protease-like proteins (encoded by *splABCDEF*), zinc metalloprotease aureolysin (*aur*), cysteine proteases staphopain A (*scpA*) and staphopain B (*sspB*), and serine protease (*sspA*, also known as V8) (Ramirez *et al*. 2020). Protease production usually happens during post-exponential phase after the synthesis of cell wall proteins is completed (Karlsson *et al*. 2001). Although there are many protease regulators, SarA, Agr and SaeRS systems play an important role in quorum sensing-dependent lung colonization (Pivard *et al*. 2021). Agr is a positive regulator of proteases also responsible for migration factors while SarA is a negative

regulator responsible for adhesins, so the switch from one system to the other may reflect the change from early colonization to late invasive stage (Karlsson *et al*. 2001; Zhang *et al*. 2015).

The four major proteases, aureolysin, serine protease V8, staphopain A and B, are important for bacterial infection and survival in host (Zhang *et al*. 2015). These secreted proteases have a variety of different functions to promote lung colonization: they can degrade proteins like fibrinogen, evade the immune system by inhibiting the complement system (Gimza *et al.* 2019; Jusko *et al*. 2014), aid in nutrient acquisition (Ramirez *et al*. 2020), damage the extracellular matrix, inhibit neutrophil chemotaxis, and induce apoptosis of neutrophils (Hall *et al*. 2015). While secreted proteases are known to cleave host proteins, they also can degrade the bacterium's own proteins such as surface protein A and fibrinogen-binding proteins (Gimza *et al.* 2019). It has been suggested that this role of extracellular proteases in cleaving the pathogen's own surface proteins represents a transition from adhesive to invasive phenotype (Kolar *et al*. 2013).

Since secreted proteases are potent, in order for their activity to be strictly controlled, the four main proteases (aureolysin, V8, SspB and ScpA) are produced as zymogens that need to be pre-processed (Pietrocola *et al*. 2017). The protease explored in this work, SspB, is a cysteine protease part of the transcriptional unit *sspABC* controlled by one promoter. For maturation of SspB, aureolysin matures serine protease SspA, which then matures SspB into its active mature form (Shaw *et al.* 2005). However, this is not a strict requirement as aureolysin mutants are still able to produce SspA and SspB (Ramirez *et al*. 2020). Finally, as another regulator of SspB activity, SspC works as the inhibitor of SspB (Shaw *et al.* 2005; Hall *et al*. 2015). SspB can cleave fibronectin, fibrinogen (Massimi *et al*. 2002), and kininogen in the host (Burlak *et al*. 2007), which aids in translocation of the pathogen. SspB can work in tandem with staphopain A to promote

vascular leakage and induce septic shock (Burlak *et al*. 2007). SspB also has an important role in *S. aureus* avoiding immune cells by digesting opsonins (Hall *et al*. 2015). SspB can also bind to monocytes, repressing its ability to chemotax (Singh *et al*. 2019). To further divert immune cells away from *S. aureus* activity, SspB can cleave CD11b and CD31 on phagocytes. Cleavage of these receptors can result in apoptosis or efferocytosis by macrophages (Smagur *et al*. 2009; Tam *et al*. 2019).

This study found that cysteine protease SspB was secreted upon growth in palmitate. This observation prompted us to form the hypothesis that SspB may play an important role in promoting *S. aureus* lung pathogenesis. SspB reduced cytotoxicity of *S. aureus* toxins against neutrophils and affected blood lysis. We hypothesized that SspB may be lowering cytotoxicity through cleaving *S. aureus* toxins, however, whether the protease cleaves toxins or neutrophil chemokines could not be determined. Furthermore, SspB's cleavage efficiency of various lung surfactant proteins remains to be established, and future studies will need to investigate the direct role of this cysteine protease in lung infections.

Materials and Methods

Bacteria Strains and Culture Conditions

Staphylococcus aureus PFGE-type USA300 strain LAC (Diep *et al.* 2006) was used in experiments. *S. aureus* strain JE2 was also used (Fey et al. 2013). LAC mutants used for this study have been generated previously, and LACΔ*sspB* and JE2Δ*sspB* (Fey *et al*. 2013) mutants were generated through methods described before (Nygaard *et* al. 2018). For all experiments except ones with normal human serum, overnight and sub-cultured bacteria cultures were grown in 20 mL of tryptic soy broth (TSB; EMD Millipore) supplemented with 0.5% glucose (1:100 dilution)

with shaking (250 rpm) at 37°C. Optical density at 600 nm (OD_{600}) was measured using a NanoDrop 2000c Spectrophotometer (ThermoFisher Scientific) and colony forming units (CFUs) were determined by plating diluted samples on tryptic soy agar (TSA; EMD Millipore) and enumerated the following day.

Reagents and Preparations

Palmitate (sodium palmitate) was purchased from TCI (CAS: 408-35-5). Before each experiment, palmitate was dissolved in 1 mL of 50:50 ethanol and DPBS at 60°C to a final concentration of 0.18 M. Subsequent dilutions were dissolved in DPBS.

S. aureus recombinant SspB protein (Aviva, CAT: OPCA04162) was at 100 μg/mL stock concentration. Purified *S. aureus* recombinant LukAB (IBT Bioservices, CAT: 0510-001) was at 1.812 mg/mL. Purified *S. aureus* PVL protein (AntibodySystem, CAT: YXX09801) was at 0.1 mg/mL.

E-64 protease inhibitor was purchased from Sigma-Aldrich (CAS:66701-25-5). Pierce Protease Inhibitor Tablets (ThermoFisher, CAT: A32963) were diluted in 1 mL DPBS to make protease inhibitor cocktail.

Surfactant Extraction

Pulmonary surfactant isolation was performed using an adaptation of the method described previously (Inselman *et al.* 2004). Briefly, murine (C57BL/6) or rat (F344BN) lung tissue (0.2 g) was homogenized in 3 mL of ice-cold Dulbecco's phosphate buffered saline (DPBS) using a tissue grinder. Homogenate slurry was passed through a 70 μM cell strainer followed by centrifugation at 300 x g for 10 minutes at 4°C. The supernatant was collected and placed in microcentrifuge tubes for centrifugation at 18,000 x g for 30 minutes at 4°C. The resulting supernatant was

aspirated and discarded leaving a pellet. The surfactant pellet was resuspended in 1 mL of DPBS at 60°C. For experiments, this was considered 100% surfactant. Research grade Infasurf® was purchased from Onybiotech. For experiments investigating growth in surfactant, 1 mL of surfactant (at varied concentrations) was added to 4 mL of TSB pre-warmed to 37°C and inoculated with 50 µL of overnight culture. Samples were incubated for five hours at 37°C with shaking (250 rpm).

Gels

For gel experiments, samples were resuspended with 30 µL of SDS-Laemmli buffer and boiled at 95°C for 10 min. Proteins were separated using 15% SDS-PAGE gels and stained with Silver stain (BioRad).

For liquid chromatography-mass spectrometry (LC-MS/MS) identification of gel band, the supernatants were separated by SDS-PAGE, band was excised and sent to Stanford University Mass Spectrometry facility.

Growth Assays

For experiments investigating growth in 180 µM palmitate, 0.5 mL of DPBS or 1,800 µM palmitate was added to 4.5 mLs of TSB pre-warmed to 37°C. Growth in surfactants were done in 10% mouse surfactant, 2% rat surfactant, or 1% Infasurf. Sub-cultures were inoculated with 50 µL of overnight culture. Samples were incubated for five hours at 37°C with shaking (250 rpm).

For experiments with resuspension, supernatants from *S. aureus* sub-cultured for 5 h in TSB with glucose were sterile-filtered (0.22 µm, Avantor) and diluted in DPBS. Then, supernatants were incubated with various compounds and PMNs for 1 hour at 37°C.

Relative Quantitative Real Time RT-PCR

Transcription of *S. aureus* genes was assessed using TaqMan™ real time reverse transcriptase-PCR (RT-PCR) as previously described previously (Nygaard *et al.* 2010; Voyich *et al.* 2005; Voyich *et al.* 2009). Briefly, sub-cultured strains were harvested at mid-exponential (ME; OD_{600} = 1.5) or early stationary (ES; OD_{600} = 3.0) phase of growth, mechanically disrupted using a FastPrep FP120 cell disrupter (ThermoFisher Scientific), and RNA purified using RNeasy Kit (Qiagen). TaqMan™ real-time RT-PCR was performed. Primers and probes of *sarA*, *sarR*, and *sspB* are listed in Table 1 below.

Table 1. Primer and Probe sequences of *sarR*, *sarA*, and *sspB*.

Gene & Probe/Primer	Sequence
sarR Forward Primer	ACTTGAAATGTTGCGTTGACTA
sarR Reverse Primer	CATGTGAACCTTGCTACAACA
sarR Probe	AAACCACTCCTCTGATGCACATCT
sarA Forward Primer	CGTTGTTTGCTTCAGTGATTCGTTTA
sarA Reverse Primer	CGAGCAAGATGCATCAAATAGGGAGG
sarA Probe	CTTTCTCTTTGTTTTCGCTGATGTATGTC
sspB Forward Primer	CTGTATCCCAAGGATTCCAGTAAA
sspB Reverse Primer	TCCTTGCACAAAGTGTATCTCA
sspB Probe	AGCGCATGTCCTAAATGTGGGTCA

Human PMN or PBMC Plasma Membrane Integrity Assays

Heparinized venous blood from healthy donors was collected in accordance with a protocol approved by the Institutional Review Board for Human Subjects at Montana State University. All donors provided written consent to participate in the study. Human polymorphonuclear leukocytes (neutrophils or PMNs) and/or peripheral blood mononuclear cells (PBMCs) were isolated under endotoxin-free conditions (<25.0 pg/ml) and cell viability and purity of preparations were assessed using a FACSCalibur Flow cytometer (BD Biosciences) as described previously (Nygaard *et al.* 2010; Voyich *et al.* 2005). Assays intoxicating PMNs with extracellular *S. aureus* proteins were performed as previously described (Nygaard *et al.* 2012; Flack *et al.* 2014). Briefly, supernatants from *S. aureus* sub-cultured for 5 h in TSB with glucose were sterile-filtered (0.22 μm, Avantor) and diluted in DPBS. PMNs or PBMCs ($1x10^6$) were exposed to varied dilutions of *S. aureus* supernatant. Samples were incubated at 37°C for 60 min then stained with propidium iodide (PI; ThermoFisher Scientific) following the manufacturer's protocol and then analyzed with a FACSCalibur Flow cytometer.

For PMN chemokine experiments, PMNs were isolated and 0.2 mL FMLF (N-formylmethionine-leucyl-phenylalanine, stock at 0.5 mg/mL in methanol) at 0.005 mg/mL concentration in RPMI was added to 2 mLs of PMNs. PMNs were incubated on ice for 20 minutes after which 0.2 mL of LPS (stock in water at 5 mg/mL) was added at a concentration of 0.05 mg/mL and incubated for 2 hours on ice. PMNs were centrifuged at 500 x g and supernatant was extracted.

Blood Lysis Experiments

S. aureus was grown as described above with glucose and sub-cultured for 5 hours. Samplees (5 μL) were spotted on a sheep blood agar plate (BD Diagnostics) and incubated at 37°C for 24 hours, and then at 4°C for 24 hours, after which lysis area was photographed and measured with ImageJ.

Results and Discussion

Cysteine Protease SspB Regulates *S. aureus* Pathogenicity

Since palmitate, a component of lung surfactant, suppressed *S. aureus* virulence just like lung surfactants, we investigated secreted *S. aureus* proteins in response to the presence of palmitate. Among the few proteins secreted by early stationary phase, SDS-PAGE analysis demonstrated a band at approximately 20 kDa size (Figure 1), that was seen in supernatants exposed to palmitate but not in control samples. LC-MS/MS identified this band as SspB, a *S. aureus* cysteine protease which is 22 kDa in its mature form (Massimi *et al*. 2002). In its precursor form, its size is 40 kDa (Massimi *et al*. 2002), which we have also observed in other gels when *S. aureus* was grown with palmitate (data not shown). This finding is confirmed by earlier work which demonstrated that growth in the presence of fatty acids promotes the production of *S. aureus* proteases like SspA and SspB (Arsic *et al*. 2012).

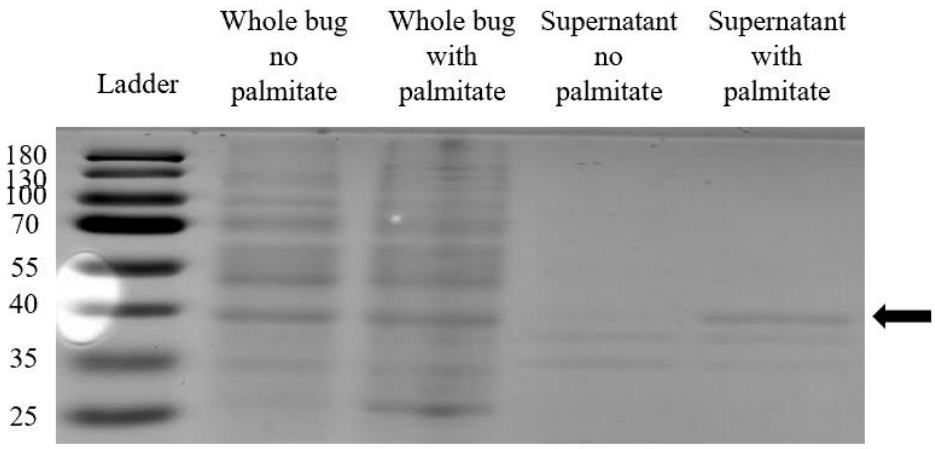


Figure 1. Growth in palmitate induced secretion of a protein at ~ 20 kDa. LAC overnight cultures were grown in TSB with glucose then sub-cultured to early stationary phase in media with or without 180 μM palmitate. 15% SDS-PAGE gel with culture with whole bug or with just supernatants was stained Silver stain and representative gel shown.

To investigate why this protease is consistently produced in response to palmitate, SspB knock out strains were created in the wildtype *S. aureus* USA300 background (strain LAC) as well as in transposon library JE2 strain which derives from the USA300 background but does not have its native plasmids (Miller *et al*. 2019). Because the plasmids contain some virulence factors like PVL toxin (Diep *et al*. 2006), without the plasmids, JE2 is comparatively less virulent than LAC. Comparing LAC and LACΔ*sspB* cytolytic capacity against PMNs at different supernatant dilutions demonstrated that LAC supernatants are very cytotoxic, and an effect due to lack of SspB could only be discerned at 1:200 (Fig 2A). Yet, because JE2 is less virulent, a higher supernatant concentration can be used to see the same effect (Figure 2C). PMNs exhibited more membrane damage from supernatants from strains lacking *sspB* gene, indicating that SspB lowers the cytotoxicity of *S. aureus* (Figure 2B and 2C). It is curious that JE2 without *sspB* has similar cytotoxicity level as LAC without *sspB*, but this may be explained by differences in strain backgrounds. Growth was not influenced by lack of *sspB*, but due to small inhibitory effect of

palmitate at the concentration used, CFU matching was done for all experiments to account for this (Figure 2D and 2E). Previously, literature has observed that cysteine protease ScpA promoted intracellular cytotoxicity of *S. aureus* JE2 strain towards epithelial cells whereas SspB had no effect on intracellular cytotoxicity (Stelzner *et al*. 2021). Here, we are finding that SspB may be involved in cytotoxicity against immune cells. Since SspB is known to interact with monocytes (Singh *et al*. 2019; Tam *et al*. 2019), we initially thought that SspB would also affect *S. aureus* cytotoxicity against peripheral blood mononuclear cells (PBMCs). However, we found that *sspB* deletion strain did not induce more membrane damage in PBMCs at 1:5 dilution (data not shown) as it does with PMNs.

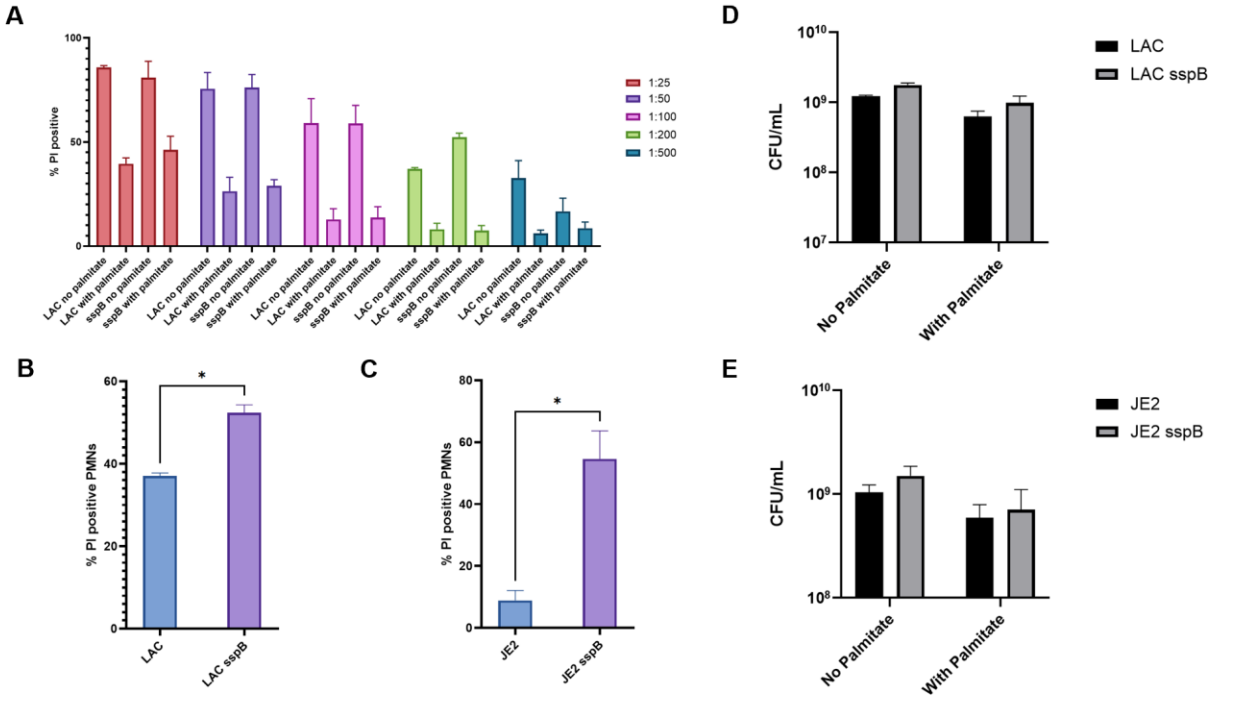


Figure 2. SspB Modulates *S. aureus* cytotoxicity against PMNs. *S. aureus* strains LAC and LAC∆*sspB* were grown to early stationary phase with and without 180 µM palmitate. *S. aureus* supernatants were harvested, diluted to final concentrations for LAC background and JE2 background, respectively, and incubated with human PMNs for one hour (A). *S. aureus* strains LAC and LAC∆*sspB* for (B) or JE2 and JE2∆*sspB* for (C) were grown to early stationary phase. *S. aureus* supernatants were harvested, diluted to 1:200 and 1:25 final concentration for LAC background and JE2 background, respectively, and incubated with human PMNs for one hour. Plasma membrane damage was assessed using propidium iodide uptake and analyzed by flow cytometry for A, B, and C. CFUs collected after growth for LAC strains (D) and JE2 strains (E). Data are from 3 biological replicates for A, B, C, D, and E. *P <0.05, **P < 0.005 Paired t-test for B and C.

To continue investigating the impact of SspB on *S. aureus* pathogenesis, blood lysis of various protease mutants was assessed using sheep blood agar plates. Wildtype and mutant strains were grown for five hours and then plated on sheep blood plates to see if there was an effect on blood lysis. While the lysis areas of LAC and protease mutants *spl* and *aur* mutants were similar, the lysis area of *sspB* mutant in LAC background compared to other protease mutants was consistently smaller (Figure 3), indicating that SspB may play a role in blood cell lysis. Indeed,

there have been reports hinting at a relationship between fatty acid kinase, Hla, and SspB (Ridder *et al*. 2020). More experiments are needed to understand the interaction between SspB and blood lysis.

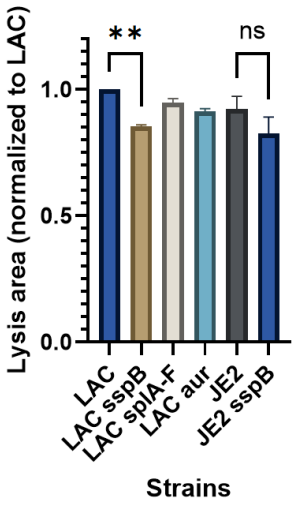


Figure 3. *sspB* may be involved in *S. aureus* blood lysis. *S. aureus* mutants were sub-cultured for 5 hours in TSB with or without 180 μM palmitate. Cultures were spotted onto sheep blood agar which was incubated for 24 hours in 37°C and then 24 hours in 4°C. Lysis area was measured and normalized to LAC. Data are from 3 biological replicates with error bars showing mean ± SEM. *P <0.05, **P < 0.005 and ****P < 0.0001 one-way ANOVA followed by Tukey's multiple comparison test. NS = not significant.

sspB Transcript is Increased in the Presence of Palmitate and Natural Lung Surfactants

A variety of elements regulate protease production: including RNAIII SarS, SarR, SarA, SarV, SarX, SarZ, ArlRS, CodY, Rot, MgrA, and SaeRS (Gimza *et al*. 2019). Among these, SarA is a prominent protease regulator which down-regulates production of proteases, while SarR is a repressor of SarA. To investigate how *sspB* and protease transcription factors *sarA* and *sarR* would be affected upon exposure to PMNs compared to palmitate, *S. aureus* strains were exposed to PMNs or palmitate. Exposure to just PMNs only slightly modulated expression of *sarR*, *sarA*, or

sspB (Figure 4). Exposure to palmitate increased expression of these genes. Only in the case of *sspB*, the expression was upregulated after exposure to palmitate but downregulated when exposed to PMNs.

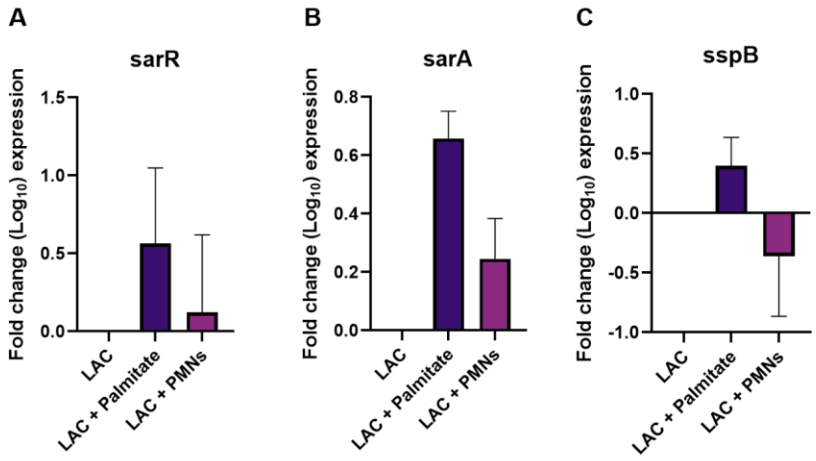


Figure 4. Modulation of *sarA*, *sarR*, *sspB* in presence of palmitate or PMNs. *S. aureus* was grown to mid-exponential phase and exposed to 180 μM palmitate or PMNs for 1 hour at 37°C. RNA was harvested and subjected to TaqMan® RT-PCR. Gene transcripts were normalized to *gyrB*. Data displayed are the mean fold-change of indicated gene relative to treatment with *S. aureus* only. Data displayed show mean ± SEM of 3 biological replicates.

To understand the relevance of SspB in context of lung infections, transcriptional responses of *sspB* were investigated in the presence of palmitate or lung surfactants. During growth in palmitate, *sspB* was slightly up-regulated by mid-logarithmic phase and strongly induced by early stationary phase (Figure 5A). This is to be expected as SarA regulates transcription most profoundly during later growth phases. However, mouse, rat, and the commercially available surfactant Infasurf® promoted *sspB* up-regulation earlier at mid-logarithmic phase and down-regulated *sspB* by early stationary phase (Figure 5B and 5C). The difference between *sspB* regulation in the presence of lung surfactant versus palmitate may point to different roles of SspB

under the conditions tested or different concentrations of palmitate (as well as other molecules that may influence *sspB*) in the lung surfactants.

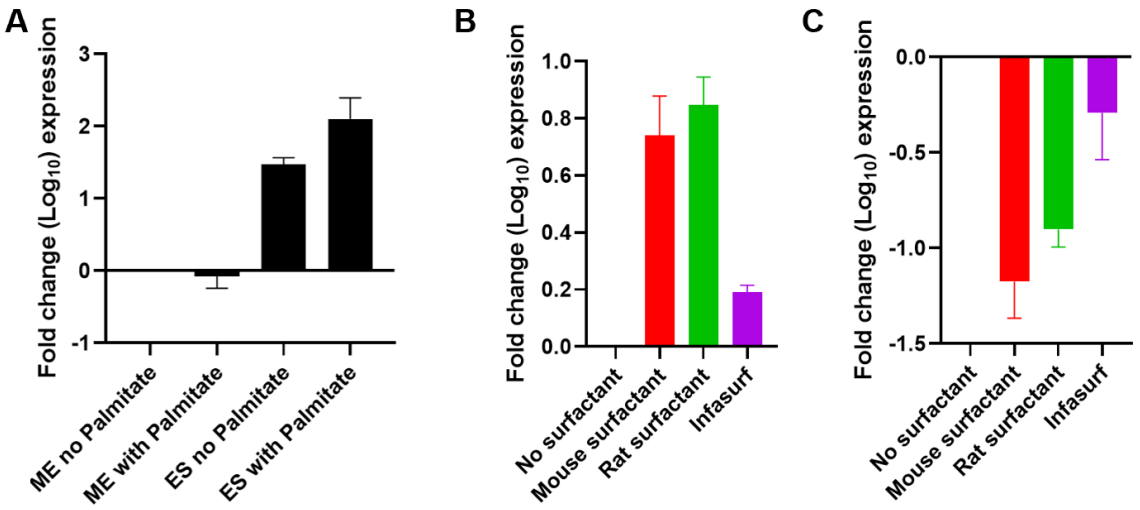


Figure 5. Gene expression of *sspB* in various surfactants. *S. aureus* was grown in 180 µM palmitate (A), or 10% mouse surfactant, 2% rat surfactant, and 1% Infasurf® to mid-logarithmic (B) or early stationary phase (C). RNA was harvested and subjected to TaqMan™ RT-PCR. Gene transcripts were normalized to *gyrB*. Data shown are the mean fold-change of *S. aureus sspB* relative to treatment with *S. aureus* only. Error bars indicate mean ± SEM of 3 biological replicates for A, B, and C.

Investigating if SspB Digests Bacterial or Host Proteins

SspB was involved in reducing *S. aureus* cytotoxicity against PMNs (Figure 2). Studies have suggested that proteases may cleave secreted toxins to regulate virulence in specific niche environments (Kolar *et al*. 2013; Shaw *et al*. 2004). Upon full deletion of proteases, an increase in abundance of toxins, such as alpha-toxin, PSMs, LukAB, PVL, and gamma-hemolysin have been observed (Kolar *et al*. 2013). A serine protease was suggested to degrade alpha toxin (Lindsay *et*

al. 1999), and aureolysin is known to control the stability of PSMs and alpha toxin (Gimza *et al*. 2019). Also, cysteine proteases in other bacteria like the Streptococcal pyrogenic exotoxin B produced by *Streptococcus pyogenes* can cleave its own secreted virulence factors (Carroll *et al*. 2011).

Given this background, we hypothesized that presence of palmitate induces protease secretion, specifically SspB, to reduce viable toxins in the supernatant. Supernatants from LAC, JE2 and respective mutant strains showed no difference in secretion patterns when analyzed by SDS-PAGE (data not shown). Addition of purified SspB protease to supernatants produced by strains also did not show any differences (data not shown). Next, we tested if purified SspB was able to cleave purified toxins of interest, LukAB and PVL. A previous study demonstrated that SspB activity happened within 16 minutes for some of its known substrates (Ohbayashi *et al*. 2011). Neither an instantaneous combination of SspB with the two toxins or a two-hour pre-incubation of the cysteine protease with each toxin did anything to either of the toxins (Figure 6). Many other experiments were conducted to determine if SspB was directly impacting toxin production, e.g. LAC and LACΔ*sspB* were grown in palmitate and surfactants (no data shown), purified SspB was incubated with palmitate (Supplemental Figure 1), and assays were done to inhibit cysteine protease activity (Supplemental Figure 2). However, no mechanism was identified.

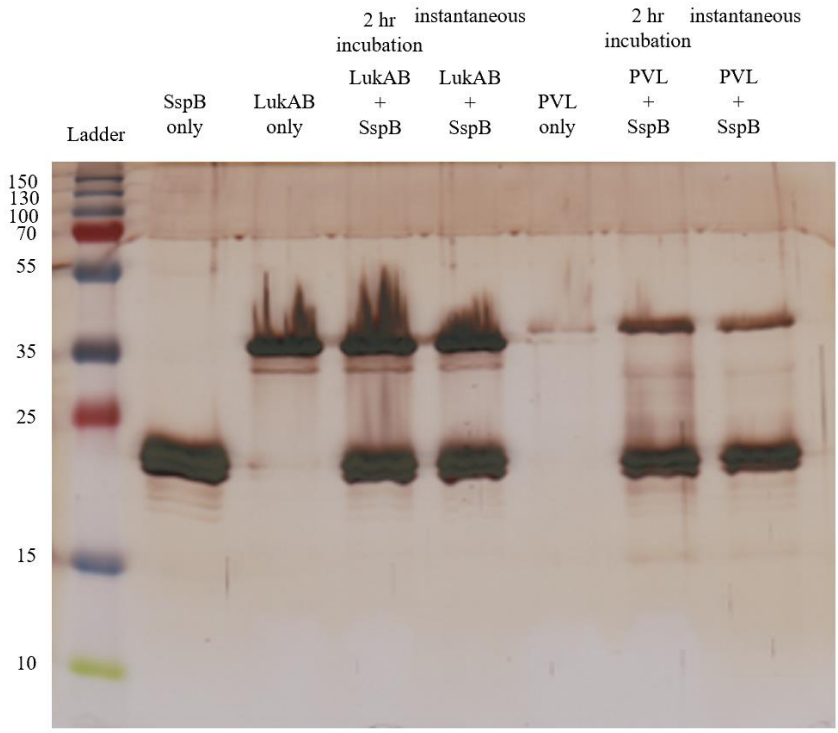


Figure 6. Purified SspB does not interact with LukAB or PVL. Purified SspB (1:20 dilution) was incubated with LukAB or PVL (at 1:40 dilution) for 2 hours at 37°C or samples were run without incubation instantaneously. Silver stain was performed on the 15% SDS-PAGE gel. N=1.

Given we could not find how SspB interacts with *S. aureus* toxins, we were then interested in seeing if it cleaves any host proteins. Based on previous reports, there are many host targets of SspB. SspB cleaves CD31 receptor which has an antagonistic effect on neutrophils, and staphopain A can cleave the CXCR2 chemokine receptor on neutrophils (Pietrocola *et al*. 2017). Similarly, a *Streptococcus pyogenes* protease can cleave CXCL8 (Kurupati *et al*. 2019). This prompted us to test if SspB may also be cleaving cytokines produced by neutrophils themselves. Unfortunately, we could not see conclusive differences between PMN secreted factors which have been incubated with or without purified SspB, although there are slight differences in 15-25 kDa sized proteins which are around sizes of several chemokines (Figure 7).

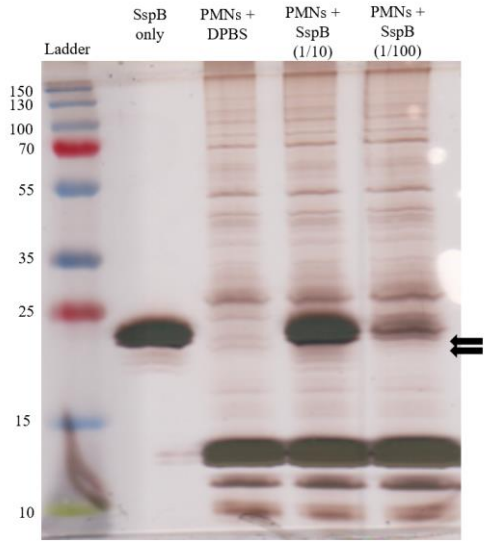


Figure 7. Purified SspB may cleave chemokines produced by PMNs. Human PMNs were exposed to FMLF for 20 minutes on ice, stimulated for 2 hours on ice with LPS. PMN supernatant was extracted and incubated with purified SspB at 1/10 or 1/100 concentrations for 2 hours at 37°C. Silver stain was performed on the 15% SDS-PAGE gel.

One final thought was that if palmitate signals to *S. aureus* that it is in the lung environment, it is possible that SspB is produced to cleave something in the lung surfactant itself. Degradation of lung surfactant proteins is not well studied but may have implications for pathogenesis since degradation of SP-A allows *S. aureus* to aggregate and attach to pulmonary epithelium (Pivard *et al*. 2021). Previous literature identified that SspB cleaves SP-A partially (Kantyka *et al*. 2013), and a cysteine protease has been indicated to cut SP-D (Zhang *et al*. 2015). Lung surfactant is composed of 4 proteins, which are SP-A, SP-B, SP-C, and SP-D, which are 26-36, 14, 6, and 43 kDa in size, respectively (Possmayer 1988; Kishore *et al*. 2006; Crouch *et al*. 1994). They are able to form various multimers (Rubio *et al*. 2004). To investigate if SspB can cleave proteins from lung surfactant, purified SspB was incubated with mouse and rat surfactants. However, any changes were difficult to notice due to the amount of bands present (Figure 8). There seem to be

lighter bands for lanes with purified SspB on mouse surfactant at about 27 kDa which may be SP-A, on rat surfactant at 43 kDa which may be SP-D, 35 kDa which may be SP-A, and between 15-25 kDa which may be SP-B or SP-C (Figure 8). Additional methods are necessary to determine SspB activity against lung surfactant proteins starting with investigating activity of SspB on purified SP-B, SP-C, and SP-D.

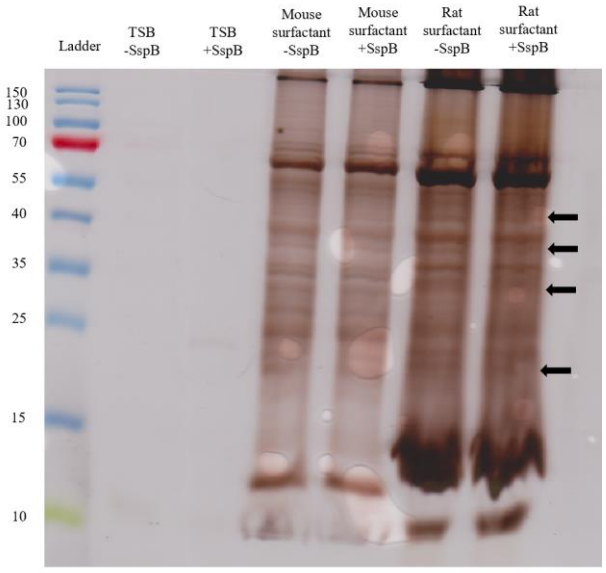


Figure 8. Purified SspB may be cleaving lung surfactant proteins. Purified SspB (1:10) was incubated in TSB, TSB with 10% mouse surfactant, 2% rat surfactant, or 1% surfactant for 5 hours at 37°C. Silver stain was performed on the 15% SDS-PAGE gel.

Conclusions

Bacterial pneumonia is a severe infectious disease in terms of incidence, morbidity and mortality, and impact on quality of life. *S. aureus* is a prominent lung colonizer known for causing pneumonia. From its normal presence in the anterior nares, *S. aureus* gets trafficked into the lungs where it encounters lung surfactant. Although lung surfactant and its components possess antibacterial properties which suppress *S. aureus* virulence, *S. aureus* still produces some factors

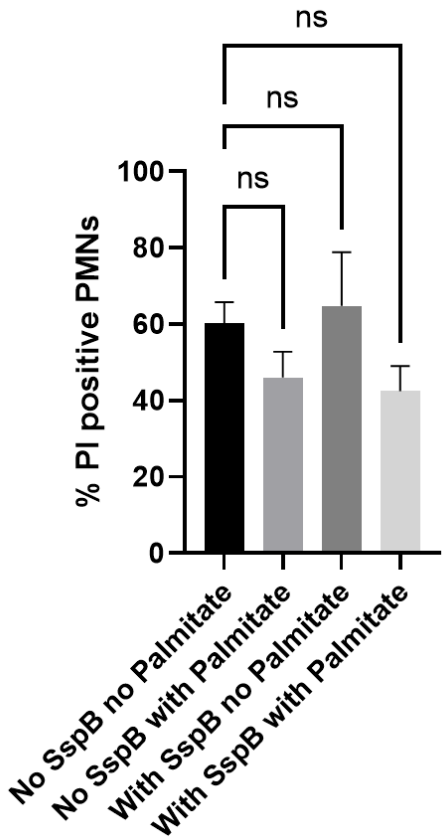
which ensure its survival in the lung. Upon investigating palmitate, a component found in lung surfactant, we discovered that palmitate stimulated *S. aureus* to secrete cysteine protease SspB. While SspB was not involved in managing to survive in palmitate, this cysteine protease reduced the potency of *S. aureus* supernatants against PMNs. Potentially, *S. aureus* uses SspB to lower its own virulence to avoid being found by patrolling immune cells until there is enough bacteria in the lung. SspB was also found to have a role in blood lysis which could be a reflection of how *S. aureus* gains nutrients or travels in the bloodstream. Future studies will investigate *sspB* mutant strains in mouse models, including sepsis and pneumonia models, to further understand its impact on pathogenesis.

Data demonstrated SspB was up-regulated early during growth in lung surfactants which points to its role in early lung pathogenesis. To further elucidate how SspB impacts *S.* aureus-neutrophil interactions by reducing cytotoxicity, we investigated if SspB was degrading *S. aureus* toxins. Our methods did not give conclusive results. Although SspB did not cleave LukAB or PVL, we were unable to completely rule out that SspB cleaves *S. aureus* toxins. It may be that only aureolysin plays a role in cleaving key *S. aureus* toxins as it has been shown to reduce alpha toxin quantity and overall cytotoxicity (Ramirez *et al*. 2020). Since SspB has been shown to work with other proteases (Burlak *et al*. 2007), it is also possible that more complex protease interactions need to be investigated to determine a mechanism.

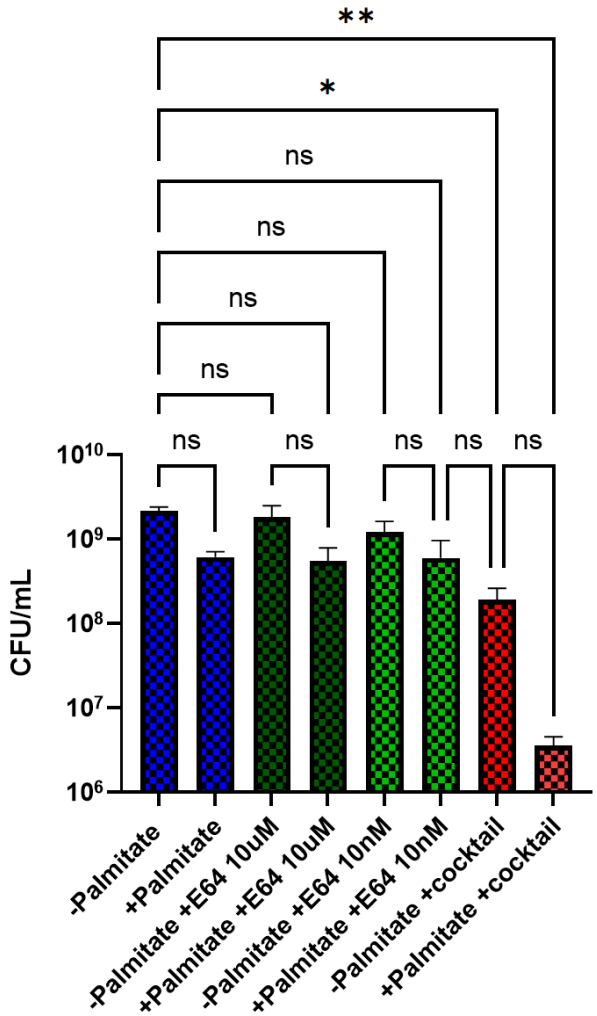
Finally, we could not determine if SspB affects neutrophil signaling by cleaving cytokines. Given past literature findings and suggestions that bacterial proteases or phospholipases may degrade lung surfactant during pneumonia (Bouhafs *et al*. 1999), we. also investigated if SspB cleaved various lung surfactant proteins. Our methods were not able to pinpoint exactly which

lung surfactant proteins SspB can digest. However, we suspect that one of the reasons why SspB is produced during lung infection is that SspB can impact the integrity of lung surfactant by digesting the proteins which maintain the structure or provide antimicrobial activity. Future studies will continue to investigate SspB to identify a mechanism.

Supplemental Data



Supplemental Figure 1. Purified SspB pre-incubated with palmitate has no effect on cytotoxicity. Purified SspB (final dilution of 1:100) was preincubated with or without 180 µM palmitate for 1 hour at 37°C. *S. aureus* was grown to early stationary phase. Supernatants were harvested and diluted into pre-incubated samples to final dilution of 1:50. Following this, samples were incubated with human PMNs for one hour. Plasma membrane damage was assessed using propidium iodide uptake and analyzed by flow cytometry. Data are from 3 biological replicates. Error bars indicate mean ± SEM. *P <0.05 one-way ANOVA followed by Dunnett's multiple comparison test. NS = not significant.



Supplemental Figure 2. LAC grown in palmitate with and without protease inhibitors. *S. aureus* was grown to early stationary phase with palmitate, E64 at varied concentrations which have been used for inhibiting SspB previously (Karlsson *et al*. 2001, Zhang *et al*. 2015), and protease inhibitor cocktail. E64 works against general cysteine proteases and protease cocktail inhibitor blocks all action of serine proteases, cysteine proteases, aspartic acid proteases, and aminopeptidases. CFUs were collected the next day. Data are from 3 biological replicates with error bars showing mean ± SEM. *P <0.05, **P < 0.005 one-way ANOVA followed by Tukey's multiple comparison test. NS = not significant.

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CHAPTER FIVE

CONCLUSIONS AND FUTURE STUDIES

Conclusions

Bacterial pneumonia continues to complicate many respiratory illnesses and claims four million lives globally (Sattar *et al*. 2024). The situation is further worsened by the decreasing susceptibility of lung pathogens like MRSA to antibiotics which comprise the treatment available, highlighting the need to study lung infections to come up with more effective treatment strategies. This work investigates preventative lung defenses and establishes the importance of endogenous pulmonary surfactant in protecting the host from bacterial pathogens. The studies give insight into the mechanisms behind the protective action, identified that surfactant and its components repress bacterial virulence, and give merit to further explore exogenous surfactant as a therapeutic approach.

While it was previously known that lung surfactant proteins have opsonization and antibacterial properties, not many reports assessed the impact of whole lung surfactant on bacterial pathogenesis. In Chapter 2, we demonstrated that lung surfactants protect the host by suppressing *S. aureus* virulence and cytotoxicity against immune cells. This is achieved by pulmonary surfactant repressing SaeRS-regulated virulence factors. Data demonstrated that rat and mouse surfactants and a commercially available bovine-derived surfactant, reduced *S. aureus* cytotoxicity against human neutrophils and PBMCs.

In Chapter 3, we identified one of the potential effectors in lung surfactant behind the protective effect of whole surfactants. We used the same methods to assess if a common fatty acid

found in lung surfactants, palmitate, was able to recapitulate the protective phenotype shown by lung surfactants. Similar to surfactants, palmitate was also able to down-regulate key toxins and protect neutrophils from *S. aureus* cytotoxicity. Although previous reports have assessed fatty acids (palmitate included) for antibacterial properties and their effect on SaeRS system, our work demonstrates transcriptional effects of palmitate on *S. aureus* virulence and further elucidates how this action impacts host-pathogen interactions. Palmitate was also shown to have a direct effect on *S. aureus* supernatants which is a novel finding that should be further studied.

The finding of the bacterial suppression by palmitate in Chapter Three prompted us to study the pathogen's response to palmitate. This research expanded into identification of a cysteine protease SspB produced by *S. aureus* in response to the presence of palmitate. This protease appears to be important in the context of lung infections and host-pathogen interactions, as it was also able to reduce cytotoxicity of *S. aureus*. Although tested, it is not clear whether this protease is able to digest *S. aureus* toxins, however, it appears to be important in digesting host surfactant proteins. Further research is needed to expand which host and pathogen substrates are digested by SspB.

All in all, this data combines to create a model behind the interaction between lung surfactant and *S. aureus*. Figure 1 shows how the presence of pulmonary surfactant suppresses bacterial virulence by influencing the SaeRS system and this is observed in whole lung surfactants and in lung surfactant components like palmitate. In response to the lung environment, *S. aureus* produces virulence factors like SspB that may aid in lowering virulence or affecting the lung surfactant. Collectively, this data underscores the importance of lung surfactant and helps

understand why secondary bacterial pneumonias emerge once initial viral infections alter surfactant composition and abundance.

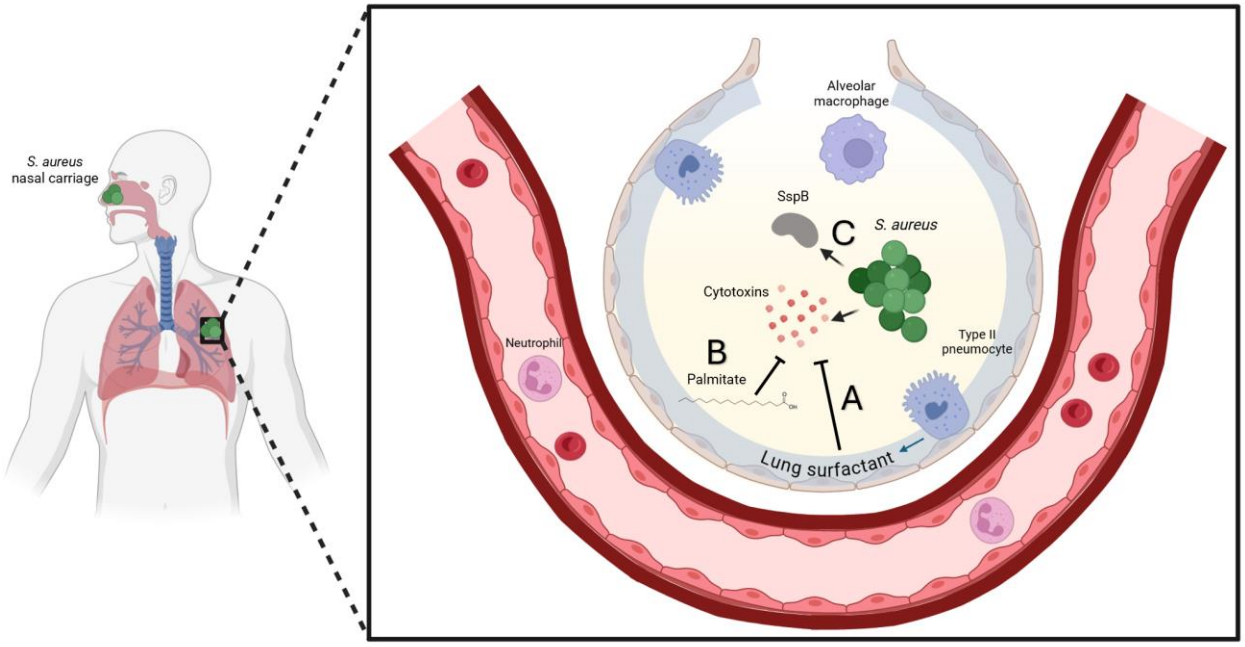

Figure 1. Lung surfactant suppresses *S. aureus* virulence. Lungs have several natural protective defenses against bacterial pathogens. In this work we define a protective role of lung surfactant which coats alveoli and is produced by type II pneumocytes. Chapter 2 establishes that lung surfactant reduced cytotoxicity of *S. aureus* toxins by impacting the regulation of toxins (A). To find which part of pulmonary surfactant achieves this, Chapter 3 investigated palmitate, a component of lung surfactant, and determined that it is also able to reduce cytotoxicity as well and directly impact toxins (B). Finally, Chapter 4 explores how a *S. aureus* cysteine protease SspB secreted in response to the presence of palmitate may have a function for lung colonization (C).

Future Studies

Having discovered that lung surfactant plays a critical role in protecting the host from lung infections, there are several avenues to expand in this research.

Determining the Active Components in Lung Surfactant Responsible for the Protection

Although we have identified palmitate's capability to mirror the protective effect of lung surfactant, the goal to narrow down the components of lung surfactant responsible for the protective action still remains. As reviewed in Chapter One, lung surfactant is composed of various lipid components as well as proteins. Previously, we hypothesized that it is a lipid component behind surfactants' protective effect as observed in Chapter Two and demonstrated in Chapter Three that palmitate was a component of lung surfactant able to reduce *S. aureus* toxicity. However, surfactant is complex, and data presented did not investigate other fatty acids and proteins of lung surfactant. To begin to identify more components of surfactant that may impact *S. aureus* virulence we inactivated the surfactant proteins by treating surfactants with heat before adding the surfactant to growth media. Inactivating the proteins did not change the effectiveness of the protection of PMNs from *S. aureus* supernatants by lung surfactants (Figure 2), indicating that it is the non-protein compounds in lung surfactants that are able to protect the immune cells. Another group that has previously shown that surfactant fraction of rat alveolar lining material had anti-pneumococcal activity used trypsin and found that the antibacterial effects they observed were due to lipids rather than proteins (Coonrod *et al*. 1983), corroborating our findings.

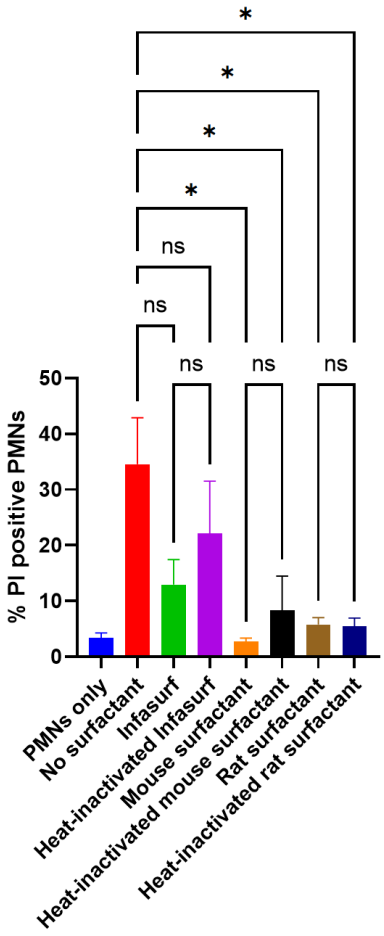

Figure 2. Heat inactivation of the proteins in surfactants does not reduce surfactant protectivity. To heat-inactivate the proteins in surfactants, 10% mouse, 2% rat surfactant, or 1% Infasurf were incubated at 56°C for 30 minutes. *S. aureus* was grown to early stationary phase in TSB with untreated or heat-treated 10% mouse, 2% rat surfactant, or 1% Infasurf. *S. aureus* supernatants were harvested, diluted to 1:50 final dilution, and incubated with human PMNs for one hour at 37°C. Plasma membrane damage was assessed by PI staining and flow cytometry. Data are from 3 biological replicates. Error bars indicate mean ± SEM. *P <0.05 one-way ANOVA followed by Tukey's multiple comparison test. NS = not significant.

Having established that it is not due to proteins, we would like to investigate if there are other major effectors in the lung surfactant behind the protective effect. In Chapter Three, we investigated palmitate for this role due to its abundance in lung surfactant. Although palmitate showed similar effects as lung surfactants themselves, there may be more and better effectors

behind this effect. To determine possible lipid targets, previous work investigated lipid components of mice following influenza A viral infection (Borgogna 2019). Herein, we analyzed the raw data to find which lipids were affected by an influenza A viral infection with the help of BioPAN, which is a web-based tool to explore lipidome metabolic pathways on LIPID MAPS (O'Donnell *et al*. 2019). With this tool, we have gathered the molecular species which were affected by influenza A virus (Figure 3A). We have found major trends such as alkyl ether linkage change to (1Z)-alkenyl ether (Figure 3B) and glycerophosphocholines being majorly converted to diradylglycerolipids (Figure 3C). In other words, in major species affected by influenza A virus, the phosphate and choline heads were detached. Choline is important for growth and function in the lungs and lipids with choline serve central roles in membrane structuring and reduction of surface tension (Bernhard *et al*. 2022). Although this may just be a trend of influenza A virus lung infection, it is possible that species with choline heads have important antibacterial properties as well. This method had significant limitations as not all lipid species were identified and only a group of molecular species were actually processed by the bioinformatic workflow.

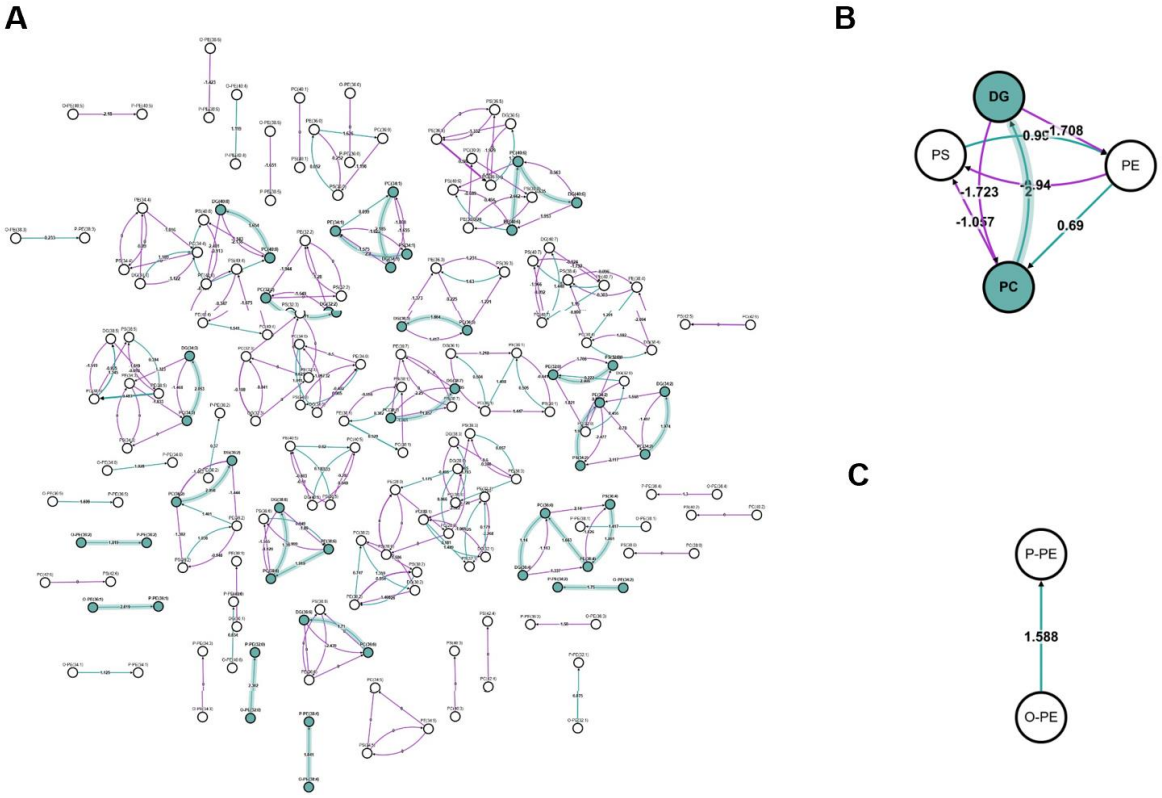

Figure 3. BioPAN lipidomic trends of mock and IAV infected murine lungs. Mock and IAV infected lungs were harvested 6-days post infection and snap frozen (n=3 mice per treatment group). Whole lungs were provided to Avanti Polar Lipids, Inc. (Alabaster, AL) and subjected to a modified Bligh Dyer extraction followed by LC-MS/MS analysis and separation by HILIC (Borgogna 2019). Raw data was processed through BioPAN. Of 1040 total molecular species, 177 molecular species were processed. Circles represent glycerolipids or glycerophospholipids. Clear circles have no status while those in teal are changed. Purple edges have a negative Z-score whereas teal have positive Z-score. A shows trends across the 177 species processed. B and C show main trends.

While palmitate is a free fatty acid, lung surfactant is mostly composed of more complex lipid structures. It is possible that these protective properties may be held by most if not all lipids that have palmitate. Furthermore, there are other lipids like cholesterol and other major phospholipids in lung surfactants which may be even more effective. A previous group also demonstrated that diacylphospholipids including dipalmitoyl phosphatidylcholine (DPPC), phosphatidylglycerol, phosphatidylinositol, and phosphatidylethanolamine did not have

antibacterial effects while lysophospholipids like palmitoyl lysophosphatidylcholine, myristoyl lysophosphatidylcholine, and palmitoyl lysophosphatidylethanolamine had dose-dependent antibacterial activity which the authors attributed to bacterial permeability (Coonrod *et al.* 1983). Hence, fatty acids and lysophospholipids found in lung surfactant appear to be prime targets. For future studies, to determine if there are multiple various effectors in lung surfactant, *in-vivo* murine models of secondary *S. aureus* infection post influenza A virus infection will help identify lipid species as targets potentially responsible for the protective effect. For this, lipidomic analysis comparing healthy mouse lung to influenza-infected lungs, to *S. aureus* infected lungs, to influenza-and-*S. aureus*-infected lungs will help identify lipid species responding to type of infection. From this list, we would then be able to test these various components for their activity against *S. aureus*.

Understanding the Mechanism(s) of Protection

Although we had seen that virulence genes were down-regulated in the presence of lung surfactants and palmitate, we must understand by which mechanisms lung surfactants and their components affect *S. aureus*. It may be that lipids interact with *S. aureus* membrane, or they are a signal for a two-component sensory system. It is also of interest to determine what surfactants do to the secreted factors. Palmitate was shown to have a direct effect of secreted virulence factors of *S. aureus* in Chapter Three. It is unknown if this interaction also happens with natural surfactants and what the nature of this interaction is.

Establish if Exogenous Surfactant can be used as Therapy

It still needs to be determined if commercially available surfactants currently used for neonates with respiratory distress syndrome may serve as a treatment or preventative measure

against bacterial lung infections. Data presented suggest surfactant could be used as a treatment. In Chapter Two, Infasurf was shown to reduce *S. aureus* cytotoxicity against PMNs and PBMCs when the bacterium was grown in the surfactant. Although we observed a lesser protective effect by Infasurf compared to natural surfactants, other commercially available surfactants may have a greater effect and should be investigated. Hence, it remains to be seen whether this is a viable treatment option. For these experiments, mice with a secondary bacterial infection will be given commercially available surfactants at different stages of infection to see if the surfactant addition can prevent or even treat the bacterial infection.

Overall, this work broadens our understanding of how lung surfactant protects the host. The protective effects of lung surfactants that we have observed may be extrapolated to other lung pathogens, such as *Streptococcus pneumoniae* and *Haemophilus influenzae* and should be investigated in future studies. Regardless data in this dissertation help explain how lungs become susceptible to bacterial pneumonia and introduce lung surfactant therapy as a potential treatment option. Thus, we hope to prompt future studies that will bring us to a world with better treatments for bacterial pneumonia.

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APPENDIX

CO-AUTHORED PUBLICATIONS

microorganisms

MDPI

Article

The Relative Importance of Cytotoxins Produced by Methicillin-Resistant *Staphylococcus aureus* Strain USA300 for Causing Human PMN Destruction

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Abstract: *Staphylococcus aureus* (*S. aureus*) is a prominent Gram-positive bacterial pathogen that expresses numerous cytotoxins known to target human polymorphonuclear leukocytes (PMNs or neutrophils). These include leukocidin G/H (LukGH, also known as LukAB), the Panton–Valentine leukocidin (PVL), γ-hemolysin A/B (HlgAB), γ-hemolysin B/C (HlgBC), leukocidin E/D (LukED), α-hemolysin (Hla), and the phenol-soluble modulin-α peptides (PSMα). However, the relative contribution of each of these cytotoxins in causing human PMN lysis is not clear. In this study, we used a library of cytotoxin deletion mutants in the clinically relevant methicillin-resistant *S. aureus* (MRSA) isolate LAC (strain ST8:USA300) to determine the relative importance of each for causing human PMN lysis upon exposure to extracellular components as well as following phagocytosis. Using flow cytometry to examine plasma membrane permeability and assays quantifying lactose dehydrogenase release, we found that PVL was the dominant extracellular factor causing human PMN lysis produced by USA300. In contrast, LukGH was the most important cytotoxin causing human PMN lysis immediately following phagocytosis with contributions from the other bicomponent leukocidins only observed at later time points. These results not only clarify the relative importance of different USA300 cytotoxins for causing human PMN destruction but also demonstrate how two apparently redundant virulence factors play distinctive roles in promoting *S. aureus* pathogenesis.

Keywords: *Staphylococcus aureus*; MRSA; USA300; neutrophil; leukocidin; cytotoxicity; virulence; pore-forming toxin; PVL; LukGH

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1. Introduction

Staphylococcus aureus is a common Gram-positive bacterium that is a major cause of human morbidity and mortality, associated with more than 1.1 million deaths worldwide [1] and responsible for over 1.7 billion US dollars in medical costs in the United States alone in 2019 [2,3]. Widespread antibiotic resistance and the lack of an effective vaccine limit our ability to treat and prevent infections caused by this pathogen. In particular, methicillin-resistant *S. aureus* (MRSA) identified by pulsed-field gel electrophoresis (PFGE) as USA300 is currently the dominant clinical isolate in the United States [4–9].

The capacity of *S. aureus* to cause a wide variety of disease in both humans and animals is attributed to the diverse and seemingly redundant array of virulence genes expressed by this organism [10,11]. These include numerous adhesins that bind to specific host molecules, pore-forming toxins that impair the integrity and function of different host cells, and immunomodulatory proteins that directly manipulate the host immune response. For example, the USA300 genome encodes more than seven prominent pore-forming toxins known to be active against different human cell types [12–18]. These

include five bicomponent leukocidins - leukocidin G/H (LukGH, also known as LukAB), Panton–Valentine leukocidin (PVL), γ-hemolysin A/B (HlgAB), γ-hemolysin B/C (HlgBC), and leukocidin E/D (LukED) - as well as α-hemolysin (Hla) and the phenol-soluble modulin-α peptides (PSMα). Given the multitude of *S. aureus* virulence genes with apparent overlapping function, parsing out their relative importance in promoting different aspects of disease has remained difficult.

Polymorphonuclear leukocytes (PMNs or neutrophils) are the most common circulating immune cells in humans and play an important role curtailing *S. aureus* pathogenesis [19–21]. Previous studies have shown that human PMNs are susceptible to intoxication by HlgAB [22–30], HlgCB [22–27,29–33], PVL [22–24,29–38], LukGH [22,24,28–30,35,39–45], LukED [22–24,30,46], Hla [39,47], and PSMα [37,39,48–53]. However, the majority of this research has examined the cytotoxicity of single virulence factors and often relied upon purified proteins used in excess to what is normally produced by *S. aureus*. An unbiased comprehensive analysis comparing relevant concentrations of each of these virulence factors has been lacking. As such, the contribution of each pore-forming toxin produced by *S. aureus* towards lysing human PMNs is not clear.

In this study, we used a library of cytotoxin deletion mutants in USA300 to determine the relative importance of each in causing human PMN destruction. Our results show that PVL is the dominant extracellular cytotoxic factor causing PMN lysis that is produced by USA300, while LukGH is the primary cause of initial PMN destruction following phagocytosis of USA300. These findings show the potency of PVL, and LukGH largely depends upon the context of intoxication and indicate these bicomponent leukocidins play distinct roles in promoting pathogenesis.

2. Materials and Methods

2.1. Bacteria Strains and Culture Conditions

Bacteria were cultured at 250 rpm and 37 °C. Overnight cultures grown in tryptic soy broth (TSB; EMD Millipore, Burlington MA, USA) were used to start subcultures in a 14 mL culture tube containing 5 mL TSB (1:100 dilution) unless otherwise stated. *S. aureus* PFGE-type USA300 strain LAC used in this study has been described previously [54]. Genomic mutations of mutants used in this study (Table 1) were performed as previously described [55–59] using primers listed in Table 2. All mutants used in this study underwent whole-genome sequencing and the breseq computational pipeline [60] to confirm the desired mutation as well as verify that no off-target mutations have occurred or that these strains have lost endogenous plasmids. To generate complementary plasmids, PCR amplification was performed using primers listed in Table 2 with the indicated restriction enzyme sites and cloned into pRB473 as previously described [61].

Table 1. USA300 isolates used in this study.

Strain	Genes Deleted; Complemented	Toxin Genes Present (↓ = Downregulated)
USA300 strain LAC	none	*hlgABC, lukED, lukGH, pvl, hla,* and *psmα* [54]
USA300Δ*agrABCD*	*agrABCD*	↓*hlgABC,* ↓*lukED,* ↓*lukGH,* ↓*pvl,* ↓*hla,* and ↓*psm* [11,13,16]
USA300Δ*saePQRS*	*saePQRS*	↓*hlgABC,* ↓*lukED,* ↓*lukGH,* ↓*pvl,* ↓*hla,* and *psmα* [13,59]
USA300Δ*arlRS*	*arlRS*	↓*hlgABC, lukED,* ↓*lukGH,* ↓*pvl, hla,* and *psmα* [62]
USA300Δ*hlgABC*	*hlgABC*	*lukED, lukGH, pvl, hla,* and *psmα*
USA300Δ*lukGH*	*lukGH*	*hlgABC, lukED, pvl, hla,* and *psmα*
USA300Δ*lukED*	*lukED*	*hlgABC, lukGH, pvl, hla,* and *psmα*
USA300Δ*pvl*	*pvl*	*hlgABC, lukED, lukGH, hla,* and *psmα*
USA300Δ*psm-a*Δ*hla*	*psm-a* and *hla*	*hlgABC, lukED, lukGH,* and *pvl*
USA300Δ*hlgABC*Δ*lukGH*Δ*pvl*	*hlgABC, lukGH,* and *PVL*	*lukED, hla,* and *psmα*
USA300Δ*hlgABC*Δ*lukGH*Δ*lukED*	*hlgABC, lukGH,* and *lukED*	*pvl, hla,* and *psmα*

USA300Δ*pvl*Δ*lukGH*Δ*lukED*	*pvl*, *lukGH*, and *lukED*	*hlgABC*, *hla*, and *psmα*
USA300Δ*pvl*Δ*hlgABC*Δ*lukED*	*pvl*, *hlgABC*, and *lukED*	*lukGH*, *hla*, and *psmα*
USA300⊕*hlgABC*⊕*lukGH*⊕*pvl*⊕*lukED*	*hlgABC*, *lukGH*, *PVL*, and *lukED*	*hla* and *psmα*
USA300Δ*hlgABC*Δ*lukGH*Δ*pvl*Δ*lukED*Δ*hla*Δ*psm*	*hlgABC*, *lukAB*, *pvl*, *lukED*, *hla* and *psm-a*	none
USA300Δ*hlgABC*Δ*lukGH*Δ*pvl*Δ*lukED* pRB473-*pvl*-comp	*hlgABC*, *lukGH*, *pvl*, *lukED*; *pvl* complemented	*pvl*, *hla*, and *psmα*
USA300Δ*hlgABC*Δ*lukGH*Δ*pvl*Δ*lukED* pRB473-*hlgABC*-comp	*hlgABC*, *lukGH*, *pvl*, *lukED*; *hlgABC* complemented	*hlgABC*, *hla*, and *psmα*
USA300Δ*hlgABC*Δ*lukGH*Δ*pvl*Δ*lukED* pRB473-*lukED*-comp	*hlgABC*, *lukGH*, *pvl*, *lukED*; *lukED* complemented	*lukED*, *hla*, and *psmα*
USA300⊕*hlgABC*⊕*lukAB*⊕*pvl*⊕*lukED* pRB473-*lukGH*-comp	*hlgABC*, *lukGH*, *pvl*, *lukED*; *lukGH* complemented	*lukGH*, *hla*, and *psmα*

Table 2. Primers used in this study.

Primer	Sequence
agrABCD-Top_fwd	5′ - GGG GAC AAG TTT GTA CAA AAA AGC AGG CGA AGC GCC CGA AAT AAT ATT TAA CAC - 3′
agrABCD-SphI-Top_rvs	5′ - GGT GGT GCA TGC CTC CTC ACT GTC ATT ATA CGA TTT AG - 3′
agrABCD-SphI-Bot_fwd	5′ - GGT GGT GCA TGC GTC AGT TAA CGG CGT ATT CAA TTG - 3′
agrABCD-Bot_rvs	5′ - GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG TAA GCC CTC TGC TGA TAT G - 3′
SaePQRS-Top_Fwd	5′ - GGG GAC AAG TTT GTA CAA AAA AGC AGG CGA AGG GGA AGT CAT TAC ACA AAC - 3′
SaePQRS-SphI-Top_Rvs	5′ - GGT GGT GCA TGC CTC CCA TTA ATG AGG GCT TC - 3′
saePQRS-SphI-Bot_fwd	5′ - GGT GGT GCA TGC CTC GGA GAG ATT GCA ATT GG - 3′
saePQRS-Bot_Rvs	5′ - GGG GAC AAG TTT GTA CAA AAA AGC AGG CGT CAT ATG GCC GTT AAA CCA CA - 3′
arlRS-SalI-Top_fwd	5′ - TGT CGA CCT CAT ATT ACG ACT TTT TC - 3′
arlRS-PstI-Top_rvs	5′ - CTG CAG TAA ACC TAA AGT GTC GTA AG - 3′
arlRS-SacI-Bot_fwd	5′ - TCA CTA TTG AGC TCT TTG TTA AAG TAG - 3′
arlRS-BamHI-Bot_rvs	5′ - AAA TGG ATC CTA TCA TAA AAT TAG TCG AAG - 3′
hlgABC-SphI-Top_Fwd	5′ - GGG GAC AAG TTT GTA CAA AAA AGC AGG CGT TCG TCA TGA TGA GCG TG - 3′
hlgABC-SphI-Top_rvs	5′ - GGT GGT GCA TGC GGT CGC AGG CGT TTA TAT AG - 3′
hlgABC-SphI-Bot_Fwd	5′ - GGT GGT GCA TGC GTG ACG ACC GTG - 3′
hlgABC-SphI-Bot_rvs	5′ - GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG CGC TAA ATC AAG GGA TG - 3′
lukGH-SphI-Top_fwd	5′ - GGG GAC AAG TTT GTA CAA AAA AGC AGG CCA ATC AGG GTG GGA CAA AAC - 3′
lukGH-SphI-Top_rvs	5′ - GGG GGT GGT GCA TGC GAC GTG CAG TGT ATG AAT CTT G - 3′
lukGH-SphI-Bot_fwd	5′ - GGT GGT GCA TGC GAT TGA TAT TTG TTG ATA TGT ATC GAC ATG TG - 3′
lukGH-SphI-Bot_rvs	5′ - GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC AAT GAT TTG AAC ATA GGC GCA AC - 3′
lukED-SphI-Top_fwd	5′ - GGG GAC AAG TTT GTA CAA AAA AGC AGG CGA AGT TAA GGC CTA CTT CAA TTG TC - 3′
lukED-SphI-Top_rvs	5′ - GGT GGT GCA TGC GAA ACT AAT CCT GGA GTA TAA CTG TTA G - 3′
lukED-SphI-Bot_fwd	5′ - GGT GGT GCA TGC CTA CTG ACA AAG TTG CAG CTA AC - 3′
lukED-SphI-Bot_rvs	5′ - GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG TGC TCG TCG TCA AGA C - 3′

PVL-SphI-Top_fwd	5′ - GGG GAC AAG TTT GTA CAA AAA AGC AGG CCT CAT ATC ATC GCC TTT GTC C - 3′
PVL-SphI-Top_rvs	5′ - GGT GGT GCA TGC GGA ATC AAC TTC ACT GGA TAG G - 3′
PVL-SphI-Bot_fwd	5′ - GGT GGT GCA TGC CTA ACG ACA ATG TTG CAG CTA ATA G - 3′
PVL-SphI-Bot_rvs	5′ - GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG AGA AAG CGC AAG TGG TG - 3′
PSMa-Top_fwd	5′′ - GGG GAC AAG TTT GTA CAA AAA AGC AGG CGT CGT CTA CCT TTC CAT GC - 3′
PSMa-SphI-Top_rvs	5′ - GGT GGT GCA TGC CTC AGG CCA CTA TAC CAA TAG - 3′
PSMa-SphI-Bot_fwd	5′ - GGT GGT GCA TGC CAG CGA TGA TAC CCA TTA AGA TTA CC - 3′
PSMa-Bot_rvs	5′ - GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC GAA TGC AAG CCA ACC AC - 3′
hla-Top_fwd	5′ - GGG GAC AAG TTT GTA CAA AAA AGC AGG CGA AGT CCA TAC AAA ATC CGC ATC - 3′
hla-BamHI-Top_rvs	5′ - GGT GGT GGA TCC CTA TCT ACT TGA TTT GCT TTC CTG AC - 3′
hla-BamHI-Bot_fwd	5′ - GGT GGT GGA TCC CAA TTT CGA GGG TTA GTC AAA GTT G - 3′
hla-Bot_rvs	5′ - GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG CAA TAC TTT ATT GTC CCA TGA TTA GTG - 3′
pvl-EcoRI-comp_fwd	5′ - AGG AGG GAA TTC GTT TGG TAA TGA ACG GGT TTT TTT CG - 3′
pvl-BamHI-comp_rvs	5′ - GGT GGT GGA TCC CAA TTA AGA CGT GGT TAC CCT AAT ATA G - 3′
hlgABC-SacI-comp_fwd	5′ - GGT GGT GAG CTC CAG TTA ATT CGA AAA CGC TTA CAA ATG G - 3′
hlgABC-BamHI-comp_rvs	5′ - GGT GGT GGA TCC CTG TTG GCG ACC GTG - 3′
lukED-SacI-Comp_fwd	5′ - GGT GGT GAG CTC CCA TGA GAG TAG AAG CTT CAG - 3′
lukED-BamHI-Comp_rvs	5′ - GGT GGT GGA TCC GAA GTT AAG ACC CAC TTC AAT TGT C - 3′
lukGH-EcoRI-comp_fwd	5′ - GGT GGT GAA TTC GTA TCA ACG ATC TTA TTA ACG CTG - 3′
lukGH-BamHI-comp_rvs	5′ - GGT GGT GGA TCC CTA CAT TCT ATG TAG CAG GCA AC - 3′

2.2. *Human PMN Purification*

Human polymorphonuclear leukocytes were isolated under endotoxin-free conditions (<25.0 pg/mL) using freshly drawn heparinized venous blood from healthy donors with written informed consent as previously described [56–58,61–66]. Cell viability and purity of preparations were assessed using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA) or SE520EON flow cytometer (Stratedigm, San Jose, CA, USA) to ensure that only preparations containing ≥95% PMNs with ≥95% viability were used. Human PMNs were used immediately following isolation.

2.3. *Cytotoxicity Assays*

Intoxication of PMNs with extracellular *S. aureus* proteins was performed as previously described [56–58,61,63,65,66]. Briefly, *S. aureus* strains subcultured for five hours in TSB were centrifuged (5000× *g* for 5 min), and the collected supernatant was immediately tested for PMN cytotoxicity. To examine the cytotoxicity of *S. aureus* supernatants grown in different media types, *S. aureus* was subcultured in Luria–Bertani broth (LB), Todd–Hewitt broth with 0.2% yeast extract (THY), or brain–heart infusion broth (BHI) where indicated. To intoxicate PMNs, 20 µL of freshly collected *S. aureus* supernatant was combined with 100 µL Roswell Park Memorial Institute (RPMI) 1640 Medium (Corning Cellgro, Corning, NY, USA) containing 5×10^5 freshly purified human PMNs in a serum-coated well of a 96-well plate. Intoxicated PMNs were incubated at 37 °C for 60 min, or other times where indicated, and then examined for plasma membrane permeability to propidium iodide (PI; ThermoFisher Scientific, Waltham, MA, USA) using a FACSCalibur (BD Biosciences) or SE520EON (Stratedigm) flow cytometer. Lactate dehydrogenase (LDH) release was measured using a Cytotoxicity Detection KitPLUS (Roche Diagnostics, Indianapolis, IN, USA) with an Epoch2 microplate spectrometer (BioTek Instruments, Winooski, Vermont, USA) following the manufacturer's protocol.

2.4. Phagocytosis Assays

An examination of human PMN plasma membrane permeability following phagocytosis of live *S. aureus* was performed as previously described [56,57,61,63,65,66]. Briefly, subcultured *S. aureus* was harvested at mid-exponential (ME) growth by centrifugation (5000× *g* for 5 min) and opsonized with 20% normal human serum for 15 min at 37 °C. Freshly cultured and opsonized bacteria were washed with DPBS and then 1×10^7 colony-forming units (CFUs) in 100 µL of Dulbecco's Phosphate-Buffered Saline (DPBS) was combined with 100 µL of RPMI containing 1×10^6 freshly purified human PMNs in a serum-coated well of 96-well plate (10:1 ratio of bacteria to PMN). Phagocytosis was synchronized by centrifugation (500× *g* for 5 min at 4 °C) in an Allegra X-15R centrifuge (Beckman Coulter, Indianapolis, IN, USA) and samples were incubated at 37 °C for 90 min unless otherwise stated. Following incubation, human PMNs were analyzed for PI plasma membrane permeability and LDH release as described above.

3. Results

3.1. Bicomponent Leukocidins Are the Primary Extracellular Cytotoxic Component Produced by USA300 Against Human PMNs

S. aureus produces numerous proteins reported to be cytotoxic against human PMNs. The SaePQRS and AgrABCD two-component systems are both known to regulate the production of most of these cytotoxic factors including the bicomponent leukocidins PVL, HlgAB, HlgCB, LukED, and LukGH as well as Hla [11,13,16,59,61]. AgrABCD also regulates additional exotoxins including PSM*α* [67]. In addition, the ArlRS two-component system has been shown to upregulate PVL, HlgCB, and LukGH [68]. To initially examine the relative importance of these different cytotoxins for causing human PMN destruction, we measured the plasma membrane permeability of primary human PMNs intoxicated with supernatants from wild-type USA300 or deletion mutants of USA300 in the SaePQRS (ΔsaePQRS), AgrABCD (ΔagrABCD), or ArlRS (ΔarlRS) two-component systems (Figure 1A). Congruent with previous findings [56,57], loss of the SaePQRS two-component system completely abrogated plasma membrane permeability caused by USA300 supernatants. As expected, the deletion of AgrABCD also eliminated the cytotoxicity of USA300 supernatants. We also noted a smaller but significant decrease in PMN plasma membrane permeability caused by USA300 extracellular factors when ArlRS was removed, similar to recently published findings by others [68].

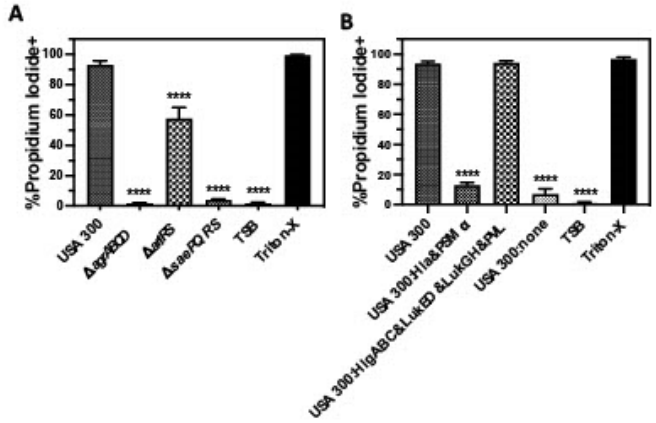

Figure 1. Human PMN plasma membrane permeability caused by extracellular factors produced by USA300 is primarily mediated by bicomponent leukocidins. Flow cytometry was used to assess the percentage of purified human PMNs permeable to propidium iodide after exposure to supernatants

from (**A**) USA300, a deletion mutant of the AgrABCD two-component system in USA300 (USA300Δ*agrABCD*), a deletion mutant of the ArlRS two-component system in USA300 (USA300Δ*arlRS*), or a deletion mutant of the SaePQRS two-component system in USA300 (USA300Δ*saePQRS*) as well as (**B**) USA300, a deletion mutant of the bicomponent leukocidins *hlgABC*, *lukED*, *lukGH*, and *pvl* in USA300 but that still expresses Hla and PSMα (USA300:Hla&PSMα), a deletion mutant of the pore-forming toxins hla and psmα but that still expresses the bicomponent leukocidins (USA300:HlgABC&LukED&LukGH&PVL), or a deletion mutant of both the bicomponent leukocidins and pore-forming toxins in USA300 (USA300:none). For both panels, PMNs were also treated with tryptic soy broth (TSB) alone or TSB with 0.5% Triton X-100 (Triton-X). All data are the mean ± SEM of at least 3 independent experiments with **** $p \leq 0.0001$ relative to USA300 as determined by repeated measures one-way ANOVA with Tukey's multiple comparison test.

To determine which class of cytotoxins is the most responsible for human PMN lysis, we examined the cytotoxicity of extracellular proteins produced by a USA300 mutant lacking all of in the bicomponent leukocidins but that still produces Hla and PSMα (USA300:Hla&PSMα), a USA300 mutant lacking Hla and PSMα but that still expresses HlgAB, HlgCB, PVL, LukGH, and LukED (USA300:HlgABC&LukGH&PVL&LukED), as well as USA300 that does not express Hla, PSMα, HlgAB, HlgCB, PVL, LukGH, or LukED (USA300:none). We found that PMN plasma membrane permeability following intoxication with extracellular proteins was primarily driven by the bicomponent leukocidins while no significant increase ($p = 0.3696$ relative to USA300:none) in plasma membrane permeability was observed with Hla and PSMα expression (Figure 1B).

3.2. PVL Is the Prominent Cytotoxic Extracellular Factor Produced by USA300 That Causes Human PMN Destruction

To determine the contribution of each of the bicomponent leukocidins towards PMN lysis caused by extracellular factors produced by USA300, we examined USA300 deletion mutants that express only one bicomponent leukocidin (Figure 2). Human PMNs intoxicated with extracellular proteins produced by a USA300 deletion mutant of *hlgABC*, *lukGH*, and *lukED* but that still has *pvl* (USA300:PVL) exhibited plasma membrane permeability (Figure 2A) and lactate dehydrogenase (LDH) release (Figure 2B) equivalent to wild-type USA300. In contrast, the expression of any other single bicomponent leukocidin did not have an observable influence on PMN destruction caused by USA300 supernatants. Only after intoxication for 120 min could a modest increase in plasma membrane permeability and LDH release be detected in PMNs exposed to supernatants from USA300 expressing HlgAB, HlgCB, LukGH, and LukED, while the expression of PVL alone caused PMN lysis equivalent to the USA300 wild-type at all the times examined (Figure 2A,B). In addition, only the complementation of USA300 lacking all bicomponent leukocidins with a plasmid encoding *pvl* rescued the cytotoxicity of extracellular proteins against human PMNs while the reintroduction of the other bicomponent leukocidins had no observable influence on cytotoxicity (Figure 2C). PVL remained the dominant extracellular cytotoxic factor causing PMN lysis during growth in tryptic soy broth (TSB), Luria–Bertani broth (LB), Todd–Hewitt broth with yeast extract (THY), and brain–heart infusion broth (BHI), though a significant increase in LukGH-mediated cytotoxicity was observed during growth in LB (Figure 3A, 3B, 3C, and 3D, respectively). Taken together, these findings show that PVL is the dominant extracellular cytotoxic factor causing PMN lysis that is produced by USA300.

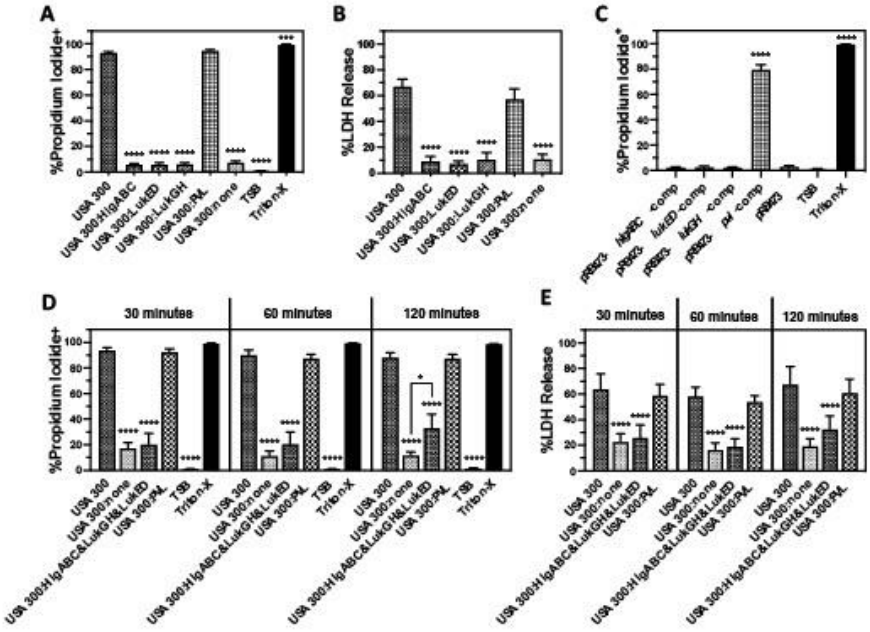

Figure 2. PVL is the major extracellular factor produced by USA300 causing human PMN lysis. Purified human PMNs intoxicated with supernatants from USA300, deletion mutants of multiple bicomponent leukocidins in USA300 but that still express HlgAB and HlgCB (USA300:HlgABC), LukED (USA300:LukED), LukGH (USA300:LukGH), PVL (USA300:PVL), or none of the bicomponent leukocidins (USA300:none) for 60 min were then assessed for (**A**) plasma membrane permeability to propidium iodide and (**B**) lactate dehydrogenase (LDH) release. (**C**) USA300 lacking all of the bicomponent leukocidins was transformed with the pRB473 control or pRB473 encoding *hlgABC*, *lukED*, *lukGH*, or *pvl* and supernatants from these strains examined for the ability to cause human PMN plasma membrane permeability. Purified human PMNs intoxicated with supernatants from USA300, a deletion mutant of all the bicomponent leukocidins in USA300 (USA300:none), a deletion mutant of *pvl* that still expresses the other bicomponent leukocidins (USA300:HlgABC&LukED&LukGH), or a deletion mutant of all the bicomponent leukocidins except for *pvl* (USA300:PVL) for 30, 60, or 120 min were assessed for (**D**) plasma membrane permeability to propidium iodide and (**E**) LDH release. For all panels, PMNs were also treated with tryptic soy broth (TSB) alone or TSB with 0.5% Triton X-100 (Triton-X). All data are the mean ± SEM of at least 3 independent experiments with * $p \leq 0.05$, *** $p \leq 0.001$, and **** $p \leq 0.0001$ relative to USA300 (Panels (**A**), (**B**), (**D**), and (**E**)) or pRB473 (panel (**C**)) as determined by repeated measures one-way ANOVA with Tukey's multiple comparison test.

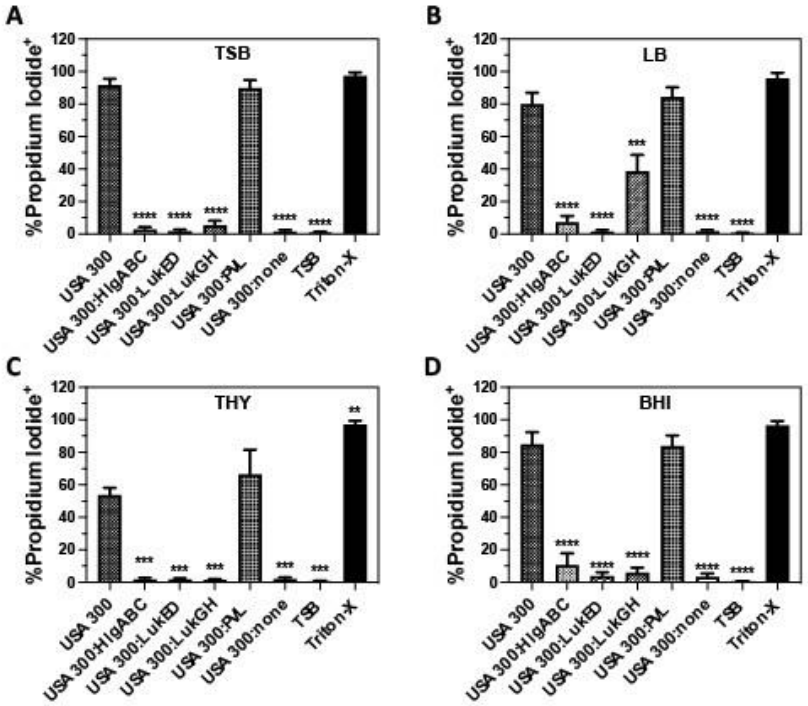

Figure 3. The influence of different culture media on PMN plasma membrane permeability caused by extracellular factor produced by USA300. Flow cytometry was used to assess the percentage of purified human PMNs permeable to propidium iodide after exposure to supernatants from USA300, deletion mutants of multiple bicomponent leukocidins in USA300 but that still express HlgAB and HlgCB (USA300:HlgABC), LukED (USA300:LukED), LukGH (USA300:LukGH), PVL (USA300:PVL), or none of the bicomponent leukocidins (USA300:none) that were subcultured in (**A**) tryptic soy broth (TSB), (**B**) Luria–Bertani broth (LB), (**C**) Todd–Hewitt broth with 0.2% yeast extract (THY), or (**D**) brain–heart infusion broth (BHI). All data are the mean ± SEM of 3 independent experiments with ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$ relative to each strain grown in TSB as determined by repeated measures one-way ANOVA with Tukey's multiple comparison test.

3.3. Lysis of Human PMNs Following Phagocytosis of USA300 Is Primarily Mediated by Bicomponent Leukocidins

To further examine the relative impact of different cytotoxins produced by *S. aureus* on PMN viability, we measured PMN plasma membrane permeability following phagocytosis of live USA300 deletion mutants (Figure 4). Parallel to cytotoxicity assays using extracellular factors produced by USA300, we found that loss of the SaePQRS two-component system caused a major decrease in human PMN plasma membrane permeability following phagocytosis of USA300 as previously reported [56,57] (Figure 4A). Deletion of the AgrABC or ArlRS two-component systems also decreased plasma membrane permeability following phagocytosis as previously shown by others [47,68] but to a lesser degree than loss of SaePQRS. As with the cytotoxicity of extracellular proteins produced by USA300, the bicomponent leukocidins were essential for compromising PMN plasma membrane integrity following phagocytosis with no observed contribution from Hla or PSMα (Figure 4B).

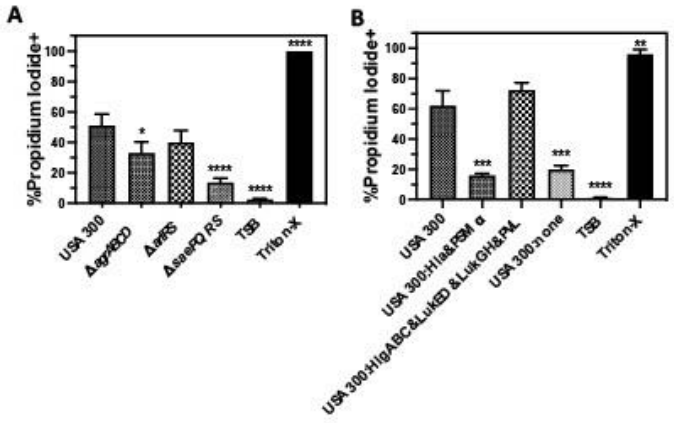

Figure 4. Human PMN plasma membrane permeability following phagocytosis of USA300 is primarily mediated by the bicomponent leukocidins. Flow cytometry was used to assess the percentage of purified human PMNs permeable to propidium iodide after phagocytosis of (**A**) USA300, a deletion mutant of the AgrABCD two-component system in USA300 (USA300Δ*agrABCD*), a deletion mutant of the ArlRS two-component system in USA300 (USA300Δ*arlRS*), or a deletion mutant of the SaePQRS two-component system in USA300 (USA300Δ*saePQRS*) as well as (**B**) USA300, a deletion mutant of the bicomponent leukocidins *hlgABC*, *lukED*, *lukGH*, and *pvl* in USA300 that still expresses Hla and PSMα (USA300:Hla&PSMα), a deletion mutant of the pore-forming toxins *hla* and *psmα* that still expresses the bicomponent leukocidins (USA300:HlgABC&LukED&LukGH&PVL), or a deletion mutant of *hlgABC*, *lukED*, *lukGH*, *pvl*, *hla*, and *psmα* in USA300 (USA300:none). For both panels, PMNs were analyzed 90 min after phagocytosis and included cells treated with tryptic soy broth (TSB) alone or TSB with 0.5% Triton X-100 (Triton-X) as controls. All data are the mean ± SEM of at least 3 independent experiments with * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$ relative to USA300 as determined by repeated measures one-way ANOVA with Tukey's multiple comparison test.

3.4. LukGH Is the Primary Initial Cause of Human PMN Destruction Following Phagocytosis of USA300

To elucidate the relative importance of individual bicomponent leukocidins on PMN lysis following phagocytosis of *S. aureus*, we examined the cytotoxicity of USA300 deletion mutants that express only one of these leukocidins using human PMN phagocytosis assays (Figure 5). In contrast with previous assays showing that PVL is the primary extracellular cytotoxic factor produced by USA300 against human PMNs, we found that LukGH was the most important cytotoxin causing initial human PMN plasma membrane permeability and LDH release following phagocytosis of USA300 (Figure 5A,B). The expression of PVL alone or HlgAB and HlgCB alone also increased PMN lysis, though this change was not significant. However, the combined expression of PVL, HlgAB, HlgCB, and LukED significantly increased PMN destruction 120 min following phagocytosis of USA300 and caused cytotoxicity nearing LukGH expression alone by 180 min (Figure 5D,E). These results were further supported by complementation of USA300 lacking all the bicomponent leukocidins with a plasmid encoding *lukGH* that rescued PMN lysis following phagocytosis, while plasmids reintroducing the other bicomponent leukocidins had no observable impact (Figure 5C). Collectively, these results demonstrate that LukGH is the most important cytotoxic factor causing human PMN lysis immediately following phagocytosis.

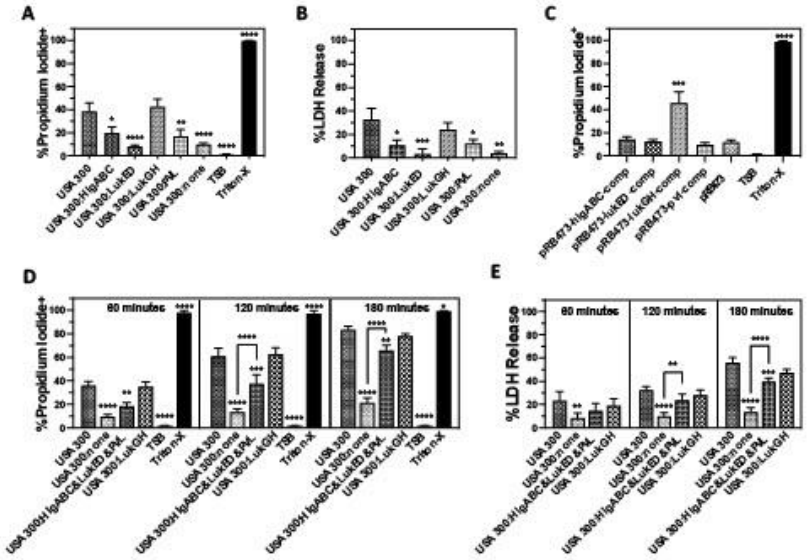

Figure 5. LukGH is the major factor causing human PMN lysis following phagocytosis of USA300. Phagocytosis by purified human PMNs of USA300, different USA300 deletion mutants of bicomponent leukocidins in USA300 that express only HlgAB and HlgCB (USA300:HlgABC), LukED (USA300:LukED), LukGH (USA300:LukGH), or PVL (USA300:PVL), as well as a USA300 deletion mutant of all the bicomponent leukocidins (USA300:none) for 120 min followed by quantification of (**A**) plasma membrane permeability to propidium iodide and (**B**) lactate dehydrogenase (LDH) release. (**C**) USA300 lacking all of the bicomponent leukocidins was transformed with the pRB473 control or pRB473 encoding *hlgABC*, *lukED*, *lukGH*, or *pvl* and examined for the ability to cause human PMN plasma membrane permeability following phagocytosis by human PMNs. Phagocytosis assays using purified human PMNs and USA300, a deletion mutant of all the bicomponent leukocidins in USA300 (USA300:none), a deletion mutant of LukGH that still expresses the other bicomponent leukocidins (USA300:HlgABC&LukED&PVL), or a deletion mutant of all the bicomponent leukocidins except for LukGH (USA300:LukGH) that were assessed at 60, 120, or 180 min for (**D**) plasma membrane permeability to propidium iodide and (**E**) lactate dehydrogenase (LDH) release. For all panels, PMNs were treated with tryptic soy broth (TSB) alone or TSB with 0.5% Triton X-100 (Triton-X) as controls. All data are the mean ± SEM of at least 4 independent experiments with * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$ relative to USA300 (Panels (**A**), (**B**), (**D**), and (**E**)) or pRB473 (panel (**C**)) as determined by repeated measures one-way ANOVA with Tukey's multiple comparison test.

4. Discussion

A large body of research has demonstrated the cytotoxicity of numerous pore-forming toxins produced by *S. aureus* against human PMNs [17–48]. However, a direct and comprehensive analysis comparing their relative contribution to PMN lysis using relevant concentrations naturally produced by *S. aureus* has been lacking. In this study, we used a library of pore-forming toxin deletion mutants in the clinically relevant MRSA strain USA300 to examine the relative cytotoxicity of each using two different PMN intoxication assays; the first measures the cytotoxicity of extracellular proteins produced by *S. aureus*, while the second examines lysis following phagocytosis of live bacteria. We found that PVL is the dominant extracellular cytotoxin causing PMN lysis produced by USA300, while initial PMN lysis following phagocytosis of USA300 is driven primarily by LukGH.

Although PVL was the first bicomponent leukocidin to be purified and characterized [69,70], this cytotoxin is encoded in the genome of only 36% of clinical *S. aureus* isolates in the United States [71]. Notably, these PVL-positive strains include exceptionally virulent

community-associated MRSA such as USA300 that have emerged within the last several decades as a prominent cause of skin and soft tissue infections [72]. While strong species specificity of this toxin has limited the usefulness of some animal models of infection to examine its importance [32,33,37,73], numerous studies have demonstrated that PVL is a potent cytotoxin against human PMNs [31–33,36–38]. The results in this study found that PVL was the dominant cytotoxic factor against human PMNs produced by USA300 under all the growth conditions tested.

We found the other pore-forming toxins had a relatively minor contribution to human PMN lysis caused by extracellular components expressed by USA300 as compared to PVL. We suspect that these virulence factors are also important but play dominant roles during other stages of pathogenesis that require specific experimental approaches to highlight their relevance. This is illustrated by the minimal cytotoxicity induced by LukGH during intoxication assays using extracellular components, in contrast to the prominent impact of this bicomponent leukocidin on PMN destruction following phagocytosis as discussed below. We propose that alternate experimental approaches will also demonstrate the context-dependent importance of the other pore-forming toxins for USA300 virulence. For example, it has been shown that *hlgABC* is highly upregulated by USA300 immediately following exposure to human blood [27], but the translation of HlgC in this strain is strongly reduced relative to HlgB due to a single point mutation in the 5′ untranslated region of the *hlgCB* operon [74]. This indicates that stimuli associated with human blood triggers HlgAB-mediated cytotoxicity to enhance bacterial survival, and assays that include this trigger and examine cell types susceptible to HlgAB may be needed to elucidate a dominant role for this bicomponent leukocidin in USA300 pathogenesis.

As opposed to the majority of pore-forming toxins expressed by *S. aureus*, LukGH was only recently identified by genomic sequencing [11]. This is somewhat surprising given that this bicomponent leukocidin is encoded in the genome of almost all *S. aureus* strains [15]. The sequence of LukGH is only 30% homologous with other bicomponent leukocidins [11], suggesting it has functions that are distinct from other cytotoxins. The first published study characterizing LukGH by Ventura et al. [45] demonstrated it is one of the most abundantly expressed proteins on the surface of USA300 and plays a significant role in causing human PMN lysis following phagocytosis, findings supported by subsequent research [41,42,75]. This led some to speculate that this bicomponent leukocidin plays a primary role during initial contact of *S. aureus* with host cells [12]. It has also been shown that unlike the other *S. aureus* pore-forming toxins, LukGH is pre-assembled in dimers prior to engagement with the host cell membrane [29,75,76]. Our findings demonstrate that LukGH is the primary factor causing human PMN lysis immediately following phagocytosis of USA300, with a significant contribution from the combined influence of all other USA300 cytotoxins only observed at later times. Based on these previously published reports and results from this study, we hypothesize that LukGH is poised on the bacterial surface in pre-assembled active form to immediately compromise the plasma membrane of human PMNs upon initial contact with *S. aureus*.

The importance of PVL for extracellular cytotoxicity against human PMNs and LukGH for causing PMN destruction following phagocytosis suggests they each play significant roles during different aspects of *S. aureus* disease. For example, the concentration of extracellular cytotoxins produced by *S. aureus* would be minimal immediately following inoculation into human tissue, yet survival following initial engagement with phagocytes is critical for subsequent pathogenesis. Under these conditions, expression of LukGH would give *S. aureus* a significant advantage in surviving phagocytosis and disseminating into host tissue to initiate disease. In contrast, the expression of high concentrations of extracellular cytotoxins by *S. aureus* during human infection will occur in an established abscess where PMNs and other immune cells have surrounded and isolated concentrated *S. aureus* [77]. High levels of PVL are likely produced by USA300 under these conditions where it would play an important role destroying incoming human PMNs.

Indeed, expression of PVL is strongly correlated with human skin and soft tissue infections generally characterized by abscessed *S. aureus* [72,78].

This research profiles the relative susceptibility of human PMNs to *S. aureus* pore-forming toxins produced extracellularly and following phagocytosis. Taken together, our results show that PVL is the primary extracellular cytotoxic factor compromising PMN cell membrane integrity produced by USA300. In contrast, LukGH was the major cause of PMN destruction immediately following phagocytosis of USA300. These findings demonstrate that what appears to be redundant pore-forming toxins in the *S. aureus* arsenal actually play very different parts in promoting pathogenesis.

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Assessing the Effects of Surgical Irrigation Solutions on Human Neutrophil Interactions with Nascent *Staphylococcus aureus* Biofilms

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Abstract: *Staphylococcus aureus* (*S. aureus*) is the leading cause of surgical site infections (SSIs) and is capable of biofilm growth on implanted foreign devices. The use of surgical irrigation solutions has become a common strategy to combat bacterial contamination events that occur during surgery. Despite their antimicrobial activity, SSI rates remain consistent, suggesting that low-level contamination persists. In these cases, circulating neutrophils must traffic from the blood to contamination sites to aid in bacterial clearance. The influence of irrigation solutions on neutrophils' ability to engage with bacteria has not been explored. The effects of three commonly used irrigation solutions: Xperience (sodium lauryl sulfate), Irrisept (chlorhexidine gluconate), and Betadine® (povidone-iodine) on nascent *S. aureus* biofilms alone and in the presence of human neutrophils were assessed at manufactured and diluted concentrations. All three solutions, at a 10% dilution, inhibited bacterial growth as demonstrated by culture assays and confocal video microscopy of bacterial aggregate formation. The effects of 10% dilutions of each of these solutions on neutrophil membrane integrity (by flow cytometry and propidium iodide staining) and motility (by confocal video microscopy of neutrophil track length) were investigated with differing outcomes for each irrigation solution. At this concentration only Irrisept preserved neutrophil membrane integrity and motility. Together, this study examines an overlooked aspect of surgical irrigation solutions by investigating their impact on innate immunity and highlights the feasibility of formulations wherein solution effectiveness is complemented by neutrophil function to reduce risks of infection.

Keywords: *S. aureus*; biofilm; neutrophil; irrigation; surgical site infection; implant; aggregates

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1. Introduction

The ability to combat infection, repair damage, and alleviate discomfort through surgical intervention is one of the triumphs of the modern medical era. While the benefits of most surgical interventions outweigh the associated risks, 1–5% of all surgeries are complicated by surgical site infections (SSIs) [1–3]. SSIs are defined as an infection occurring at the surgical site within 30 days of surgery [4,5]. Implantation of foreign materials can serve as scaffolding for bacterial biofilm growth and delay SSI detection for up to one year [5]. These infections often remain recalcitrant to interventions and require explantation for infection resolution. Collectively, patients affected by SSIs display an increased risk of mortality greater than 10-fold, expect an average extended hospitalization length of 9.7 days, and accrue an increased cost greater than USD 20,000 [4]. The substantial burden imposed by SSIs warrants evaluations and the development of strategies aimed at reducing the risk of SSI occurrence.

Bacterial contamination events causing SSIs most often occur at the time of surgery [6,7]. Unlike many hospital-acquired infections (HAIs) where etiologies were linked to exogenous sources such as hospital plumbing systems, poor instrument sterilization, or contaminated surfaces, during SSIs an individual's own skin is often the primary reservoir for bacteria implicated in SSIs [6,8–10]. *Staphylococcus aureus* (*S. aureus*) is commonly described as a skin commensal but often serves as an opportunistic pathogen and was identified as the most frequent cause of SSIs [5,7,11]. *S. aureus* maintains a diverse array of immune evasion factors and has a high propensity for biofilm growth on abiotic surfaces [12,13]. In immune-competent hosts, infections with *S. aureus* are first confronted by neutrophils. Neutrophils are the most abundant leukocytes in circulation and are critical to early detection and clearance of staphylococcal infections [14,15]. Delayed neutrophil recruitment and subsequent *S. aureus* growth significantly impede bacterial clearance [16,17].

Efforts to prevent SSIs focus on the maintenance of a sterile environment during surgery. Given the threat of endogenous infection, eradication of possible contaminating organisms from the skin and incision are the predominant risk reduction strategies [18,19]. Antiseptic irrigation solutions are often used to flush the intraoperative space and aim to cleanse the wound from debris and contaminating microorganisms. This strategy is demonstrated to significantly reduce bacterial numbers; however, unless sterility is achieved, the risk of infection persists. In cases such as these, the prevention of SSIs may depend on the ability of circulating neutrophils to extravasate to the site of contamination and destroy remaining bacteria through potent intra- and extracellular killing mechanisms. Thus, irrigation solutions that inhibit bacterial growth while maintaining an environment permissive to neutrophil function may enhance the clearance of contaminating microorganisms.

In this study, we assessed three irrigation solutions for bactericidal capacity in the presence of human neutrophils. Betadine (povidone-iodine) was first introduced as an antiseptic in 1955 and remains the most widely used surgical site irrigation solution [20,21]. Iodine solutions function as potent oxidizers and cause severe damage to bacterial cell membranes, proteins, and nucleic acids [22]. While this mechanism of action kills a broad spectrum of microorganisms, its effects are not restricted to surgical contaminants and host cell toxicity is often observed [21–23]. Irrisept is an antimicrobial wound lavage containing 0.05% chlorhexidine gluconate [24]. Chlorhexidine gluconate was first developed in the 1940s and was recognized for displaying antibiofilm activity in the 1970s [25]. Cationic chlorhexidine molecules destabilize bacterial cell walls through charge–charge interactions leading to a disruption in osmotic equilibrium and cell death [22,25]. These solutions are demonstrated to reduce bacterial numbers of organisms commonly associated with SSIs and are reported to cause minimal host cell damage [22,24,26,27]. XPerience (XP) is a novel irrigation solution formulated with citric acid, sodium citrate, and sodium lauryl sulfate [24]. These components function in combination by disrupting biofilm matrices and lysing exposed bacterial cells. In contrast to other irrigation solutions, XP is designed as a "no-rinse" solution, suggesting XP confers minimal off-targeted toxicity to host cells; however, no studies have examined the effects of XP on neutrophils.

While the concern of irrigation solution toxicity on host cells and leukocytes was raised, there is a paucity of data examining these effects on primary leukocytes. To date, no studies have directly investigated the effects of these solutions on neutrophils [28]. Using confocal microscopy, we directly visualized interactions between *S. aureus* nascent biofilms and human neutrophils in the presence of irrigation solutions. Collectively, these data demonstrate the plausibility of irrigation solution formulations that retain antimicrobial activity while remaining conducive to neutrophil function. Moreover, our findings suggest irrigation solution effectiveness may be complemented by neutrophil-mediated bacterial killing, leading to fewer contamination events and an overall reduction in SSI occurrences.

2. Materials and Methods

2.1. Bacterial Strains

Staphylococcus aureus (*S. aureus*) strain AH2547 was used in these studies. This strain constitutively expresses green fluorescence protein (GFP) through the addition of pCM29 to methicillin-susceptible strain *S. aureus* HG001 [17]. Nascent *S. aureus* biofilms were grown and attached as described in Ghimire et al. [16]. Briefly, overnight cultures of *S. aureus* were grown in tryptic soy broth (TSB) supplemented with 10 μg/mL of chloramphenicol. Aliquots were centrifuged, washed, and serial diluted in Dulbecco's Phosphate-Buffered Saline (DPBS) to adjust the optical density (OD_{600}) to 0.1. To attach bacteria to 96-well (Greiner Bio-one, Monroe, NC, USA) flat bottom plates or four-chambered glass bottom petri dishes (Cellvis, Mountain View, CA, USA), 10 μL of the diluted bacteria was applied directly to the surface followed by incubation at 37 °C for 30 min. At the end of the 30 min incubation, unattached bacteria were removed by gently rinsing in DPBS. Hank's Balanced Salt Solution with Ca^{2+} and Mg^{2+} (HBSS), XPerience (XP) (Next Science LLC, Jacksonville, FL, USA), Irrisept (Irrimax Corporation, Lawrenceville, GA, USA), or Betadine (Avrio Health L.P., Stamford, CT, USA) was added to each well at the appropriate percentage and supplemented with 10% of freshly isolated autologous normal human serum (NHS).

2.2. Neutrophil Preparations

Heparinized venous blood from healthy donors was collected in accordance with an Institutional Review Board for Human Subjects approved protocol at Montana State University. Human neutrophils were isolated under endotoxin-free conditions as previously described [14,29]. Neutrophil purity (<2% PBMC contamination) and viability (<5% propidium iodide positive) were assessed using BD FACS Calibur flow cytometer.

2.3. Irrigation Solution Titration Experiments

S. aureus AH2547 nascent biofilms were attached to 96-well plate bottoms as described above. Solutions were serially diluted 1:2 from 100% to 0.1% in HBSS containing a final in-well concentration of 10% NHS. Control wells contained HBSS + 10% NHS only. All wells contained a final volume of 100 μL. Dark-walled 96-well plates (Greiner Bio-one, Monroe, NC, USA) were used to capture relative fluorescent intensities. A Cytation 5 Imaging reader (BioTek, Winooski, VT, USA) was used to quantify GFP fluorescence at 37 °C for four hours at 5-min intervals by excitation at 485 nm and emission at 528 nm. Bacterial growth was determined by GFP detection over time. Minimum inhibitory concentrations (MICs) were identified with a custom protocol based on detected changes in the overall GFP fluorescence. MIC was taken as the lowest solution percent resulting in <2.5-fold increase in GFP detection following four-hour incubations. Fold increase was calculated by dividing the GFP fluorescence intensity at four hours by the GFP fluorescence intensity at time zero.

2.4. Microscopy

A Leica DMi8 inverted confocal laser scanning microscope was utilized for all imaging. An Okolab (Ambridge, PA, USA) Uno Stage Top Incubator with a stand-alone humidity controller was utilized to maintain 5% CO_2, 20% O_2, 90% humidity, and 37 °C for sample incubation and during imaging. For all microscopy studies, experiments were performed in four-chambered glass-bottom petri dishes. For experiments analyzing solution effects on aggregate number and size following four-hour growth, images of each well were collected using a Leica 10×/0.4 NA dry objective lens to obtain merged 4 × 5 tile scans with a z-stack height of 30 μm with 3 μm z-intervals. GFP-tagged bacteria were excited with a 488 nm laser.

For microscopy experiments using human neutrophils, the neutrophils were kept on ice after isolation until stained with LysoBrite™ Red (AAT Bioquest, Pleasanton, CA, USA, Cat no. 22645) according to the manufacturer's instructions. In neutrophil challenge experiments, the diluted bacteria were grown in the four-chambered dish with 10% of the

appropriate solution and 10% NHS for four hours at 37 °C, 5% CO_2, and 90% humidity. Post the four-hour incubation, the LysoBrite™ Red stained neutrophil cells were immediately added to each chamber surface followed by the addition of 5 µg/mL propidium iodide (PI). Cells stained with LysoBrite Red were excited with a 590 nm laser and the damaged cells stained with PI were excited with a 510 nm laser along with additional TauGating to differentiate the excitation time for the different neutrophil stainings. Image stacks (20 µm) with 1 µm z-slices were recorded sequentially at 5 minute intervals over a four-hour time course using a Leica 20×/0.7 NA dry objective lens. One field of view was imaged per chamber in each experiment.

To enumerate the surviving bacteria, bacteria were detached from the chamber-well surface by repeated pipetting of the well contents. The solutions were removed from the wells, placed in 1.5 mL tubes, and sonicated for 5 min at 60 Hz in a bath sonicator. Following sonication, tubes were vortexed for 30 s before being serially diluted. Diluted bacteria were plated on tryptic soy agar (TSA) and incubated overnight at 37 °C with 5% CO_2. Resulting colonies from the overnight incubations were manually enumerated.

2.5. Image Analysis

At the end of the four-hour incubation, merged 4 × 5 tile scan images were analyzed as maximum projection z-stacks. MetaMorph v 7.8.13 (Molecular Devices) image analysis software was used as described in Pettygrove et al. to quantify aggregate object numbers [30]. The Integrated Morphometry Analysis tool was used to measure the object diameter and area.

Time-lapse movies were prepared with Imaris version 10.01 (Oxford Instruments, Abingdon, UK). For the analysis of individual neutrophil–bacteria interactions, the Imaris Spots feature was used to identify neutrophils and PI staining events. The tracks of the neutrophils were mapped in each condition and track length was calculated using the average total length of the neutrophil displacements within the track.

2.6. Flow Cytometry

Neutrophil viability experiments were completed in 96-well flat bottom plates that were pretreated with autologous NHS for 30 min and rinsed prior to the addition of neutrophils. Neutrophils were resuspended at 1×10^7 cells/mL in HBSS and 100 µL was aliquoted into wells. Cells were allowed to settle for 5 min. Irrigation solutions were diluted and added to appropriate wells. All wells were supplemented with 10% NHS. Plates were incubated at 37 °C for one hour. Following incubation, the contents of each well were transferred into flow cytometry tubes. Cells were washed and stained with Zombie Violet (Pac Blue) viability dye (BioLegend) at a 1:250 dilution for 20 min at room temperature in the dark. After staining, cells were washed, resuspended in a final volume of 300 µL of FACS buffer, and immediately analyzed. All neutrophil flow cytometry experiments were performed on a BD LSR Fortessa and resulting FCS files were analyzed using FlowJo 10.8.1.

3. Results

3.1. Irrigation Solutions Inhibit the Growth of S. aureus at Low Concentrations

Antiseptic surgical irrigation solutions are formulated to combat microbial contamination and can be applied during preoperative, operative, and postoperative procedures [3,31,32]. Though effective at microbial killing, manufactured concentrations are often toxic to host cells and are frequently diluted prior to use [33–35]. To that end, we first sought to identify the lowest concentration that retained growth inhibitory effects (Figure 1). Inhibitory growth concentrations of XP, Irrisept, and Betadine towards *S. aureus* nascent biofilms were measured using an adaptation of the biofilm growth model described in Ghimire et al. [16]. Briefly, small *S. aureus* aggregates were pre-attached to multiwell plates followed by incubation in each solution and corresponding dilution for four hours. Consistent with reports and directed uses, the use of all solutions at manufactured concentrations resulted in no detectible growth of *S. aureus* (Figure 1). As expected, serial

dilutions of each irrigation solution resulted in a concentration-dependent loss of growth inhibition. The minimum inhibitory concentration (MIC), expressed as a percentage of the full-strength solution, was 6.25% for XP and 0.78% for Irrisept and Betadine (Figure 1A–C) (Supplemental Table S1). Notably, all solutions impacted bacterial growth at <10% of the manufactured concentrations.

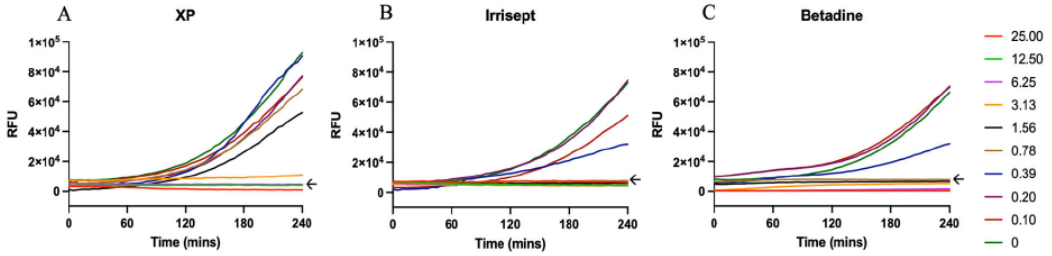

Figure 1. Irrigation solutions below 10% manufacturer concentrations inhibit nascent *S. aureus* biofilm growth. *S. aureus* aggregates were attached to wells in 96-well plates. Aggregates were incubated for four hours in irrigation solutions serially diluted 1:2 in HBSS supplement with 10% NHS. GFP mean fluorescence intensity was detected over time (**A**) XPerience, (**B**) Irrisept, or (**C**) Betadine. Data displayed are representative of three biological replicates.

3.2. Visualization and Quantification of Irrigation Solution Effects on Nascent S. aureus Biofilms

After identifying that the 10% solutions inhibited or suppressed *S. aureus* growth, we sought to directly visualize the effects of these irrigation solutions on nascent biofilms at this concentration. Using confocal microscopy, time-lapse images of *S. aureus* growth in corresponding solutions were collected for four hours as described in Pettygrove et al., 2021 [17]. In the HBSS control solution, the number of bacterial objects or aggregates remained stable over the observation period while the size of aggregates increased due to bacterial growth (Figure 2B,C). No significant changes in aggregate numbers were observed when comparing the HBSS control solution to Irrisept and XP solutions at four hours (Figure 2A). In contrast, incubation in the 10% Betadine solution eliminated nearly all visible GFP signals and abolished aggregate growth. Only two aggregates were detected in one of three biological replicates. The total number of detected objects did not significantly differ among the XP, Irrisept, and HBSS control solutions; however, the average aggregate diameter and area were significantly smaller in XP (3.7 μm, 18.4 μm^2) and Irrisept (2.2 μm, 8.3 μm^2) solutions as compared to the HBSS (8.2 μm, 72.1 μm^2) control (Figures 2B,C and S1). In these experiments, treatment with Irrisept consistently resulted in smaller aggregate formation compared to both the control and XP solution. Taken together, these data suggest that treatment of *S. aureus* nascent biofilms with 10% solutions of XP and Irrisept resulted in bacteriostatic effects, whereas treatment with 10% Betadine was bactericidal. To ensure that the reduced bacterial detection in diluted irrigation solutions was due to growth inhibition and not interference of the solutions with GFP detection, viable colony plate counts were obtained following incubation with irrigation solutions or control for four hours (Figure 2D). Colony forming units (CFUs) collected from these experiments confirmed that the 10% concentrations of all solutions significantly impact bacterial growth as compared to the control (Figures 2D and S2).

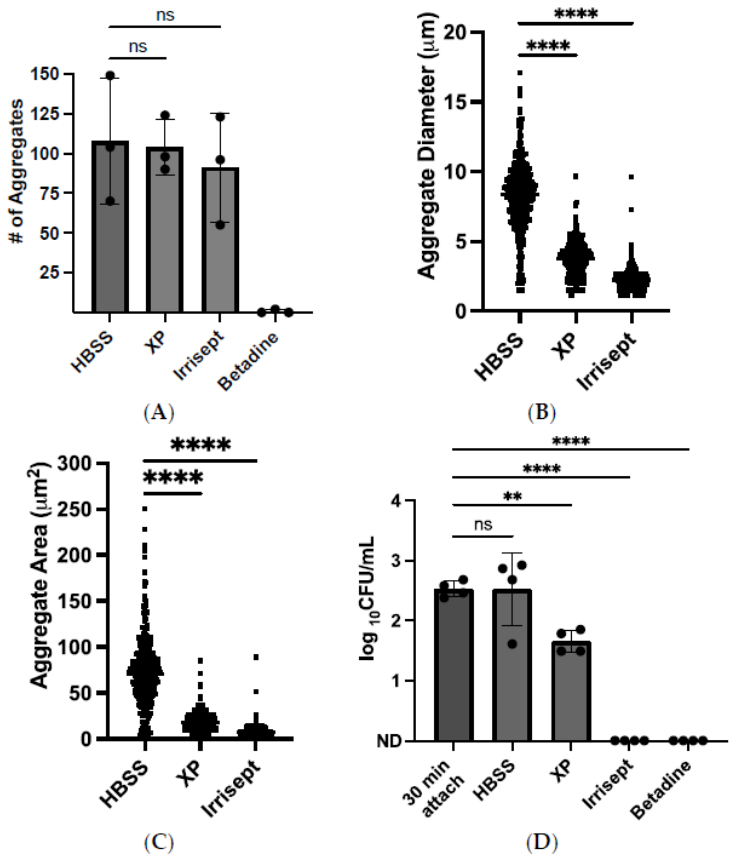

Figure 2. Direct visualization and quantification of 10% irrigation solution on *S. aureus* growth. Confocal images were taken 30 min post-attachment and following four-hour growth in the specified condition. Maximum projection z-stack quantification of (**A**) total aggregates detected (**B**) aggregate diameter and (**C**) aggregate area. Data are from three biological replicates. (**D**) Colony forming units (CFUs) of *S. aureus* after four-hour growth in solutions. Colonies were manually counted following overnight growth on TSA (ND = not detected). Data displayed are from four separate experiments. ns: not significant ** $p < 0.005$, **** $p < 0.0001$ as analyzed by one-way ANOVA followed by Dunnett's multiple comparisons test. Error bars indicate mean ± SD.

3.3. Neutrophil Membrane Integrity at Low Irrigation Solution Concentrations

The timely migration of circulating neutrophils to infection sites is essential for the clearance of *S. aureus* [15,17]. Recent in vitro studies demonstrated that neutrophil killing of *S. aureus* is significantly impaired if bacterial aggregates have a chance to enlarge prior to neutrophil arrival [16,17,36]. The relatively consistent rate of SSI occurrence, despite the application of irrigation solutions, implies that adequate disinfection of the intraoperative space is not always achieved. In cases where small numbers of contaminating organisms persist, the prevention of an SSI is influenced by host immune responses and the ability of neutrophils to find and kill remaining contaminant bacteria. Taken together, we hypothesized that irrigation solutions that are capable of arresting growth or disrupting biofilm formation while maintaining an environment conducive to neutrophil function may be key to wound disinfection and preventing SSIs.

To begin to explore the possible complementary contributions of neutrophils towards bacterial clearance, we first assessed the effects of the irrigation solutions on neutrophil

viability by measuring plasma membrane damage. Neutrophils were incubated for one hour in varied doses of each irrigation solution. Following incubation, flow cytometry was used to quantify the intracellular accumulation of Zombie Violet (Pac Blue), a membrane-impermeable amine-reactive dye (Figures 3 and S3). Treatment of human neutrophils with all solutions at 0.1% and 1% concentrations resulted in average viability across replicates >98% (Figures 3E and S3). At the 10% concentration, XP and Irrisept solutions had minimal effects on neutrophil membrane permeability and retained an average viability >90% (Figure 3A–C,E). Despite no effects on membrane integrity, a slight cellular condensation was observed for the 10% XP-treated neutrophil population as indicated by a small shift forward scatter (FSC-A) [37]. In accordance with previous studies, treatment with 10% Betadine severely disrupted neutrophil membrane integrity (increase in Pac Blue fluorescence) resulting in plasma membrane damage causing drastic shifts in both cell size and granularity and reducing viability to an average of 8.5% (Figure 3D,E) [21,38].

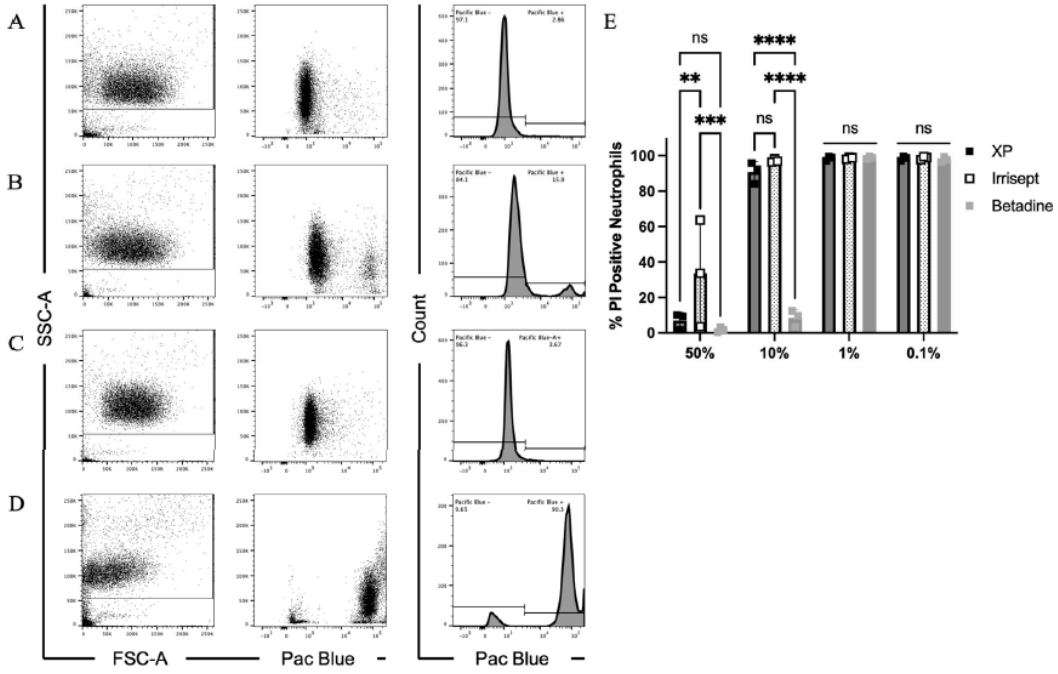

Figure 3. Effects of irrigation solutions at low doses on neutrophil plasma membrane damage. Flow cytometric analysis of neutrophil membrane integrity. Neutrophils were incubated in 10% irrigation solutions. Representative plots showing forward (FSC-A) and side scatter (SSC-A) or membrane permeability as measured by increase in Pac Blue staining (dot and histogram plots) of (**A**) HBSS only, (**B**) XP, (**C**) Irrisept, or (**D**) Betadine. (**E**) Quantification of neutrophil plasma membrane damage following incubation with irrigation solutions. Data displayed are from three biological replicates. ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$ as determined by two-way ANOVA followed by Tukey's multiple comparisons test. Error bars indicate mean ± SD.

Given the minimal toxicity observed for XP and Irresept at 10% concentrations, we were unsure if concentrations more closely resembling the commercially manufactured concentrations would negatively impact neutrophil viability. To directly address the toxicity of commercially manufactured concentrations, irrigation solutions and neutrophils were combined at equal volumes yielding an in-well solution concentration of 50%. These samples were incubated for one hour followed by analysis using flow cytometry. Incubation of neutrophils in XP, Irrisept, and Betadine in 50% of the commercially manufactured

concentrations caused significant plasma membrane damage. Intact plasma membranes were observed in only 7%, 33.6%, and 1.7% of neutrophils treated with the respective solutions (Figures 3E and S3). Collectively, these data demonstrate that 10% Betadine solution is damaging to human neutrophils; however, 10% XP and Irrisept solutions do not significantly compromise neutrophil membrane integrity. Whereas these data strongly imply incubation in 10% XP or Irrisept solutions is not cytotoxic to neutrophils, they do not address impacts on function such as neutrophil motility or bacterial killing.

3.4. Neutrophil Motility and Engagement of Bacteria in Irrigation Solutions

Using time-lapse confocal microscopy, we assessed neutrophil motility and discovery of bacteria in 10% irrigation solutions (Figure 4). To best replicate the application and sequence of irrigation solution used, nascent biofilms were first incubated in the presence of each 10% irrigation or control solution for four hours (Figures 2 and S1). At four hours post aggregate growth, neutrophils were added to each chamber. Propidium iodide (PI) was aliquoted into the chambers and images were collected at five-minute intervals for an additional four hours (Videos S1–S4). After four hours neutrophil motility was quantified in all solutions (Figure 4A,B). For these experiments, motility was expressed as the total track length, which is defined as the average distance traveled by all neutrophils within one condition.

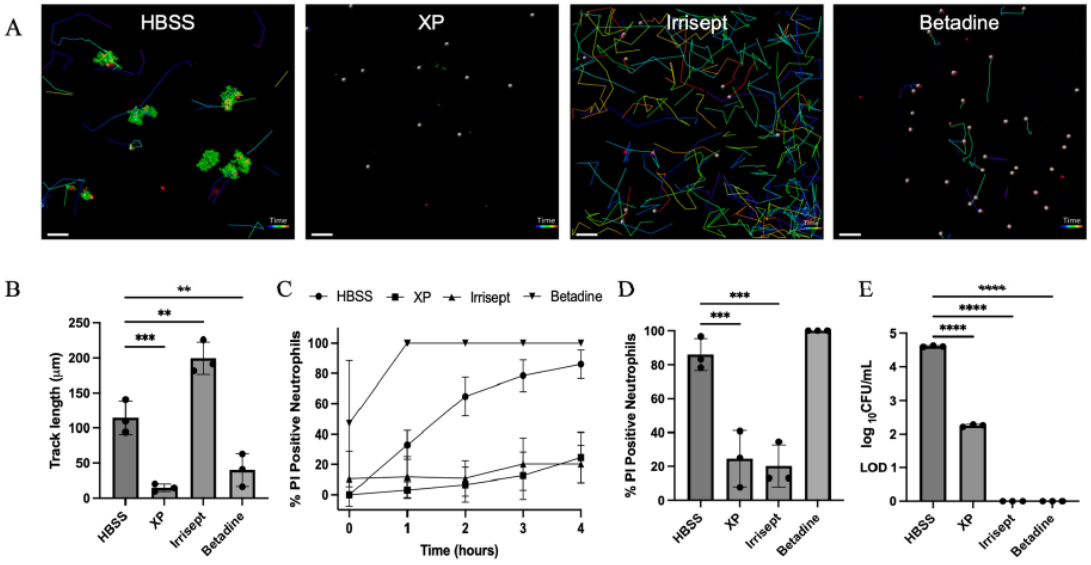

Figure 4. Neutrophil motility, bacterial engagement, and clearance in 10% irrigation solutions. (**A**) Representative images of the neutrophil track length following four-hour incubation of *S. aureus* in HBSS, XP, Irrisept, or Betadine, respectively. Neutrophils stained with LysoBrite Red (blue) and propidium iodide (PI, red) following four-hour interactions with *S. aureus* aggregates (green) grown with 10% concentration of the displayed irrigation solution with 20 µm z-stacks. (**B**) Quantification of neutrophil track lengths post four-hour incubation in respective solutions. (**C**) Accumulation of PI-positive neutrophils during interactions with nascent biofilms in irrigation solutions. (**D**) PI-positive neutrophils after four-hour interactions with nascent biofilms in irrigation solutions. (**E**) Quantification of remaining *S. aureus* CFUs following four-hour growth in solutions in the presence of neutrophils (ND = not detected). LOD = 10 CFU/mL. Scale bar = 50 µm. Images displayed are representative of one field of view and of three biological replicates. ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$ as analyzed by one-way ANOVA with Dunnett's multiple comparisons test. Error bars indicate mean ± SD.

While mobilization of neutrophils to sites of infection is a requisite of function, the presence of neutrophils is not a correlate of bacterial clearance or wound healing. For these reasons, we directly visualized neutrophil interaction with *S. aureus* aggregates. Consistent with previous studies, Neutrophils are highly motile in HBSS but were unable to effectively kill bacterial aggregates given the size of aggregates due to the head-start growths [17] (Figure 4B). Large bacterial aggregates were resistant to neutrophil offenses and caused significant membrane damage as evidenced by the accumulation of PI-positive neutrophils at the aggregate interface (Figure 4A,C,D, Video S1) [17]. Despite no effects on membrane permeability, treatment with a 10% XP solution abolished neutrophil motility and resulted in no interaction with remaining *S. aureus* aggregates (Figure 4A–D, Video S2). Treatment with 10% Irrisept had no negative effects on neutrophil motility (Figure 4B, Video S3). Indeed, neutrophils readily patrolled and engaged remaining bacterial aggregates with minimal accumulation of PI in neutrophils contacting aggregates (Figure 4A,C,D). Treatment with a 10% Betadine solution caused significant damage to neutrophil membranes (Figure 3D,E). Not surprisingly, these cells demonstrated no motility and were immediately positive for PI (Figure 4A–D, Video S4). Bacteria CFUs were enumerated from each chamber following four-hour incubations with neutrophils (Figure 4E) and confirmed previous observations that a significant reduction in bacteria was observed in all irrigation solutions compared to HBSS control. Taken together, these data demonstrate that a 10% Irrisept solution can promote bacterial clearance by disrupting bacterial growth while maintaining aspects of neutrophil function. Moreover, these findings highlight an overlooked strategy to combat SSIs wherein enhanced bacterial clearance is achieved through formulations of irrigation solutions that promote synergism with host immune defenses.

4. Discussion

In this study, we investigated whether the antibacterial effects of three commonly used irrigation solutions could be complemented by neutrophil-mediated bacterial killing. Given the high prevalence and severity of SSIs resulting from *S. aureus* contamination on implanted surfaces, our study focused on irrigation solution effectiveness during the early interactions between *S. aureus* and neutrophils. Using an established model of neutrophil interactions with nascent *S. aureus* biofilms on abiotic surfaces, we directly compared the effects of commonly used irrigation solutions on bacterial growth and neutrophil clearance [16,30].

Irrigation solutions target contaminating microbial organisms and are demonstrated safe for topical use. Despite this, off-target host cell toxicity is reported at commercially manufactured concentrations [21–23,26]. Given these reports and our emphasis on neutrophil health in irrigation solutions, we sought to identify a maximum concentration common among the irrigation solutions that inhibited bacterial growth while preserving neutrophil viability. Furthermore, despite the effects of solutions on planktonic cultures being well-defined, the potency of these solutions against nascent *S. aureus* biofilms remained understudied. To center on a productive solution concentration for these studies we first diluted each solution and measured *S. aureus* growth by detection of GFP. All solutions were highly effective at inhibiting *S. aureus* growth well below the commercially manufactured concentration. Following this initial concentration screening, 10% solutions were chosen for further analysis due to similar potencies at this concentration across all the solutions. It should be noted that while irrigation solutions are at times diluted prior to use, the low concentration of 10% used in these studies may not be indicative of in vivo efficacies.

The effects of each 10% solution on nascent biofilms were visualized using confocal microscopy. In these experiments, treatment with Betadine largely reduced bacterial aggregates below the threshold of detection. Among three independent biological replicates, only two distinct *S. aureus* aggregates were detected. Given the potency of Betadine and the inability to recover bacterial CFUs from any of these replicates, we are confident that the reduction in the number of aggregates is due to the bactericidal activity of Betadine under

these conditions. Both XP and Irrisept are utilized for their antibiofilm properties [24,39]. Following four-hour incubations, no significant changes in the number of aggregates were detected between these solutions and the control; however, aggregate size was significantly reduced. Reductions in CFUs recovered from wells containing XP were observed. CFUs were unable to be recovered from wells treated with Irrisept. Given that aggregates were detected in these samples, it is more likely that treatment with Irrisept reduced bacterial numbers below the limit of detection rather than resulted in sterilization of the well.

The reduced bacterial aggregate size caused by XP and Irrisept solutions highlights a fundamental aspect of complemented bacterial clearance by neutrophils. Neutrophils are highly adapted to engage and kill *S. aureus* at low numbers and small aggregate sizes [16,36]. The effectiveness of neutrophil-mediated *S. aureus* clearance is largely determined by the multiplicity of infection (MOI) [12,40]. Increases in *S. aureus* cell numbers or aggregate size can overwhelm neutrophil offensives leading to continued bacterial growth. *S. aureus* aggregates $\geq$50 μm^2 not only resist neutrophil killing but demonstrate an enhanced ability to lyse nearby neutrophils [16,36]. Taken together, the ability of these solutions to restrict bacterial growth likely enhances neutrophil efficiency in these environments.

In order for neutrophils to contribute to bacterial clearance in the presence of irrigation solutions, host cell toxicity must be minimized. We used flow cytometry to assess neutrophil membrane permeability in each solution. At the 10% concentration, both XP and Irrisept preserved neutrophil membrane integrity, whereas treatment with Betadine caused significant membrane damage. Given previous studies examining the effects of Betadine on multiple cell types, off-target toxicity from povidone-iodine-induced oxidative stress from these solutions was not surprising [21,22,41,42]. The preservation of membrane integrity observed in the XP and Irrisept solutions suggested that the 10% concentration may not affect neutrophil motility or function.

Despite no significant increases in membrane permeability, we noted a slight reduction in the FSC-A of the neutrophil population treated with 10% XP indicating a reduction in cell size. Commercial manufactured solutions of XP maintain a pH of ~ 4.0 and it was unaltered by reducing the concentration to 10%. Consistent with our observations, studies examining the impact of low pH environments on neutrophils have reported a condensation in cell size and a reduction in motility [43,44]. Time-lapse imaging analysis of neutrophils in the presence of the XP solution demonstrated that while some neutrophils remain viable, nearly all neutrophils were immobile (Figure 4A, Video S2). To confirm the reductions in neutrophil motility observed in the 10% XP solutions were due to the acidic pH, solutions were titrated with NaOH to pH 7.0 representing a more optimal pH for neutrophils. Neutralization of the XP solutions restored neutrophil motility but abolished bacteriostatic effects (Video S5).

In direct contrast to the reduced neutrophil motility observed following XP treatment, treatment with Irrisept increased neutrophil motility metrics above the HBSS control. Consistent with previous reports, head-start bacterial growth in the HBSS for four hours leads to *S. aureus* aggregates >50 μm^2 [16,17,36]. At this size, leukocidins likely accumulate at the aggregate surface at concentrations that interfere with neutrophil efficacy and lead to toxin-mediated neutrophil cell death. Treatment with Irrisept restricted *S. aureus* growth. At four hours, all observed aggregates remained at <50 μm^2 and were unable to cause neutrophil damage. As a result of these effects, neutrophils readily surveyed the abiotic surface and engaged the remaining aggregates. The reduced bacterial growth and increased neutrophil motility conferred by treatment with 10% Irrisept demonstrate the feasibility and possible benefits of host-permissive irrigation solutions.

In the current study, the effects of Irrisept on neutrophil-mediated bacterial killing were not directly explored. It is possible that neutrophil killing mechanisms such as the generation of the phagolysosome and production of reactive oxygen species were negatively impacted. However, the ability of neutrophils to successfully chemotax and interact with remaining aggregates without significant increases in PI uptake suggests the neutrophil function is unchanged. Future studies will directly test the functionality and efficiency of

neutrophil bacterial killing mechanisms in chlorhexidine gluconate solutions. In summary, these findings highlight an overlooked strategy to combat SSIs wherein enhanced bacterial clearance is achieved through formulations of irrigation solutions that promote synergism with host immune defenses.

5. Conclusions

The premise behind this study was that an ideal surgical irrigation solution would combine antimicrobial properties with innate immune system compatibility. A solution that is bacteriostatic or bactericidal while also preserving the normal function of frontline phagocytic cells has logical appeal for an infection control application. We explored the potential of this concept by measuring the effects of three commercial irrigation solutions on bacterial growth as well as on human neutrophil viability and motility. Even in this preliminary investigation with just three products, we discovered a range of qualitative outcomes. Betadine exhibited bacteriostatic and bactericidal activity but strongly permeabilized neutrophil cell membranes and strongly suppressed neutrophil motility. The potent antimicrobial activity of this solution may be offset in vivo by its interference with neutrophil function. XP exhibited modest inhibition of bacterial growth without reducing neutrophil viability but eliminated neutrophil motility. This solution, therefore, may also suppress innate immune cell performance. Irrisept combined effective inhibition of bacterial growth while maintaining neutrophil viability and motility.

These diverse outcomes suggest that it may be valuable and important to assess compatibility with innate immune system function when developing antimicrobial or antibiofilm products. Two technologies with similar antimicrobial activity, one of which compromises innate immune cell function and another that is innate immune compatible, could plausibly have differing efficacy in the clinic. Of course, this conjecture will ultimately need to be validated with animal models and human clinical data.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/microorganisms12101951/s1, Figure S1: Nascent *S. aureus* biofilm growth in 10% irrigation solutions; Figure S2: CFUs recovered following attachment and growth in irrigation solutions; Figure S3: Irrigation solution effects on neutrophil membrane permeability; Table S1: Mean fold-change in GFP intensity at four hours; Video S1: Representative time-lapse videos showing neutrophil interactions with *S. aureus* aggregates in HBSS. Video S2: Representative time-lapse videos showing neutrophil interactions with *S. aureus* aggregates in 10% XP. Video S3: Representative time-lapse videos showing neutrophil interactions with *S. aureus* aggregates in 10% Irrisept. Video S4: Representative time-lapse videos showing neutrophil interactions with *S. aureus* aggregates in 10% Betadine. Video S5: Representative time-lapse showing neutrophil interactions with *S. aureus* aggregates in 10% XP adjusted to a pH of 7.0 using NaOH.

Author Contributions: Conceptualization and study design, G.J., P.S.S. and T.R.B.; formal analysis of experiments, G.G. and T.R.B.; experimental investigation, G.G. and T.R.B.; neutrophil resources, M.P. and J.M.V.; data curation, G.G. and T.R.B.; writing—original draft preparation, T.R.B.; writing—review and editing, G.G., M.P., J.M.V., G.J. and P.S.S.; supervision, J.M.V. and P.S.S.; project administration T.R.B.; funding acquisition, J.M.V., G.J. and P.S.S. All authors have read and agreed to the published version of the manuscript.

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