

**Real-time Monitoring of *Staphylococcus Aureus* Biofilm Formation Under
Flow Conditions in Microfluidic Chambers**

by

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Abstract

Staphylococcus aureus (*S. aureus*) causes most of life-threatening infections such as endocarditis, osteomyelitis and sepsis. Since biofilm development is often a key factor in the development of an infection of indwelling medical devices, we sought to study biofilm formation in real-time using a lab-made microfluidic device with the goal of assessing the efficacy of therapeutic agents. In this study, we monitored the bacterial growth of *S. aureus* in side-by-side microfluidic channels. The total volume of an individual microfluidic channel was 0.148 mm³. Each independent micro-channel has three separated ports (two inlets and an outlet for waste) to allow for constant flow of both growth medium and the bacterial cells. Biofilm coverage of *S. aureus* was characterized under various flow conditions ranging from 0.1 to 1 $\mu\text{L}/\text{min}$. Optimal biofilm formation was evident at 0.5 $\mu\text{L}/\text{min}$, therefore that flow rate was used in all subsequent experiments. To more closely mimic the human plasma environment, the effect of fibrinogen (Fbg) supplementation was tested. We found that fibrinogen enhanced the process of biofilm formation. To determine if we were monitored true biofilm formation rather than simple bacterial grow, we infused calcofluor white (CFW) into the channels and imaged by a real-time fluorescence microscope. We verified true biofilm formation by visualizing the production of exopolysaccharides matrix by *S. aureus* indicated by positive CFW staining. By using this microfluidic-based model, we assessed the possibility of inhibiting the biofilm development by testing the anti-staphylococcal activity of a potential probiotic bacilli strain, called *B. velezensis* AP183, and the bacteriolytic activity of lysostaphin, an extracellular enzyme secreted by *staphylococcus simulans* strain. These results may help to better understand the biofilm formation process and discover new drugs targets for the treatment of staphylococcal infections.

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Dissertation Dedication

This dissertation is dedicated to my grandfather (Abdulaziz A. Al Mouslem). Although he was my inspiration to pursue my PhD degree, he was unable to see my graduation. This is for him.

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List of Abbreviations

SSTI	Skin and soft tissue infections
ECDC	European Centers for Disease Control and Prevention
FAO/WHO	Food and Agriculture Organization and World Health Organization
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
VRE	Vancomycin-resistant enterococci
MRSA	Methicillin-resistant <i>S. aureus</i>
MSSA	Methicillin-susceptible <i>S. aureus</i>
USA	United States of America
CDC	Centers for Disease Control and Prevention
IE	Infective endocarditis
EPS	Extracellular polymeric substances
eDNA	Extracellular DNA
NIH	National health institution
CWA	Cell-wall-anchored
IsdA	Iron-regulated surface protein A
SasG	<i>S. aureus</i> surface protein G
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
FnBPA	Fibronectin-binding protein A
FnBPB	Fibronectin-binding protein B
ClfA	Clumping factor A
ClfB	Clumping factor B
Cna	Collagen-binding adhesin

ECM	Extracellular matrix
Fbg	Fibrinogen
Fn	Fibronectin
Bap	Biofilm-associated protein
PIA	Polysaccharide intercellular adhesin
PNAG	Poly- β (1,6)-N-acetyl-D-glucosamine glycans
Ica	Intercellular adhesion
PSM	Phenol soluble modulins
ScpA	Staphylococcal cysteine proteinase staphopain A
SspB	Staphylococcal cysteine proteinase staphopain B
SspA	The serine protease A of <i>Staphylococcus aureus</i> strain V8
SpA	Surface protein A
Aur	Metalloproteinase aureolysin
Nuc	Nuclease
Nuc2	Nuclease2
Eap	Extracellular adherence protein
Atl	Murein hydrolases
agr	Accessory gene regulator
Esp	<i>Staphylococcus epidermidis</i> serine protease
AIP	Autoinducing peptide
SpsB	Type I signal peptidase
MIC	Minimum inhibitory concentration
VRSA	Vancomycin-resistant <i>S. aureus</i>

VISA	Vancomycin-intermediate <i>S. aureus</i>
SrtA	<i>S. aureus</i> transpeptidase sortase A
DNase I	Deoxyribonuclease I
RIP	RNAIII inhibiting peptide
MBIC	Minimum biofilm inhibitory concentration
RAP	RNAIII-activating peptide
TRAP	Target of RNAIII-activating peptide
LAB	Lactic Acid Bacteria
<i>E. coli</i>	<i>Escherichia coli</i>
<i>S. oralis</i>	<i>Streptococcus oralis</i>
<i>S. salivarius</i>	<i>Streptococcus salivarius</i>
<i>ECN1917</i>	<i>E. Coli Nissle 1917</i>
<i>L. fermentum</i>	<i>Lactobacillus fermentum</i>
<i>L. plantarum</i>	<i>Lactobacillus plantarum</i>
<i>L. reuteri</i>	<i>Lactobacillus reuteri</i>
<i>L. rhamnosus</i>	<i>Lactobacillus rhamnosus</i>
GI	gastrointestinal tract
ATP	Adenosine triphosphate
ROS	Reactive oxygen species
<i>B. velezensis</i> AP183	<i>Bacillus velezensis</i> AP183
WGA	Wheat Germ Agglutinin
BHI	Brain heart infusion
SCVs	small colony variants

Chapter 1 Literature review

1.1 Introduction

Since the discovery of penicillin in the 1940s, the fight against Gram-positive bacterial infections has been ongoing. Several diseases, ranging from mild to life-threatening, are caused by these pathogens, including skin and soft tissue infections (SSTI), osteomyelitis, infective endocarditis, and indwelling medical device infections (European Centre for Disease Prevention and Control [ECDC], 2015; Doernberg et al., 2017; Woodford & Livermore, 2009). Among Gram-positive bacteria, enterococci and *Staphylococcus aureus* (*S. aureus*) are among the most concerning pathogens with regard to global antimicrobial resistance. Examples of antibiotic-resistance in these Gram-positive bacterial species include vancomycin-resistant enterococci (VRE) and methicillin-resistant *S. aureus* (MRSA) (Costerton et al., 1999; Hall-Stoodley et al., 2004). Together they impose a significant economic impact on public health and healthcare systems globally, with the United States of America (USA) and Europe having the highest prevalence of such infections (Doernberg et al., 2017).

In addition to bacterial resistance at the individual cell level, some of these bacteria develop a broader strategy to survive in harsh environments by forming a community of sessile cells called a biofilm (Romling & Balsalobre, 2012). The recalcitrant nature of biofilms and their growing resistance to treatments represents a major concern in our healthcare settings (Donlan & Costerton, 2002; Schierholz & Beuth, 2001). The ability to form biofilms is a virulence determinant in many bacterial species (Crump & Collignon, 2000). It has been shown that this cellular aggregation plays a crucial role in the resistance of bacterial cells as indicated by the fact these microbes can become 10-1,000 times more resistant to conventional antibiotics, host protective immune

responses, hostile environmental stresses or pressures, and shear forces than their planktonic counterparts (Donlan, 2002; Gupta et al., 2016; Kolter & Greenberg, 2006; Marić & Vranes, 2007; Otto, 2008). The burden of biofilm-related infections on healthcare/public health is more significant. In the USA, 80% of nosocomial infections are bacterial biofilm-related infections, and these infections cause approximately 500,000 deaths annually (Romling & Balsalobre, 2012; Wolcott et al., 2010). Treating these grave infections costs an estimated to be 11 billion dollars per year (Romling et al., 2014). Because of the high mortality rates and tremendous economic burden of treatment, biofilm-associated infections are considered a significant health problem. Biofilm-associated infections are categorized into two main types: (i) biotic surface-related biofilm infections and (ii) abiotic surface-related biofilm infections (Santos et al., 2018). **Table 1.1** lists some examples of these clinically relevant biofilm-related infections (Lebeaux et al., 2013). The biofilm's capability to endure and flourish under extreme environmental stress is tied to a number of factors including (i) the highly diverse mature biofilm structure containing cells with varying metabolic and growth rates, (ii) the low permeability of the extracellular matrix to antimicrobial agents, (iii) the down-regulation of antimicrobial drug targets in bacterial cells, (iv) the presence of the efflux pumps and transporter proteins to eject the intracellular antibacterial agents, (v) the up-regulation of resistance genes, and (vi) the presence of persisters, a sub-population of cells that are dormant, metabolically inactive, and highly resistant to antimicrobial exposure (Donlan, 2002; Gupta et al., 2016; Kolter & Greenberg, 2006; Marić & Vranes, 2007).

The challenges of treatment and difficulty of eradication presented by biofilm-associated infections have led to a burst in biofilm research and the role of biofilm in enhancing bacterial pathogenicity that promotes infectious diseases (Santos et al., 2018). As shown in **Figure 1.1**, the number of publications about the process of biofilm formation, agents, and strategies to combat

Table 1.1 Common sites of biofilm-related infections.

Living-tissue infections (biotic surface)	Non-living-tissue infections (abiotic surface)
Endocarditis	Central vascular catheters
Cystic fibrosis	Prosthetic cardiac valves
Otitis media	Central
Dental plaque	Orthopedic implants and Prosthetic joints
Urinary tract infections	Urinary Catheters
Chronic wounds	Peripheral vascular catheters
Osteomyelitis	Contact lenses
Gingivitis	Tissue fillers

The most common studied infections caused by a biofilm formation on living or non-living tissues.

Adapted from (Lebeaux et al., 2013) with permission.

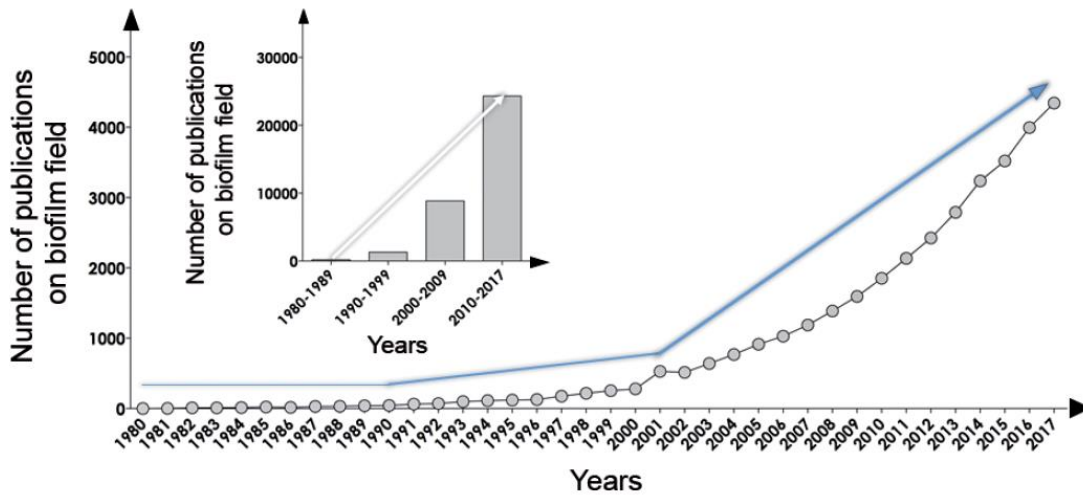


Figure 1.1 The trend of microbial biofilm publications. It shows the numbers of biofilm publications during the period 1980-2017. Adapted from (Santos et al., 2018) with permission.

this process has followed a nearly exponential increase in the last two decades, contributing to improvements in our current understanding of this virulence determinant.

Among all gram-positive species, *Staphylococci* is the most common pathogen associated with implant infections (Zimmerli et al., 2004). MRSA caused infections in an estimated 324,000 hospitalized patients and, in 2017, caused about 10,000 deaths in the US at healthcare costs of 1.7 billions of dollars (Centers for Disease Control and Prevention [CDC], 2019). Due to its ability to quickly become drug resistant, *S. aureus* remains a serious clinic problem. In my dissertation, I plan to present evidence that supports the development of a state-of-the-art microfluidic *in vitro* model for the following purposes: (i) to monitor *S. aureus* biofilm formation ability under constant flow conditions, (ii) to test factors that may affect biofilm formation by *S. aureus* under flow, (iii) to study of the effects of shear pressures on the initiation of *S. aureus* biofilm formation, (iv) to study of the production of polysaccharides by *S. aureus*-grown in biofilms, and (v) to examine novel therapies to reduce these biofilms. To date, there are some *in vitro* models used to investigate the formation of biofilms by a variety of bacterial species and identify the biofilm components in either static conditions or a dynamic environment. Presented in this chapter, I plan to review the current knowledge concerning the following: (i) bacterial biofilms formation by *S. aureus* , (ii) available therapeutic strategies to combat the biofilm formation process, and (iii) background about the current state of the art concerning lytic agents and probiotics to treat *S. aureus* infections. Overall, the goal of these studies is to develop new therapeutic strategies to fight against the biofilm structure of *S. aureus* by improving understanding of this process and its resistance to antimicrobial drugs and the immune system.

1.2 General characteristics of bacterial biofilm and the molecular mechanism of biofilm formation in Gram-positive bacteria

Until the 1970s, it was unknown that bacteria predominately exist in surfaces-attached biofilms, not free-floating (planktonic) bacteria (Reffuveille et al., 2017). Following its discovery, a general definition of “biofilm” existed for many years, but only recently has biofilm structure begun to be more thoroughly understood (Costerton et al., 1999). A bacterial biofilm is defined as well-organized aggregates of microbial cells encased in a self-secreted extracellular polymeric matrix and attached to an abiotic or biotic surface (Boudarel et al., 2018; Hoiby et al., 2011). Within the bacterial extracellular matrix, there is a complex network of pores and water channels, consisting of extracellular polymeric substances (EPS), e.g., proteins, extracellular DNA (eDNA), lipids, polysaccharides, and other molecules, as shown in **Figure 1.2** (Foulston et al., 2014). Biofilm matrix in Gram-positive bacteria and specifically *S. aureus* is heterogeneous, complex, and affected by environmental stimuli and growth conditions (Cheng et al., 2007). Bacteria tend to prefer a biofilm-based approach to growth because of the three survival advantages that EPS provides: (i) entrapment of external nutrients that are consumed by cells for sustenance by charged polysaccharides within the matrix, (ii) distribution of essential nutrients for the growth of bacterial cells (Chen et al., 1998; Goldberg, 2002; Pang et al., 2005), and (iii) protection of bacterial cells from external environmental stresses, such as damage from antimicrobials or immune cells, and dynamic environmental factors (Romling & Balsalobre, 2012). As a result of this evolutionary advantage, approximately 80% of nosocomial infections involve biofilm formation, including indwelling medical devices-related infections, in particular implanted catheters, artificial heart valves, and prostheses, according to National health institution (NIH) (Donlan, 2002; Renner & Weibel, 2011; Romling & Balsalobre, 2012). Biofilm-related infections can be caused by a singular or multiple (polymicrobial) bacterial species at the infection site. Polymicrobial biofilm infections are common and highly persistent due to the symbiotic interactions between the

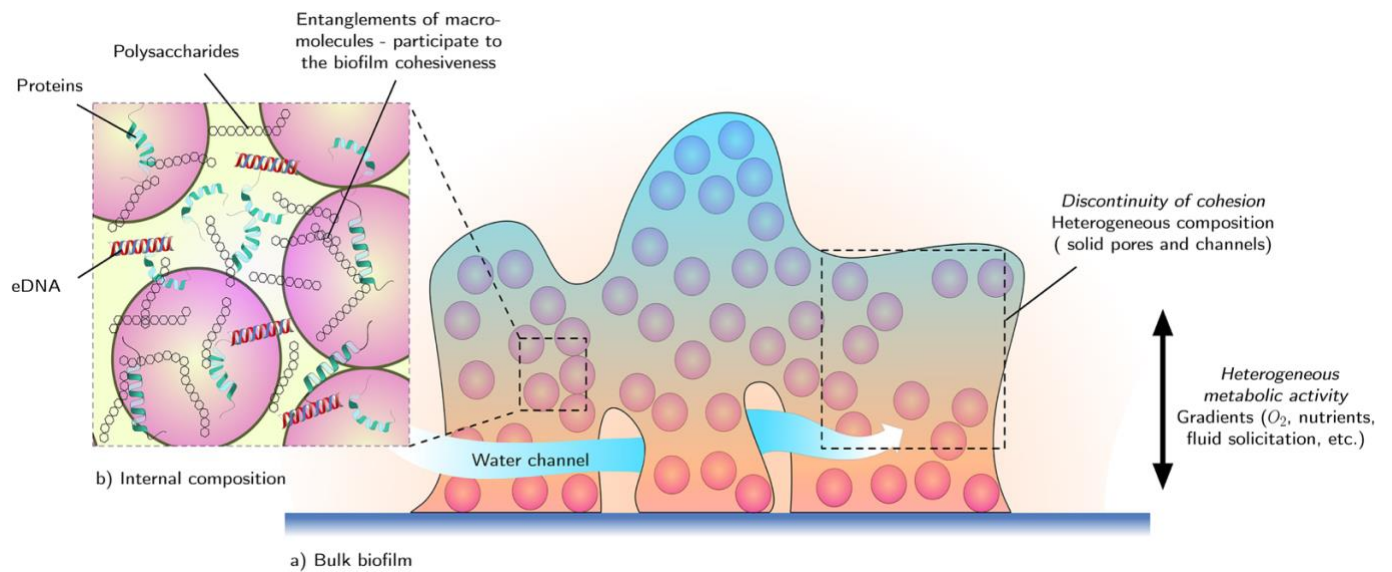


Figure 1.2 The structure of the biofilm matrix and its composition.

A. Biofilms are made of bacteria encased with matrix (EPS). **B.** A focus on the composition shows that the matrix consists of several components. Reproduced from (Boudarel et al., 2018) and it is licensed under [CC BY 4.0](https://creativecommons.org/licenses/by/4.0/).

co-existing microbial species.

Biofilm development is a dynamic multistage process that is affected by: first, the physical properties of cells such as hydrophobicity of the cell surface, signaling molecules, and appendages presence; second, the nature of the substratum such as roughness, charge, and chemical composition; and third, the growth medium characteristics, such as temperature, pH, nutrient availability, antimicrobial agent presence, and shear stress (Bos et al., 1999; Donlan, 2002). As shown in **Figure 1.3**, the stages of biofilm development are similar among microbial species, which include: (i) formation of a preconditioning film by host protein on a surface leading to reversible cells adherence to the surface through weak electrostatic interactions and Van der Waals forces (Bos et al., 1999; Donlan, 2002; Fitzpatrick et al., 2005); (ii) irreversible adherence through hydrophilic and hydrophobic forces and attachment structure-mediated interactions such as adhesive proteins to the surface (Branda et al., 2005; Flemming et al., 2007); (iii) cell proliferation and EPS matrix production consisting of eDNA, proteins, and polysaccharides (Dufour et al., 2010; Hall-Stoodley et al., 2004); (iv) mature biofilms formation with water channels to distribute signaling molecules and nutrients (Srey et al., 2013); (v) biofilm cell detachment in response to internal or external stimuli; and (vi) the dissemination of detached biofilm cells and colonization of new niches (Boudarel et al., 2018). In the following sections, we reviewed the detailed molecular mechanisms of biofilm formation stages in the human pathogenic *S. aureus* and some therapeutic strategies for preventing and treating *S. aureus* biofilm-related infections.

1.2.1 *Staphylococcus aureus* biofilm formation

S. aureus is a ubiquitous Gram-positive bacterium and is a normal part of healthy flora in humans and animals (Cheng et al., 2009). *S. aureus* is most prevalent in nasal mucous membranes and the skin of healthy people (Kluytmans et al., 1997; Pietrocola et al., 2017). *S. aureus*

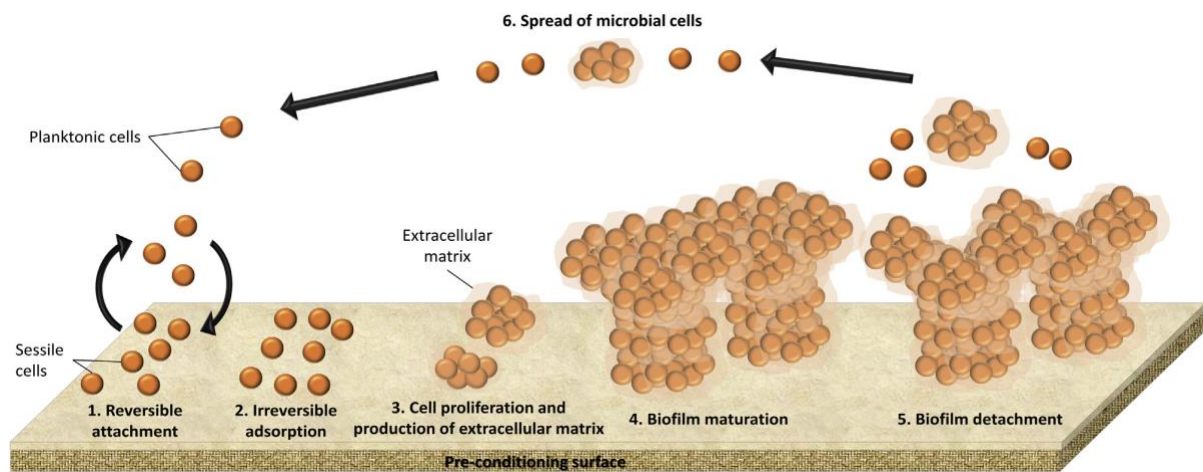


Figure 1.3 The stages involved in biofilm development.

Reprinted from (Vázquez-Sánchez & Rodríguez-López, 2018), with permission from Elsevier.

persistently colonizes 20-30% of the human population (Diep et al., 2004; Gehanno et al., 2009). However, as previously noted *S. aureus* is also a dangerous opportunistic pathogen that can cause infections involving the skin and soft tissue, bloodstream, and the lower respiratory tract (Beenken et al., 2004; Fitzpatrick et al., 2005). Once an injury or break in the nasal passages or skin epithelium occurs, *S. aureus* transmits to circulatory system and is carried to distal sites throughout the body (Foster et al., 2014). If invading *S. aureus* is not rapidly eradicated by the innate immune system, planktonic cells of pathogenic *S. aureus* will tether to available surfaces and form a biofilm, becoming more persistent through the following steps:

A. Reversible attachment

The initial attachment of *S. aureus* differs between biotic and abiotic surfaces. Specific interactions facilitated by cell-wall-anchored (CWA) proteins are required for *S. aureus* to adhere to a living surface (Foster et al., 2014). Previous studies have shown that different types of *S. aureus* CWA proteins are involved in the early attachment step to biotic surfaces, specifically the *S. aureus* surface protein G (SasG), the iron-regulated surface protein A (IsdA), and the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Clarke et al., 2009; Foster et al., 2014; Roche et al., 2003). Some examples of MSCRAMMs are the fibronectin-binding protein A (FnBPA) and fibronectin-binding protein B (FnBPB), which are associated with the attachment to extracellular matrix proteins (Burke et al., 2011; Geoghegan et al., 2013; McCourt et al., 2014). Other MSCRAMMs needed for the initial adherence to immobilized fibrinogen or collagen-rich tissues are clumping factor A (ClfA) or collagen-binding adhesin (CNA), respectively (Ganesh et al., 2008; Geoghegan, Ganesh, et al., 2010; Zong et al., 2005). Clumping factor B (ClfB) is an MSCRAMM associated with bacterial nasal colonization by binding to the cell envelope protein of squamous epithelial cells called loricrin (Ganesh et al.,

2011; Mulcahy et al., 2012; O'Brien et al., 2002; Walsh et al., 2004; Wertheim et al., 2008; Xiang et al., 2012). Other surface proteins involved in the early attachment step, such as IsdA and SasG. The IsdA can trap heme from hemoglobin in iron-restricted environments and facilitates bacterial nasal colonization. However, SasG facilitates bacterial nasal colonization by binding to nasal epithelial cells (Clarke et al., 2009; Roche et al., 2003).

In the case of abiotic surface adherence, *S. aureus* also relies on hydrophobic interactions and electrostatic forces between the cell and the surface (Busscher & van der Mei, 2012). Thus, the bacterial cell and surface's physiochemical properties play a crucial role in the initial attachment to non-living surfaces. MSCRAMMs also play a role by binding to extracellular matrix (ECM) proteins, such as fibrinogen (Fbg) and fibronectin (Fn) that have preconditioned the abiotic surface within nanoseconds (**Figure 1.4**) (Foster et al., 2014). Moreover, some *S. aureus* surface molecules, e.g., teichoic acids and autolysins, facilitate this early attachment by modifying the physiological characteristics of the bacterial surface (Gross et al., 2001; Heilmann et al., 1997).

B. Development of Biofilm:

In this stage, the irreversible adherence, EPS matrix production and biofilm maturation take place. After the bacteria initially adhere to a surface, the bacterial cells produce EPS, in which the cells become embedded and are irreversibly adhered to the surface (Flemming & Wingender, 2010). The created EPS matrix has several roles in biofilm structure integrity (Branda et al., 2005; Flemming, 2011). It protects the embedded cells from biological, physical, and chemical environmental stresses (Branda et al., 2005; Flemming, 2011). The EPS also stabilizes and maintains the mature biofilm structures and acts as surface-anchoring material (Branda et al., 2005; Flemming, 2011). Moreover, EPS plays a crucial role in preventing starvation and dehydration of the encased cells by supplying them with and helping with retention of nutrients and water,

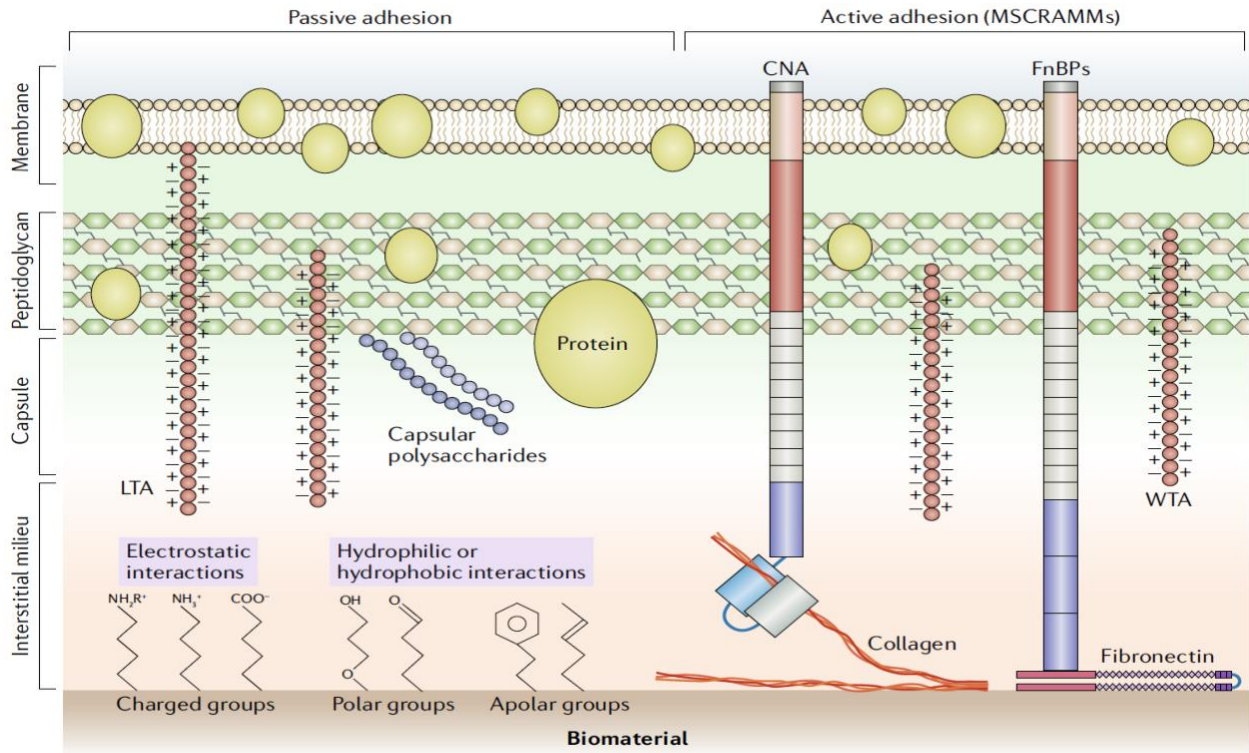


Figure 1.4 Adhesion of *S. aureus* to abiotic surfaces. The adhesion step is facilitated by a combination of the reversible passive and irreversible active mechanisms. Reprinted with permission from (Arciola et al., 2018)

respectively (Flemming & Wingender, 2010). Within a biofilm, the communication of cell-to-cell is essential to form local cooperation within the biofilm cells (Branda et al., 2005; Flemming & Wingender, 2010). This communication is facilitated by maintaining the cells in close proximity inside the EPS matrix (Fitzpatrick et al., 2005). The characterization of the *S. aureus* biofilm EPS matrix is still not entirely well-defined because of the barriers in purifying and extracting the EPS components (Flemming et al., 2007). However, published literature has determined that the extracellular matrix of *S. aureus* consists of three main components: polysaccharides, proteins, and eDNA (Lister & Horswill, 2014).

I. Polysaccharides

In *S. aureus*, polysaccharides represent a fundamental fraction of the biofilm matrix, and their main component is the polysaccharide intercellular adhesin (PIA) (Maira-Litran et al., 2002). The PIA is a poly- β (1,6)-N-acetyl-D-glucosamine glycans (PNAG) with deacetylated residues (Cramton et al., 1999; Maira-Litran et al., 2002). In most *S. aureus* strains, the chromosomal *ica* operon (also known as *icaADBC*) products synthesize PNAGs (Cramton et al., 1999; Fitzpatrick et al., 2005). This operon is mainly suppressed by the products of *icaR*, which are stimulated by the LuxS/AI-2 quorum-sensing system and the staphylococcal accessory regulator A (Cerca et al., 2008; Jefferson et al., 2003; Yu et al., 2012). A previous study has revealed that the *ica* operon's role in the *S. aureus* biofilm matrix is complicated and dependent on the strain and the environment (O'Gara, 2007). Furthermore, a number of environmental factors, e.g., antimicrobial agents, temperature, anaerobic conditions, ethanol, glucose, and osmolarity, influence the expression of *ica* operon (Cramton et al., 2001; Fitzpatrick et al., 2005). PIAs are known to play a fundamental role in the accumulation of cells and biofilm cohesion, and they are associated with the immune evasion of *S. aureus* *in vivo* models (Vázquez-Sánchez & Rodríguez-López, 2018). However,

some studies report *ica*-deletion mutants of *S. aureus* strains form robust biofilms *in vitro* and *in vivo*, which indicate that PIA is not essential for *S. aureus* biofilm formation since PIA was not produced (Fitzpatrick et al., 2005; Lister & Horswill, 2014). Another study has shown the PIA non-essentiality in the biofilm of *S. aureus* since the addition of a PNAG-degrading enzyme (Dispersin B) impaired the growth of new *S. aureus* but did not influence on the preformed biofilms (Izano et al., 2008). The exact role of PIA in *S. aureus* remains unclear as several strains exhibit different phenotypes regardless of the PIA presence.

Currently, teichoic acids, complex polysaccharides with positive net charge anchored to the cell wall, are considered an essential part of the biofilm matrix (Archer et al., 2011; Payne & Boles, 2016). They are products of the *dlt* operon, which incorporates D-alanine into the teichoic acids (Fitzpatrick et al., 2005). A published paper has shown that *dlt*-mutant strains of *S. aureus* produced modified teichoic acid structure, which leads to an electrical imbalance in the cell wall and increases its negative charge (Gotz, 2002). As a result, the biofilm formation capacity is reduced but can be restored by adding Mg^{2+} to the growth media. This result indicates that the presence of a balancing positive charge in the *S. aureus* cell wall is vital to form a biofilm (Gotz, 2002).

II. Proteins

At this time, the identified proteins in the *S. aureus* biofilm matrix are numerous, and their functionality and roles in the biomass accumulation onto surfaces and the adhesion are diverse (Lister & Horswill, 2014; Paharik & Horswill, 2016). Proteins are integral in providing a bond between the surface and the pathogen by being anchored to the peptidoglycan walls (Lister & Horswill, 2014). One of the main components of the proteins fraction in the biofilm matrix is the biofilm-associated protein (Bap), initially found in biofilm-mutant strains of *S. aureus* that cause

bovine mastitis (Lasa & Penades, 2006). Later, research groups have shown that in low pH and low Ca²⁺ environments, N-terminus-containing Bap can aggregate and form fibers into the biofilm matrix, resulting in improved stabilization of the biofilm (Di Martino, 2016; Taglialegna et al., 2016).

Surface-wall associated proteins are another critical group of structural matrix proteins that act as bacterial adhesins. MSCRAMMs are the leading group of these proteins in *S. aureus* contain tandemly linking IgG-like domains (Foster et al., 2014). The staphylococcal FnBPA and FnBPB and the ClfA and ClfB are the well-known MSCRAMMs involved in the biofilm development stage (Patti et al., 1994). The presence of both ClfA and ClfB facilitates the aggregation of bacteria to Fbg (Ganesh et al., 2008; Xiang et al., 2012). In the absence of Ca²⁺ in vitro, ClfB can promote biofilm formation if other matrix components such as eDNA and PNAGs are not functioning (Abraham & Jefferson, 2012). Additionally, elastin and Fbg-linking domains of FnBPA and FnBPB can enhance bacterial accumulation and aggregation (McCourt et al., 2014; O'Neill et al., 2008; Paharik & Horswill, 2016). During host infection of *S. aureus*, other surface-wall related proteins, SasG and SasC, are associated with the biofilm formation and the intercellular adhesion (*ica*) by a Zn²⁺-dependent manner (Corrigan et al., 2007; Geoghegan, Corrigan, et al., 2010; Geoghegan, Ganesh, et al., 2010; Schroeder et al., 2009). Furthermore, SpA protein, which is reversibly anchored to the cell wall, plays a role in biofilm-related infections (Merino et al., 2009). At elevated levels, this protein is a multifactorial virulence factor, specifically in biofilm formation and bacterial accumulation (Merino et al., 2009).

Lastly, the phenol soluble modulins (PSMs), surfactant-like proteins, control the biofilm expansion in *S. aureus* (Periasamy et al., 2012). PSMs facilitate the delivery of nutrients to deeper layers by forming channels into mature biofilms (Periasamy et al., 2012). When activated by auto-

inducing peptides, the accessory gene regulator (*agr*) of the quorum-sensing system triggers the production of these secreted surfactant-like molecules (Boles & Horswill, 2008; Lauderdale et al., 2010; Yarwood et al., 2004).

III. Extracellular DNA (eDNA)

Another critical structural component of the *S. aureus* biofilm matrix is eDNA (Izano et al., 2008). The eDNA importance within the biofilm matrix is in its negative electric charge, which facilitates the cell-host, cell-cell, and cell-surface interactions by acting as a binder (Lister & Horswill, 2014). eDNA is mainly found outside the cells because of the programmed lysis of a small population of cells within the biofilm (Thomas & Hancock, 2009). During this process, *S. aureus*'s cell wall is degraded using murein hydrolases (AtlA) and is controlled by two genes, *lgrA* and *cidA* encoding antiholins and holins, respectively (Lister & Horswill, 2014; Rice et al., 2007). Furthermore, eDNA has a dual role by participating in the formation of biofilms and also maintaining mature structures. A published study has discovered these two roles by treating *S. aureus* culture with deoxyribonuclease I (DNase I), resulting in an attenuation of biofilm formation and dispersion of the polystyrene plates-preformed biofilms (Izano et al., 2008). Other studies have shown that eDNA has a cohesive effect by interacting with other biofilm matrix components, leading to improved stabilization of biofilm structure (Huseby et al., 2010; Schwartz et al., 2016). Also, eDNA can protect *S. aureus* biofilm from antimicrobials by sequestering cationic antimicrobial agents (Izano et al., 2008). Importantly, the horizontal gene transfer (HGT) is promoted because of large amounts of eDNA residing in the biofilm matrix and acting as genetic material, specifically in multi-strains or multi-species biofilms (Molin & Tolker-Nielsen, 2003; Schwartz et al., 2016). As a consequence, the probability of the exchange of antimicrobial resistance genes between strains increases, also increasing concerns regarding biofilm-related

infections treatment (Flemming et al., 2007; Fux et al., 2005).

C. Biofilm Dispersal

The last stage of the biofilm life cycle in *S. aureus* is the dispersion of biofilm cells (Kaplan, 2010). This step permits the dispersed cells to disseminate to new sites of the host body and initiate a new life cycle of a biofilm (Kaplan, 2010). High shear stress and other physical environmental forces disperse attached biofilm cells by a passive detachment mechanism (O'Toole et al., 2000). However, some bacterial species, such as *S. aureus*, can intentionally release biofilm cells by using both broad-spectrum and specific mechanisms of active dispersion (Lister & Horswill, 2014). These self-regulated processes depend mostly on the extracellular matrix composition to promote the biofilm cells dispersal into the environment (Chaignon et al., 2007; Izano et al., 2008; Kiedrowski et al., 2011). For example, enzymatic targeting against eDNA and proteins in the matrix of polysaccharide-rich biofilms is ineffective. On the other hand, the utilization of destructive enzymes to target PIA is not effective in the matrix of polysaccharide-independent biofilms (Lister & Horswill, 2014).

The broad-spectrum mechanisms of biofilm breakdown and cell dispersal include the stringent response and D-amino acids (Lister & Horswill, 2014). The stringent response is a universal adaptive system in bacterial species activated by harsh environmental conditions such as nutrients starvation (Srivatsan & Wang, 2008). In *S. aureus*, this was demonstrated by applying a powerful antibiofilm peptide, degraded ppGpp alarmone, which regulates changes in the metabolic status of bacterial cells and to switch the metabolism balance from division and growth to stress and survival response (de la Fuente-Nunez et al., 2014). Because this response was observed during infection by *S. aureus* (Geiger et al., 2010), future studies are needed to explore the exact role of stringent response in the dispersal step in *S. aureus* biofilms infections.

Another broad-spectrum mechanism of dispersal in *S. aureus* is the production of D-amino acids, D-Proline, D-Tyrosine, and D-Phenylalanine, in the stationary phase of *S. aureus* growth (Hochbaum et al., 2011). The D-amino acids inhibit the aggregation step in biofilm formation by preventing the proteins cell-cell adhesion from being localized (Kolodkin-Gal et al., 2010). This effect seems consistent across the non-similar bacterial species (Kolodkin-Gal et al., 2010). As a consequence of D-amino acid accumulation, dispersion of aggregated biofilm cells will occur. This application has demonstrated a potential preventative strategy to inhibit biofilm formation by diminishing *S. aureus* biofilm development on polymeric surfaces (Hochbaum et al., 2011; Sanchez et al., 2014; Sanchez et al., 2013). PSMs, which, as mentioned earlier, form channels within mature biofilms, have a nonspecific action of dispersing biofilm cells and leading to a disseminated infection (Periasamy et al., 2012).

In contrast, one of the specific dispersal mechanisms in *S. aureus* biofilm is the employment of protease secretion by which the components of the proteinaceous matrix components are degraded, resulting in biofilm disruption (Shaw et al., 2004). The proteases production is regulated by the *agr* quorum-sensing system (Thoendel et al., 2011). *S. aureus* secretes proteases that include: two-related cysteine proteinases; staphopain A (ScpA) and staphopain B (SspB); V8 serine protease (SspA); and the metalloproteinase aureolysin (Aur) (McGavin et al., 1997; Shaw et al., 2004). The staphopains can cause biofilm degradation by targeting unidentified proteins (Mootz et al., 2013). While SspA has the ability to disturb the FnBPA, FnBPB, surface protein A (SpA), and Bap proteins (Marti et al., 2010; McGavin et al., 1997; O'Neill et al., 2008). Last, Aur has a disruptive activity against ClfB and Bap functionalities (Abraham & Jefferson, 2012; Marti et al., 2010; Shaw et al., 2004). The effect of these proteases

as enzymes to disperse well-established biofilms or to inhibit the initial adherence has gained an interest with the emergence of protein-rich biofilms of *S. aureus*.

Another specific mechanism of dispersal in *S. aureus* during the infection is the production of thermostable nucleases, nuclease (Nuc) and nuclease2 (Nuc2), which are involved in forming the channels within mature biofilms (Kiedrowski et al., 2014; Olson et al., 2013; Tang et al., 2008). However, it has been observed that the *nuc* gene expression downregulated during the process of the biofilm formation, which leads to an accumulation of eDNA in the matrix (Kiedrowski et al., 2011; Mann et al., 2009; Olson et al., 2013).

In addition to self-breakdown, the *S. aureus* biofilm matrix can be disrupted by a group of enzymes released from other bacterial species in the environment (Lister & Horswill, 2014; Sugimoto et al., 2013). For example, *Staphylococcus epidermidis* releases serine protease (Esp) to degrade some proteins of the *S. aureus* biofilm matrix, such as FnBPA, and extracellular adherence protein (Eap) (Sugimoto et al., 2013). Moreover, Esp has been found to disrupt Atl in order to prevent eDNA from being released into the matrix (C. Chen et al., 2013). The PIA glycosidic linkages in the *S. aureus* biofilms matrix can be cleaved by dispersin B, an enzyme secreted by the Gram-negative *Actinobacillus actinomycetemcomitans* (Kaplan et al., 2004). While this process may serve to disperse the *S. aureus* biofilm further, the utilization of these disruptive bacterial molecules is a potential strategy to prevent pathogens such as *S. aureus* from forming mature biofilms and could be the source of disruptive enzymes for clinical treatment of infections.

1.2.2 Quorum-sensing systems in *S. aureus*

Between the mechanisms that regulate the adaptation of *S. aureus* to a new environment, the quorum-sensing is one of the most important and well-studied mechanisms that control *S. aureus* pathogenicity (Le & Otto, 2015). Quorum-sensing is a process of cell-to-cell communication, by

which bacterial species regulate gene expression depending on environment and population density (Waters & Bassler, 2005). In *S. aureus*, there are two regulatory quorum-sensing systems: Agr quorum-sensing system, which is the typical regulator system of quorum-sensing in *S. aureus*, and the LuxS quorum-sensing system (Kleerebezem et al., 1997; Xue et al., 2013).

1. The Agr quorum-sensing system

The *agr* locus consists of RNAII and RNAIII, which are divergent transcriptional units. The transcription of RNAII and RNAIII is triggered by P2 and P3 promoters, respectively (Morfeldt et al., 1995; Peng et al., 1988). The RNAII locus includes the genes *agrD*, *agrB*, *agrC*, and *agrA* (Novick & Geisinger, 2008). As shown in **Figure 1.5**, the extracellular quorum signal of Agr is called an autoinducing peptide (AIP), and its peptide precursor is a product of the *agrD* gene (Ji et al., 1995). The mature form of AIP contains 7-9 amino acids and has a distinct thiolactone between the C terminus and the central cysteine (Ji et al., 1997; Ji et al., 2005; Novick & Geisinger, 2008; Otto et al., 1998). The *agrB* transcript encodes a membrane-bound endopeptidase that plays a role in the trimming and export of AIP (Saenz et al., 2000; Zhang et al., 2002; Zhang & Ji, 2004). While AIP is in the extracellular environment, it received a final cleavage by a type I signal peptidase (SpsB) (Kavanaugh et al., 2007). The *agrC* and *agrA* genes encode a two-component signal transduction system. (Lina et al., 1998). This system involves a histidine sensor kinase called (AgrC), which is a membrane-associated protein, and its response regulator (AgrA) (Lina et al., 1998). The phosphorylation of AgrC and AgrA is triggered upon the AIP binding (Lina et al., 1998; Novick et al., 1995; Queck et al., 2008). The active AgrA binds to the P2 and P3 promoters for RNAII and RNAIII transcription, respectively, with a higher affinity for P2 (Koenig et al., 2004; Novick et al., 1995). The RNAIII is the effector of the *agr* system, which regulates two classes of virulence factors: (i) factors associated with attachment to host, and

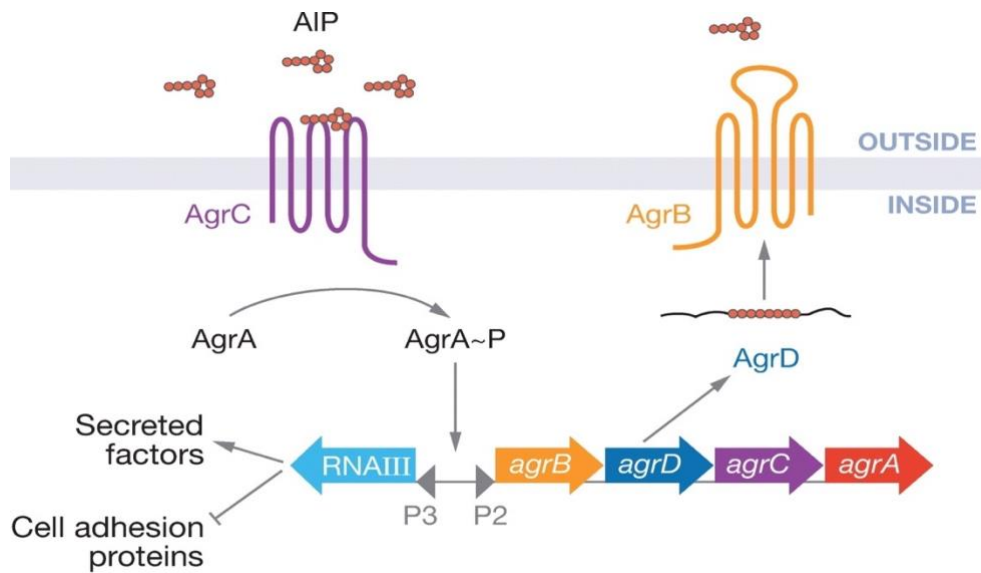


Figure 1.5 The accessory gene regulator (*agr*) quorum sensing system of *S. aureus*.

The *agr* locus consists of distinct transcripts named RNAII and RNAIII, driven by P2 and P3, respectively. The AIP signal is yielded from the AgrD precursor, while the transmembrane enzyme AgrB participates in the activation and transport of the AIP. At a critical threshold concentration of AIP, the two-component signal transduction system, AgrC–AgrA, is activated and triggers the phosphorylation of AgrA. Once phosphorylated, AgrA attaches to the P2 and P3 promoter regions, and RNA is transcribed. Reprinted with permission from (Waters & Bassler, 2005).

(ii) factors associated with invasion and toxin production (George & Muir, 2007; Koenig et al., 2004; Roux et al., 2009). Production of RNAlII increases the gene expression of proteases, toxins, and capsule, which are necessary for invasion and dissemination, and decreases the production of surface adhesins, which are essential for initiating biofilm formation and immune evasion. (Bowden et al., 2005; Novick & Geisinger, 2008; Roux et al., 2009; Yao et al., 2006)

2. The luxS Quorum-sensing system

The regulatory activity of *luxS* was first described in *Vibrio* species (Bassler et al., 1997). It was discovered in regulating the bioluminescence and then recognized as a quorum-sensing system in bacteria (Bassler, 2002). The LuxS quorum-sensing system uses an autoinducer known as AI-2, a furanosyl borate diester (Chen et al., 2002). In *S. aureus*, AI-2 regulates several phenotypes such as virulence, biofilm formation, antimicrobial resistance, and capsule synthesis (Xue et al., 2013; Yu et al., 2012; Zhao et al., 2010). Previous studies have shown that *luxS* controls *S. aureus* biofilm formation by the *icaR* locus under *in vitro* and *in vivo* experimental conditions (Yu et al., 2012). However, the exact role of LuxS as a regulatory system of quorum-sensing in *S. aureus* is still controversial.

1.3 Current therapeutic strategies for preventing and treating *S. aureus* biofilm-related infections

Treatment outcomes improve when therapeutic intervention for biofilm-related infections begins as soon as possible (Hall-Stoodley et al., 2012; Hoiby et al., 2015). Since the biofilms can persist for prolonged periods of time such as months and years without detection by the immune cells, early diagnosis and early treatment are not always possible (Burmolle et al., 2010). Currently, the methods used for biofilm infections are antimicrobial treatment and physical removal of the infection source through the surgical removal of artificial implants and necrotic tissues

(McConoughey et al., 2014). However, these interventions are not always successful in fighting biofilm-associated infections (Bhattacharya et al., 2015; Hoiby et al., 2015).

1. Antimicrobial treatment

The antibiotic resistance is rapidly developing within *S. aureus* biofilm-infections, which results in increased antibiotic minimum inhibitory concentration (MIC) compared with their planktonic counterparts (Howlin et al., 2015). This significant problem is also associated with exposing a higher number of biofilm *S. aureus* cells to antibiotic selective pressure (Koch et al., 2014). Currently, the most commonly used drug for treating biofilm *S. aureus* infections is vancomycin (Liu et al., 2011). However, there is a growing caution to administer vancomycin since the evident emergence of vancomycin-resistant *S. aureus* (VRSA) and vancomycin-intermediate *S. aureus* (VISA) strains (Howden et al., 2010). To overcome this resistance, it is recommended to combine vancomycin with a second drug such as linezolid or rifampin (Howlin et al., 2015; Salem et al., 2011; Vergidis et al., 2011). Some studies have published that this combination has inconsistent results and indicates that their synergism is only effective against biofilm infections caused by Methicillin-susceptible *S. aureus* (MSSA) strains but not MRSA strains (Salem et al., 2011; Zimmerli, 2014). Moreover, rifampin alone is the only highly effective antibiotic against most biofilm-forming *S. aureus* strains (Zimmerli, 2014).

A lipopeptide molecule called daptomycin treats infections caused by VRSA (Cha et al., 2003). The mechanism of action of daptomycin is disrupting the function of bacterial cell membrane, leading to a rapid depolarization of membrane potential. As a result, the synthesis of DNA, RNA, and proteins will be inhibited, and ultimately cell death will occur (Alborn et al., 1991; Patel & Saw, 2020). Importantly, daptomycin does not require the cells to be metabolically active to exert its action (Mascio et al., 2007). In a published study that has compared the efficacy

in clearing *S. aureus* from established biofilms of daptomycin, vancomycin, clindamycin, linezolid, and tigecycline, daptomycin was the most effective of the five drugs (Smith et al., 2009). Unfortunately, the use of antibiotics to treat biofilm-related infections will lead to the development of a subpopulation of dormant cells (called persisters) resistant to antibiotic treatment (Wood et al., 2013).

Lastly, treatment guidelines for *S. aureus*-biofilm infections are specific to the *S. aureus* strain and the type of infection (Osmon et al., 2013). These guidelines do not include all biofilms infections (Stevens et al., 2014). As a consequence, the effectiveness of most antimicrobials against biofilm-specific infections is limited (Zimmerli, 2014). Therefore, novel approaches, such as antibiotic-coated surfaces or combinational treatment are required (Zimmerli, 2014). Additionally, altering the duration of therapy and delivery method of the drug are strategies that have been found to enhance antibiotic efficacy (Howlin et al., 2015). For example, using silver nanoparticles to deliver the drugs to inside the biofilms has been shown to improve antimicrobial efficacy (Brown et al., 2012; Kho et al., 2010) However, all of these methods will require additional research and further investigation to validate their clinical applications.

2. Physical removing of the infection source

The early development of *S. aureus* biofilm infections on implants is a fundamental cause of concern (Stoodley et al., 2008). The method in treating these infections relies on surgical intervention to remove the infected implants and the damaged area (Kathju et al., 2014). This process includes completely removing the artificial implants and the surrounding damaged tissues (Peel et al., 2011). However, the retention of implants after the surgery is not recommended since the infection may reoccur and leads to the failure of treatment if not performed in the first two weeks of the onset (Brandt et al., 1997; Crockarell et al., 1998; Deirmengian et al., 2003).

Unfortunately, the physical removal strategy has achieved only limited success (Deirmengian et al., 2003). Several published studies have shown that most of the currently used surgical methods are unable to effectively remove *S. aureus* biofilms from the source of infection (Rmaile et al., 2015; Schwechter et al., 2011; Stoodley et al., 2008). As a result, more studies are needed to enhance the effectiveness of these techniques.

1.4 Recent and novel therapeutic approaches for preventing and treating *S. aureus* biofilm-related infections

Since the *S. aureus* biofilm cells are distinct from their planktonic counterparts in terms of causing diseases and drug resistance, there is a rationale to develop biofilm-specific strategies that target the stages of biofilm formation and its matrix components (Gunn et al., 2016; Hall & Mah, 2017; Koo et al., 2017). These strategies are broadly categorized into two categories, as displayed in **Figure 1.6**, and are under evaluation for treating biofilm-infections.

1.4.1 Preventing new biofilms formation

I. Inhibition of attachment step

As earlier mentioned in this review, the first step of biofilm formation is the attachment of bacteria to a surface. In a *S. aureus* biofilm, the adherence to an abiotic surface is nonspecific and facilitated by electrostatic interactions, Van der Waal's forces, and conditioning film on the surface by host proteins (Busscher & van der Mei, 2012; Fitzpatrick et al., 2005). In contrast, the attachment to biotic surfaces is specific and occurs through MSCRAMMs recognizing host proteins (Foster et al., 2014). This early-stage has a crucial role in the bacterial colonization of medical devices and the initiation of medical device-related infections (Zheng et al., 2018). Therefore, anti-infective strategies such as 1) Antiadhesive surfaces and 2) Antimicrobial-coated surfaces that target this initial step have been developed.

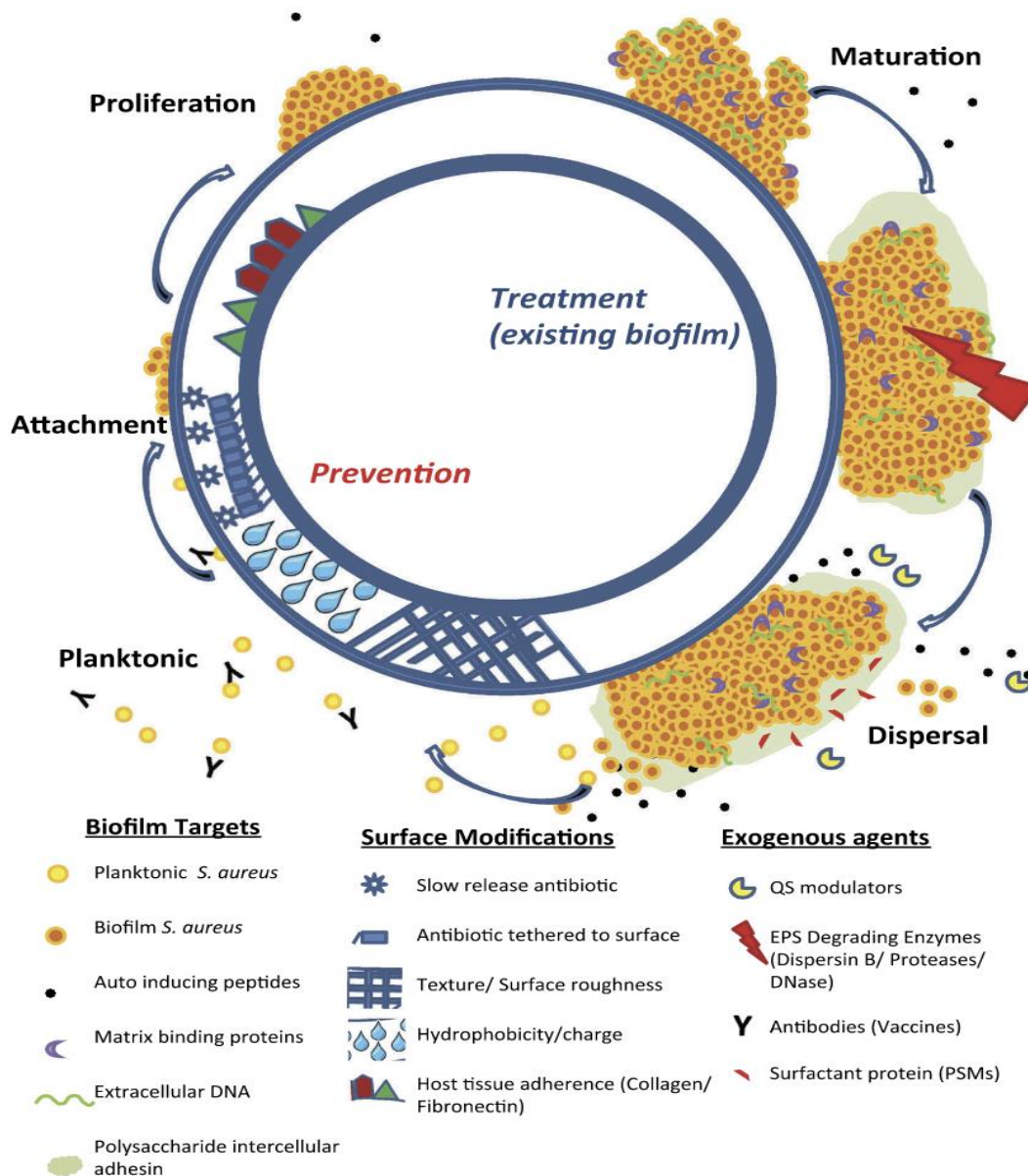


Figure 1.6 Strategies for prevention and treatment of *S. aureus* biofilms. This figure summarizes the life cycle of *S. aureus* biofilm, with showing its various stages. Each of these stages represents possibilities for therapeutic preventative and disruptive intervention strategies.

Reprinted with permission from (Bhattacharya et al., 2015).

1. Anti-adhesive surfaces:

Developing antiadhesive materials to prevent the bacterial adhesion on the implant surface is an alternative approach for inhibiting bacterial colonization (Ramachandran & Muthuvijayan, 2019). This strategy can be achieved by modifying the physical properties of the surface, such as charge, hydrophilicity, hydrophobicity, roughness, and texture (Chung et al., 2007; Ramachandran & Muthuvijayan, 2019). Furthermore, some researches have shown that by facilitating host cells attachment to the surface before bacterial attachment lowered the risk of biofilm infection development (Gristina et al., 1989). This approach also can be enhanced by directly targeting MSCRAMMs such as FnBPs and Bap, which play a role in initiating *S. aureus* biofilm infections (Speziale et al., 2014; Valle et al., 2012). Importantly, the abovementioned strategies are mainly applied against abiotic surface infections (Bhattacharya et al., 2015). However, the inhibition of *S. aureus* transpeptidase sortase A (SrtA), a protein responsible for anchoring surface proteins such as FnBPs, ClfA and ClfB proteins to the cell wall, has a potential in preventing *S. aureus* biofilm infections (Cossart & Jonquieres, 2000; Mazmanian et al., 1999; Zhang et al., 2014). A published study has shown that this inhibitory activity disrupts the surface-anchored proteins, leading to a reduction in *S. aureus* colonization of host tissues (Mazmanian et al., 2000). These findings led to the discovery that surface-anchored proteins could be promising therapeutic targets to prevent bacterial adherence and initiation the biofilm process (Cascioferro et al., 2014; Guo et al., 2015). Lastly, it is essential to preserve the primary purpose of the artificial implants in the process of anti-adhesive surfaces development and to ensure that the coating is not lethal to host cells (Jena et al., 2012; Kazemzadeh-Narbat et al., 2010; Kazemzadeh-Narbat et al., 2013; Pavlukhina et al., 2012).

2. Anti-microbial-coated surfaces:

One approach currently being evaluated is the development of coatings with bactericidal and bacteriostatic activities that utilize an engineered surfaces to inhibit bacterial adherence (Harris et al., 2004; Zhao et al., 2009). A silver nanoparticle-coated catheter is one example of this approach, which is under investigation as a means of preventing formation of *S. aureus* biofilm infections (Jena et al., 2012). However, despite the promising activity shown by in vitro studies, silver must be used cautiously since it can cause cytotoxicity to host tissues (Stevens et al., 2009; Zhang et al., 2008). To make these surfaces feasible for long-term clinical use, there are ongoing research studies that aim to improve the host compatibility of them and lower the risk of cytotoxicity (Mandakhalikar et al., 2018). Other commonly used implant materials such as stainless steel and titanium are also being coated with vancomycin and tested for their ability to inhibit *S. aureus* biofilm formation (Hu et al., 2010; Kazemzadeh-Narbat et al., 2013; Kruszewski et al., 2013). However, the drawback of this strategy is the potential of antibiotic-resistance selective pressure (Bhattacharya et al., 2015).

II. Vaccines

With the emergence of antibiotic resistance, more research has been conducted in developing vaccines that inhibit *S. aureus* biofilm infection (Salgado-Pabon & Schlievert, 2014). Unfortunately, none of the developed vaccines have passed phase III in clinical trials because of their failure to take into consideration the biofilm mode of growth, which may contribute to their inefficacy (Fowler & Proctor, 2014; Proctor, 2012; Salgado-Pabon & Schlievert, 2014). For instance, a developed vaccine was effective against planktonic *S. aureus* infections by possessing activity against the capsular polysaccharide (CP 5/8) with a non-toxic *Pseudomonas aeruginosa* (*P. aeruginosa*) exotoxin A utilized as carrier protein (Ho et al., 2006). However, since some *S. aureus* strains, such as USA300 strain, lack the capsule, this vaccine candidate is not expected to

show efficacy against several strains related to biofilm infections (Sutter et al., 2011). As a result, there is no approved vaccine to use against *S. aureus* biofilm infections.

Recently, researchers are developing vaccines specifically target biofilms (Boles et al., 2010; Harro et al., 2010). Their first targeted component of the biofilm matrix is PIA or PNAG, which is present in biofilms for aggregation (Maira-Litran et al., 2012). However, some published studies have shown that there are a high number of clinically relevant *S. aureus* strains can form biofilms independent of PIA (Fitzpatrick et al., 2005). Because of the reduced immunogenicity of polysaccharides, it is crucial to include an appropriate protein or peptide with vaccines under the investigation (Harro et al., 2010; Jansen et al., 2013). For these reasons, PIA vaccines had limited success (Jansen et al., 2013).

1.4.2 Treating and disrupting formed biofilms

Biofilms propensity to evade host immune system response paired with the commonality of late diagnosis of biofilm infections necessitate the development of new therapeutic strategies to target the established biofilms (Bjarnsholt, 2013; Gupta et al., 2016). During the stage of detachment within the cycle of biofilm development, the bacterial cells switch back to the planktonic mode of growth to colonize new sites of the host (Boles & Horswill, 2011; Costerton et al., 1999). While the cells are in this mode, they will regain susceptibility to host immune responses and antimicrobial treatment (Singh et al., 2009). Several published studies have shown that more than one mechanism is involved in the dispersal step (Boles & Horswill, 2011; Lister & Horswill, 2014). These mechanisms include: 1) enzymatic degradation of the matrix, 2) activation of biofilm dispersal, 3) induction of small molecule inhibitors, and 4) interfering with *S. aureus* signaling networks.

1. Enzymatic degradation of matrix

Synthesizing decoy proteins in the biofilm matrix, which inhibit the activity of antibiotics by immobilizing them, contributes to the antimicrobial resistance and the transfer of antimicrobial genetic tolerance (Brady et al., 2006). Therefore, some researchers have investigated strategies that target the main matrix components (Kiedrowski & Horswill, 2011). This approach is conducted by adding exogenous enzymes such as trypsin, proteinase K, dispersin B, and DNase I, that disrupt proteins, polysaccharides, and eDNA components of EPS matrix, respectively (Kiedrowski & Horswill, 2011; Lauderdale et al., 2010; Marti et al., 2010). In some studies, dispersin B has been found to degrade the polysaccharides components of *S. aureus* biofilms (Izano et al., 2008; Rohde et al., 2010). In contrast, other studies have shown that the susceptibility of *S. aureus* strains to dispersin B depends on the biofilm matrix chemical composition, and there are a high number of clinical isolates that form polysaccharide-independent biofilms (Boles et al., 2010). DNase I is another capable enzyme that disrupts the matrix and targets the *S. aureus* biofilms by degrading eDNA, which is released from the subpopulation of autolyzed cells (Bose et al., 2012; Pasztor et al., 2010). It has been found that disrupting the activity of DNase I is higher in the early stages of biofilm development, indicating that eDNA has a crucial role in the early stages of biofilm formation (Mann et al., 2009; Moormeier et al., 2014; Rice et al., 2007). Although these strategies seem promising, the use of these approaches can cause a protein-induced inflammatory response in the host. Thus, it is recommended to combine them in the treatment of medical device-associated *S. aureus* infections in a similar approach to the “antibiotic lock” approach (Fernandez-Hidalgo & Almirante, 2014; Franca et al., 2016; Justo & Bookstaver, 2014; Meije et al., 2014). In this approach, a high concentration of an antibiotic is combined with an anti-coagulant such as heparin to allow for local instillation into the lumen of catheter (Justo & Bookstaver, 2014).

2. Activation of biofilm dispersal

Because of the higher rate of antibiotic-resistance development within biofilm subpopulation compared to planktonic cells, it is vital to evaluate antibiotic-independent strategies for treating biofilm-related infections (Percival et al., 2011). One study reports that the dispersal of biofilm cells from the biofilm matrix increases their antibiotic susceptibility, suggesting that the dispersal of biofilm into single cells would be a valuable strategy to combat the antibiotic resistance (Cos et al., 2010; Hoiby et al., 2010). To evaluate this approach, some researchers have investigated dispersion-inducing factors specific for *S. aureus* (Fux et al., 2004). For instance, nonspecific dispersal agents that include surfactants such as PSMs have been found effective against most *S. aureus* biofilms (Periasamy et al., 2012; Peschel & Otto, 2013; Wang et al., 2007). Unfortunately, PSMs can also cause adverse effects, such as intrinsic inflammation and induction of phagocytic lysis (Surewaard et al., 2013). Other researchers have shown that PSMs in planktonic conditions can be soluble, but in biofilms, they form insoluble amyloid fibers (Schwartz & Boles, 2013; Schwartz et al., 2012). This insolubility, caused by the loss of surfactant properties, leads to the lysis of phagocyte cells (Schwartz & Boles, 2013). As a result, this drawback causes concern for the potential use of these molecules as dispersion inducers.

A small molecule produced by *P. aeruginosa*, *cis*-2-decenoic acid, has been found to enhance the release of single cells from *S. aureus* biofilms and has potential applicability as a disassembly agent (Jennings et al., 2012). This fatty acid messenger has been the focus of numerous research studies, but more are needed to validate these results due to its yet unknown dispersal mechanism (Marques et al., 2015).

3. Induction of small molecule inhibitors

Recently, a variety of small-molecules inhibitors has shown to have an activity *in vitro*

against *S. aureus* biofilms (Boles & Horswill, 2008; M. Chen et al., 2013; Kiedrowski & Horswill, 2011). Early studies found that the D-amino acids, such as D-proline, D-tyrosine, and D-phenylalanine, have inhibitory activity against *S. aureus* biofilms (Hochbaum et al., 2011; Kolodkin-Gal et al., 2010). These D-amino acids inhibited the mature biofilm development from cell aggregates or microcolonies, but they did not prevent the initial adherence of *S. aureus* to surfaces (Hochbaum et al., 2011). The mechanism of these small molecules is primarily targeting the protein component of the biofilm matrix and inducing biofilm disassembly (Aliashkevich et al., 2018; Ampornaramveth et al., 2018; Kolodkin-Gal et al., 2010). However, the use of D-amino acids is controversial since some *S. aureus* strains have been reported to form biofilms in the presence of D-amino acids (Sarkar & Pires, 2015). Lastly, more research is needed to better understand the mechanism of these molecules and similar effectors and to validate these findings in *in vivo* models as therapeutic strategies for *S. aureus* biofilms.

3. Interfering with *S. aureus* signaling networks

There is agreement that quorum-sensing systems in most bacterial species have a role in triggering the switch from planktonic to the biofilm mode of growth (Davies et al., 1998). Therefore, modulating quorum sensing systems, which leads to prevention of biofilm formation or induction of biofilm dispersal, is an exciting approach in combating biofilm-associated infections (McDougald et al., 2011). However, unlike other bacterial regulation of quorum-sensing systems in biofilms, the *S. aureus agr* quorum-sensing system is repressed in the biofilm mode of growth (Lauderdale et al., 2010). Instead, activation of the *agr* quorum-sensing system triggers the dispersion of *S. aureus* single cells from the biofilm (Boles & Horswill, 2008). Published studies, however, have revealed that a high concentration of AIP activates the *agr* system via RNA III, leading to the expression of several virulence factors such as PSMs and proteases (Ji et al., 1995;

Lister & Horswill, 2014; Queck et al., 2008). Therefore, it was hypothesized that developing agents to inhibit the expression of *agr* system would prevent the expression of virulence determinants, which have a crucial role in acute infections caused by *S. aureus* (Boles & Horswill, 2008). Unexpectedly, these *agr* locus inhibitors promote biofilm formation and lead to persistent bacteremia and more biofilm formation since they prevent the dispersal of biofilms (Balasubramanian et al., 2017; Fowler et al., 2004; Schweizer et al., 2011). Thus, several scientists turned to targeting the downstream factors of *agr* operon to understand the specific functions of this system (Balaban et al., 2007). For instance, targeting RNAIII by using RNAIII peptide inhibitors (RIPs) has been explored (Cirioni et al., 2006). As expected, RIPs do inhibit the *S. aureus* biofilm formation on the surface of some medical devices and reduce the minimum biofilm inhibitory concentration (MBIC) for several antimicrobial agents, such as vancomycin, imipenem, and ciprofloxacin, when combined with these compounds *in vitro* and *in vivo* (Balaban et al., 2007; Cirioni et al., 2006). The proposed mechanism of RIPs inhibitory activity is competition with RNAIII-activating peptide (RAP) for the receptor binding to target of RAP (TRAP), leading to blockage of quorum sensing-mediated virulence (Ciulla et al., 2018; Tan et al., 2018). With all conducted research on quorum sensing systems, the pleiotropic regulation of virulence factors and biofilm formation by *agr* system awaits further investigation.

1.5 Probiotics and their products: a potential alternative in fighting pathogenic biofilms

Since there are still challenges in developing effective therapies for fighting bacterial biofilm infections, recent evidence suggests the use of probiotics would be a promising approach against pathogenic biofilms (Fijan et al., 2019). Probiotics are “living microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (Food and Agriculture Organization and World Health Organization [FAO/WHO], 2006). Moreover, the beneficial

impact of probiotics on the host and their interactions with gut microbiota were revealed by high-throughput techniques (Iannitti & Palmieri, 2010; Tidjani Alou et al., 2017). Probiotics were found to modify the host mucosa, enhance the immune system, and protect the host from pathogenic microorganisms (Iannitti & Palmieri, 2010). In current probiotic preparations, the most common and essential microbial species are *Bifidobacterium* and *Lactobacillus* (Lactic Acid Bacteria, LAB) (Didari et al., 2014). Previous research has shown that these species maintain health by supporting a balanced immune response, maintaining a healthy gut microbiome, and improving the absorption of nutrients (Sanchez et al., 2017). Additionally, other reports have published that these beneficial bacterial species could inhibit the overgrowth, adhesion, and coaggregation of pathogenic microbes (Guarner et al., 2012; Monteagudo-Mera et al., 2019). These inhibitory activities of probiotics against pathogens in the gastrointestinal (GI) tract are exerted by decreasing gastric pH, producing antimicrobial molecules, and competing for nutrients and adhesion sites (Liao & Nyachoti, 2017; Sadekuzzaman et al., 2015; Vieco-Saiz et al., 2019). Therefore, probiotics could be a practical approach in treating pathogenic biofilms depending on their properties. Herein, we reviewed the current knowledge of the activity of probiotics and their products and their ability to form biofilms against pathogenic biofilms.

A. Preventing the formation of biofilms by probiotics

It is still valuable to develop novel alternative strategies to inhibit biofilms with the identified challenges and ineffectiveness of currently used guidelines (Khatoon et al., 2018). The application of probiotics in combating biofilm infections is considered a new avenue with advantages (Besser et al., 2019). The benefits of probiotics application are 1) the low risk of inducing selective pressure on resistant isolates compared to conventional antimicrobials (Imperial & Ibana, 2016), and 2) the low cytotoxicity of probiotics compared to other agents such as quorum-

sensing inhibitors (Barzegari et al., 2020; Tan et al., 2018). Furthermore, probiotics utilize multiple molecular mechanisms to limit the pathogenic activity of microorganisms and their adhesion to either biotic or abiotic surfaces (Bermudez-Brito et al., 2012). For example, probiotics produce antagonistic substances such as biosurfactants, bacteriocins, organic acids, hydrogen peroxide, and enzymes (Liao & Nyachoti, 2017; Sadekuzzaman et al., 2015). These molecules can prevent the colonization of pathogenic bacteria and biofilm formation of pathogenic bacteria on surfaces by generating unfavorable growth conditions, e.g., competing for surfaces and nutrients as well as changing environmental pH (Ahimou et al., 2000; Liao & Nyachoti, 2017; Okuda et al., 2013; Sadekuzzaman et al., 2015; Vieco-Saiz et al., 2019).

Several probiotic strains of *Streptococci*, *Lactobacilli* and *Escherichia coli* (*E. coli*), isolated from human, animals, environment, plant, and diet have been reported to have anti-biofilm activity (Chen et al., 2017; Sornplang & Piyadeatsoontorn, 2016). Among these strains, *Streptococcus oralis* (*S. oralis*), *Streptococcus salivarius* (*S. salivarius*), *E. Coli Nissle 1917* (*ECN1917*), *Lactobacillus fermentum* (*L. fermentum*), and *Lactobacillus plantarum* (*L. plantarum*) have been shown to have antibiofilm activity against *S. aureus* biofilms (Bidossi et al., 2018; Fang et al., 2018; Melo et al., 2016). *S. oralis* and *S. salivarius* were found to prevent biofilm formation by decreasing pH and biofilm biomass of *S. aureus* and dispersing established biofilms through the secretion of diffusible molecules (Bidossi et al., 2018). Another study revealed that the antibiofilm activity of *E. Coli Nissle 1917* against *S. aureus* occurs by proteins production that may have a role in inhibiting pathogenic *S. aureus* biofilms (Fang et al., 2018). Moreover, *L. fermentum* and *L. plantarum* have been reported to alter the *ica* operon, which is involved in the synthesis of the biofilm matrix, to inhibit *S. aureus* biofilm formation (Melo et al., 2016).

B. Inhibiting pathogenic biofilms by probiotic products

Lactic acid bacteria (LAB) produce many different metabolites with antimicrobial activity. Some examples of these metabolites are bacteriocins, surfactants with antibiofilm effect, and reactive oxygen species (ROS) (Merghni et al., 2017; Sharma et al., 2018; Yan et al., 2019). LAB also secrete exopolysaccharides with antibiofilm, antioxidant, and stimulatory immune system effects (Kim et al., 2009; Pan & Mei, 2010). Various studies have determined that the *Lactobacilli* exopolysaccharides have activity against Gram-positive and Gram-negative bacteria. Specifically, these exopolysaccharides are effective against *S. aureus*, *Listeria monocytogenes*, *P. aeruginosa*, and *Salmonella typhimurium* (Mahdhi et al., 2017).

Additionally, other reports have demonstrated that the antibiofilm effect of several bacteriocins (Kim et al., 2009). These bacteriocins exert their actions by mechanisms that are not known yet. It was reported that bacteriocins could eradicate biofilms either by inducing pore-formation on the cell wall, which leads to ATP efflux or by acting as proteolytic enzymes (Okuda et al., 2013). As an example, *Bacillus sonorensis* MT93 strain produces a bacteriocin called sonorensin that was found to reduce the cell viability in *S. aureus* biofilms. Moreover, it has been reported to inhibit the adhesion and formation of biofilm (Chopra et al., 2015).

Another *Bacilli* bacterium, *Bacillus subtilis*, produces subtilisin, a cyclic bacteriocin that targets the surface receptors through its net cationic charge and inhibits the growth and biofilm formation of pathogenic bacteria (Algburi et al., 2015; Turovskiy et al., 2012; van Kuijk et al., 2012). Several *Lactobacilli* can secrete biosurfactants with antimicrobial, antiadhesive effects against pathogenic biofilms (Zakaria Gomaa, 2013). For instance, *Lactobacillus rhamnosus* (*L. rhamnosus*) has been determined to produce biosurfactants that cause cell lysis of pathogenic bacteria by disrupting the membrane or protein conformations (Tan et al., 2017).

C. Influencing gene expression of biofilms in pathogenic bacteria by probiotics

Several research groups have pointed out that probiotics influence the gene expression of virulence factors, cell adhesion proteins, quorum sensing, and biofilm formation in several pathogenic bacteria (Kim et al., 2009; Kiymaci et al., 2018; Tahmourespour et al., 2011; Wasfi et al., 2018). For example, a previous study published that biosurfactants produced by *L. plantarum* and *Pediococcus acidilactici* have a dose-dependent inhibitory effect against *S. aureus* biofilm formation (Yan et al., 2019). The molecular mechanism of these bio-surfactants is thought to be targeting the expression of biofilm-related genes such as *cidA*, *sarA*, *icaA*, *dltB*, *sortaseA*, and *agrA* and the interference of the production of AI-2 (Yan et al., 2019).

D. Fighting pathogenic biofilms by probiotic biofilms

Another advantage of using a probiotic-based approach is that they can form biofilms and compete with pathogenic bacteria biofilms for space and nutrients using different mechanisms (Salas-Jara et al., 2016). If a biofilm by probiotic bacteria is established, the colonization of probiotics in the host mucosa will be enhanced, and their growth will be stimulated, which will lead to preventing the invasion of pathogenic bacteria (Jones & Versalovic, 2009). Moreover, previous studies have revealed that some strains of *Lactobacillus* can form biofilm on non-living surfaces. For example, *L. plantarum*, *L. rhamnosus*, and *Lactobacillus reuteri* (*L. reuteri*) have formed biofilms on polystyrene surfaces (Jones & Versalovic, 2009; Kubota et al., 2008; Kubota et al., 2009; Lebeer et al., 2007), suggesting their potential application to combat medical device-related infections.

There is evidence that biofilms formed by one *S. aureus* strain were eradicated by biofilms formed by *L. brevis* 104/37, *L. plantarum* 118/37, and 6E strains (Wallis et al., 2019). Additionally, it was reported that biofilms of two other strains of *L. plantarum* (WCFS1 and NA7) secreted

molecules with an inhibitory effect against the growth of food pathogens, including *S. aureus* (Aoudia et al., 2016). Lastly, several *Lactobacillus* strains in biofilm status have shown beneficial probiotic properties with strain specificity (Aoudia et al., 2016).

Concluding remarks:

The bacterial biofilm formation has been substantially explored and studied to date. Despite this, severe biofilm-related infections and their resilience to treatments still pose a tremendous challenge in the healthcare settings. Thus, it is crucial to develop models that resemble the host environment to better understand this dangerous process. Additionally, it is also necessary to investigate effective approaches and strategies to inhibit this serious issue. Among investigated methods to prevent biofilms, the use of probiotics and their relationships with the host immune system have been evidenced as a tool to use against the invasion of pathogens and deleterious biofilm formation. The impacts of probiotics on fighting against biofilms are reported through different mechanisms, such as preventing the adhesion, mediating competitive exclusion and counteraction, and downregulation of genes of virulence factors genes in pathogens.

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Chapter 2 Monitoring of Staphylococcal Biofilms in a Microfluidic System under Flow Conditions

2.1 Introduction

Staphylococcus aureus (*S. aureus*) is a ubiquitous Gram-positive pathogen that colonizes the skin and nasal mucosal membranes of 20-30% of the human population (Cheng et al., 2009; Gehanno et al., 2009; Kluytmans et al., 1997). *S. aureus* is held at bay by the protective barriers created by healthy skin, mucosal, and endothelial layers (Chavakis et al., 2005; Pietrocola et al., 2017). The transition to infection often requires damage or trauma to these protective barriers causing a few *S. aureus* cells to infiltrate and attach to basement membrane proteins. Abscesses can quickly develop and lead to life-threatening infections, such as endocarditis and sepsis (Beenken et al., 2004; Fitzpatrick et al., 2005). One clear difference between these types of *S. aureus* infections is the extent of vascular involvement and dynamic flow pressures. For *S. aureus* to cause a disease such as native valve endocarditis (defined as an infection in the absence of an artificial valve), the pathogen must attach to the host vascular surface under constant flow pressures as the microbe passes by the site of damaged or inflamed valvular endothelium. *S. aureus* biofilm initiates with the adhesion of planktonic cells to a surface (Otto, 2013). Then, the surface-adhered cells will proliferate and will switch to the biofilm mode by downregulation or upregulation a set of specific genes, followed by biofilm maturation whereby an organic polymer matrix is incorporated (Otto, 2008).

As a reminder, biofilms are defined as complex communities of cells encased by a matrix, consisted of extracellular polymeric substances (EPS) such as proteins, exopolysaccharides, and extracellular DNA (eDNA), that develops on a surface (Boudarel et al., 2018; Hoiby et al., 2011).

S. aureus can form a biofilm on biotic and synthesized surfaces (Reffuveille et al., 2017). The mature *S. aureus* biofilms consist of thick layers of cells that interconnect through water channels to supply nutrients and remove waste (O'Toole et al., 2000). The sophisticated structure of mature biofilm also enhances the stability of the biofilms and the protection against external stress such as anti-microbial agents (Otto, 2013). As the biofilm develops, some microbial cells will disperse by active or passive mechanisms contributing to the development of bacteremia *in vivo* and embolic seeding of distal sites of infection (O'Toole et al., 2000; Otto, 2008). Because of the resilient nature of the *S. aureus* biofilms couple with incomplete pathogen killing, remnant resistance bacteria emerge. Treatment of the patients with re-occurrent *S. aureus* infection is often challenging and often require removal of the indwelling device and wound debridement (Arciola et al., 2012; Wu et al., 2015). As a result, new adjunctive or preventative measures are needed to combat *S. aureus* biofilms formation. Our current understanding of the kinetics of biofilm development is limited by the lack of models that resemble vascular attachment conditions. It is only through technological advances that we will begin to understand current limitation to and develop effective treatments against biofilm production by *S. aureus*. (Costerton et al., 2007).

One common biofilm assay is the use of crystal violet or other stains in microtiter plates providing a high-throughput approach (Coenye & Nelis, 2010; Stepanovic et al., 2007). However, there is no dynamic flow control and media is not replenished leading to increased cell death and friability of the biofilm (Stepanovic et al., 2007). Other devices such as Robbins and CDC biofilm reactor provide dynamic environments and with limited-range of control over flow conditions (Coenye & Nelis, 2010; Donlan et al., 2004; McBain, 2009; McCoy et al., 1981). These systems tend to use large quantities of reagents and are vulnerable to variations (Coenye & Nelis, 2010; Jass et al., 1995; Parra-Ruiz et al., 2010). In this presented study, we describe use of a microfluidic

device of linear arrangement to directly monitor biofilm formation in real-time under fluidic conditions by light microscopy. This design and approach has been successfully used to study several slower proliferating plant pathogens and has allowed for the visualization of the development and characterization of their respective biofilms (Leonardo De La Fuente et al., 2007). Given the 2 input and 1 outlet layout, this device allows for parallel testing of the impact that a single additive has on biofilm structure and on degradation of well-established biofilms.

Herein, we describe a detailed protocol for the preparation of these sterile chambers, for their use to assess staphylococcal biofilms by light microscopy, and for the processing of the images collected. Our protocol should provide readers details enabling them to monitor the formation of biofilms over time, and strategies to begin to examine factors that may influence the integrity of the biofilm.

2.2 Protocol

2.2.1 Bacterial Cell culture preparation

NOTE: Work in a biological safety cabinet for all bacterial cell culture (opening bacterial stock tubes, culture tubes, and media flasks). Wear appropriate personal protective equipment (PPE) and disposable gloves. Use 70% ethanol to disinfect the surfaces and pipettes before starting the experiment. Use of sterile filter pipettes tips and aseptic techniques are essential.

1. Streak for isolation a stock of *S. aureus* USA300 strain on 15x100 mm blood agar (5% sheep blood in tryptic soy agar (TSA) base) plates. Use a short piece of parafilm to wrap the edges to prevent desiccation of the agar. Incubate at 37 °C overnight.
2. Select a single isolated colony from the streak plate to inoculate into 75-mL of Luria broth (LB) liquid media and incubate at 37 °C with shaking at 220 rpm for 15-16 h.

NOTE: *S. aureus* is β -hemolytic and a noticeable zone of clearance should be present.

3. Dilute the bacterial cell culture in a fresh LB growth media to 1:25 cell culture inoculum, which is equivalent to 5×10^7 cell/mL in a 50-mL centrifuge tube.

NOTE: It is recommended that you do not make the dilution of bacterial cell culture before the microfluidic device is ready to use.

2.2.2 A molded polydimethylsiloxane (PDMS) fabrication

NOTE: Conduct the following steps within a fume hood.

1. Make PDMS mixture with a 1:10 ratio of silicone elastomer curing agent: silicone elastomer base (g:g) using the scale and mix with a wooden tongue depressor in 250 mL container until the mixture becomes white.
2. Place the mixture in the vacuum desiccator to degas for 15-20 minutes. Typically, a low-grade vacuum is sufficient for this purpose.

NOTE: Sealed bottles of PDMS can be stored at -20°C for up to two months.

3. Clean dust and airborne particulates from the silicon wafer brushing across the wafer with short bursts of compressed air and pour a small amount of PDMS mixture that fills the entire mold on the silicon wafer.
4. Return the wafer to the desiccator to degas and maintain it there until all bubbles have disappeared.

NOTE: To speed up the process, release the pressure in the vacuum chamber in 15 minutes increments.

NOTE: it is optional to cover the PDMS on the wafer with the plastic press and make it is centered on the pattern (design). Placement of the cover must occur initially at one side and then travel laterally along the wafer to avoid creating air bubbles. Some bubbles may develop

after placement but there is nothing that can correct those at this point.

5. Polymerize the PDMS on the wafer by placing it in the oven at 80 °C for 1 ½ h, or until the PDMS has solidified on to the wafer.

2.2.3 Plasma bonding of PDMS and glass slide

1. Cut the PDMS from the wafer using a razor blade with being careful not to apply too much pressure to prevent damaging the wafer.
2. Punch out holes on the printed circles on the PDMS using the 2.0 mm biopsy punches.
3. Clean both sides of PDMS by using adhesive tape to remove any dust or residues.

NOTE: you should leave the tape on the PDMS if it is not used in a short time.

4. Use the plasma cleaner to attach the PDMS replica to the glass slide.
 - 1) Place both the glass slide and PDMS (pattern side up) in the back of the plasma cleaner on the same level with the longer sides facing out.
- 2) Turn on the vacuum pump switch for 2 minutes to allow the pressure to build.
 - 3) Turn on the plasma power switch for 1 minute.
 - 4) Turn the radiofrequency (RF) control dial to the “HIGH” setting for 2 minutes.

NOTE: A purple light should be visible through plasma cleaner hatch window.

- 5) When the process is done, first, turn the RF control dial back from high to off, then the plasma power switch and finally, the vacuum pump switch.
 - 6) Slightly open the 3-way valve to let the air get inside the chamber and the door will open.
5. Remove the PDMS from the plasma cleaner by a tweezer and set it down on a plastic mat or clean surface.

6. Remove the glass slide by the tweezer, flip it over, and place it on top of the PDMS **ASAP**.

NOTE: This ensures that both exposed surfaces to the plasma can bind to each other.

7. Apply pressure to ensure a good bond by putting some weight. Test edges with forceps to determine proper attachment.
8. Cut six pieces of silicon tubing 1 cm each, insert them in the preformed holes and test the chamber with sterile dH₂O.

NOTE: This determines if there is a leakage or blockage in the channels. If there is no leakage, you can proceed with the following steps.

9. Put on the top of polymerized PDMS, a supporting microscope slide that is previously bored and seal the bored spaces using unpolymerized PDMS, then put it in the oven for 20-30 min to solidify.

2.2.4 Assembly of microfluidic chip

1. Cut six pieces of extra thin tubing (ETT) 20 cm each and other six pieces of the silicon tubing to connect to female Luer lock fitting (syringe adaptor).
2. Connect the ETT six pieces to the chamber and test the flow by injecting dH₂O throughout the chamber.

NOTE: This determines the functionality of the chamber.

3. If there is no blockage, cover all syringe adaptors with foil and put the whole chamber in a beaker to autoclave it and proceed with the following steps.

2.2.5 Preparation of microfluidic channels with *S. aureus* cell culture

NOTE: Perform all following sub-steps within a biological safety cabinet. Turn on the biosafety at least 30 minutes before the experiment. Wear appropriate personal protective equipment (PPE) and disposable latex gloves. Use 70% ethanol to disinfect the surfaces and pipettes before starting the experiment.

NOTE: The size of the glass syringe is based on the desired volume and flow rate in the experiment.

1. Prepare the dilution of bacterial cell culture as in **step 1.3**.

NOTE: if the experiment includes a supplementation to the growth media, you should prepare the supplemented media before you start the following steps.

2. To prepare the microfluidic chip, you will need the following:
 - 2.1. A sterilized tray.
 - 2.2. Diluted bacterial culture.
 - 2.3. Fresh growth media and, supplemented growth media if it is needed.
 - 2.4. A sterilized microfluidic chamber.
 - 2.5. Two sterilized 5-mL glass syringes for the media
 - 2.6. Four 1-mL plastic syringes (two for the bacterial cell culture, two for infusing the growth media).
 - 2.7. Two plunger-less 3- or 5-mL plastic syringes that will be used to collect waste.
 - 2.8. Transparent adhesive tape.
 - 2.9. Sterilized Iris forceps and cotton balls.
3. Take the microfluidic chamber out of the beaker and tape the short sides of it down on to the tray.

NOTE: This will stabilize the chamber while you infuse/inject the solutions

4. Open the plastic syringes (3- or 5-mL) intended to use for collecting waste fluids and put two cotton balls inside them using the iris forceps.

NOTE: This will prevent any spill of the biological waste. Connect the syringes to the outlet adapters, which are on the side with only two outlets and put aside.

5. Open one plastic 1-mL syringe antiseptically, draw up 1 ml of fresh media, and remove any air bubbles in the syringe.

NOTE: place a thin tissue around the syringe to collect any excess media being pushed out without touching the top of the syringe to avoid contamination.

6. When you have removed all of the air bubbles, push gently to allow a small drop of media to come out and connect it to one of the central inlet adapters, which are on the side with four inlets.

NOTE: this will ensure a continuous infusion as you push gently on the syringe's plunger until the infusion reaches the other end of the channel and goes into the waste-collecting syringes.

7. Once the growth media has been thoroughly infused throughout the chamber, replace the plastic syringe with one of the 5-mL glass syringes filled with 5-mL of fresh growth media, making sure that no air bubbles are present.

8. Repeat steps 5-7 for the other channel of the microfluidic chamber with the desired growth media or bacterial cell culture if it is different.

9. Open another plastic syringe 1 ml to draw up 1 ml of the bacterial cell culture to be injected, making sure there are no air bubbles present. Gently push on the plunger to allow a drop of liquid to appear at the top of the syringe. Connect it to one of the bacteria inlets (lateral inlets).

NOTE: this will ensure a continuous injection and no air bubbles inside the tubing.

10. Repeat step 9 for the other channel with the desired bacterial solution if it is different.

NOTE: At this point, you are ready to set up your experiment under the inverted microscope.

2.2.6 Setting up the microscope for microfluidic chamber

1. Place the microfluidic chamber under the inverted microscope and hang the waste syringes up on the metal holder at the same level as the microscope.
2. Place the inlet syringes (media and bacterial culture syringes) in the syringe pumps that are used to control the flow and tighten them accordingly to stabilize them in the pumps.
3. Adjust the syringe pump setting based on the diameter of the syringe and the rate as it is needed.
NOTE: it is recommended to start a high infusion rate for the bacteria to get enough bacteria in the chamber then slow it down later on.
4. Switch microscope, the shutter, and the camera on and wait until it is warming up.
5. Set the microscope on 40X magnification; Differential Interference contrast (DIC); and Phase 1; and filter No.1 (empty) for brightfield.
6. Adjust the microscope illumination by bringing the field iris diaphragm image inside the field of view.

2.2.7 Setting up image acquisition during microfluidic chamber experiment

1. Start (NIS elements) software and click on the (DIA) button and Brightfield (BF) button and try to find the channels on the screen.
NOTE: it is recommended to be the area, in which bacterial inlet is intersected with the main channel, as a field of view in the beginning.
2. Start the time-lapse acquisition by going to (acquire >> ND acquisition >> interval: 1 minute>> duration: continuous >> name the file and choose where to save it >> click on (run now)

button).

NOTE: it is recommended to save a copy of the data file as AVI, which its size is smaller than nd2 file.

2.2.8 Large image acquisition

1. To acquire large images of the channels at specific times points to analyze them using imageJ, you can go to the drop-menu of “Acquire” >> Grab Large Image, then, a “Large Image grabbing” will be displayed.

NOTE: This tool will stitch the multiple images manually taken by the user.

2. In the “Large Image Grabbing” window, you should click on ‘transparent’ stitching assist and you should use the stage translation control in the microscope to move the field of viewing to align it with the previous taken image and chose a new area to take an image.
3. You repeat step 2 to acquire other images to create the a stitched large image.
4. Once you are done with taking images, you click on “Finish” and the large image will be displayed.
5. You should save the stitched large image as a TIFF or PNG file in order to analyze it using ImageJ software.

NOTE: it is essential to use the transparent stitching assist in the software to have a good aligned large image at the end.

2.2.9 Analyzing the acquired images

1. Open the saved image using ImageJ software by dragging the images on the software window.
2. In ImageJ, crop each channel in the saved large image to separated images (individual

channels) by clicking on the 'Image' tap and click on 'crop' after you selected the desired channel and save it as TIFF file.

3. From the 'Process' tap, click on Sharpen and then click on Find Edges.

NOTE: The contrast of the image can be enhanced by clicking on the 'Process' tap and click on 'Enhance Contrast' and choose the saturated pixels (0.3%) in the shown window.

4. The image is now ready to be converted to a Binary image, by clicking on the 'Process' and then Click on Binary > Make Binary.

NOTE: Make sure to choose 'Black Background' by clicking on the 'Process' and then Click on Binary > Options, in the displayed window, click on 'Black background' option.

5. Now you have a binary image that can be used to quantify the biofilm by clicking on the 'Analyze' tap and click on Analyze Particles, then make sure that you click on 'Display results and Summarize' options and then click OK.

NOTE: The result will be shown in a table labeled "Summary".

6. Locate the value for '%Area' and this represents the covered area of the channel by bacterial biofilms.
7. Repeat step 3-6 to analyze the other cropped image of the other channel.

2.3 Representative Results

In this study, we conducted an *in vitro* biofilm assay using the customized microfluidic chamber to visualize the growing of biofilm by *S. aureus* strain USA300 in Fbg-supplemented LB growth media in the presence of flow over time. Also, the impact of treating established biofilms of *S. aureus* strain with lysostaphin, a lytic agent, was monitored in Luria Broth growth media.

Video 1 shows the growing of biofilm formation of *S. aureus* USA300 strain in Fbg-supplemented LB growth media over time in the presence of flow. At the beginning of the video,

a small *S. aureus* biofilm is attached to the upper edge of the viewing channel. As the time progresses, the *S. aureus* biofilm grows and expands exponentially the end of the video, the *S. aureus* biofilm almost covered the viewing channel. Also, **Figure 2.1** shows this process at the indicated time points from the experiment.

Video 2 shows the treatment effect of supplementing LB growth media with 400 μ L of a mixture that contains (2 mg/ml of lysozyme plus 100 μ g/ml of lysostaphin) on established *S. aureus* biofilms over time. At the beginning of the video, the established biofilms of *S. aureus* started to detach their outer layers with the flowing media. As time progresses, more cells are dispersed, and the size of the biofilm becomes smaller, and ultimately the structure of the biofilm disappeared. At the end of the experiment, only cell residues are visualized in the viewing channel. Also, **Figure 2.2** shows the effect of lysostaphin at the indicated time points from the experiment.

2.4 Discussion

The microfluidic-based biofilm assay presented here enables the real-time visualization of the development of biofilms. Within this system, the flow rate and the temperature can be controlled for the study of biofilm formation in different bacterial strains and for the testing of the inhibitory effect of any given anti-bacterial or anti-biofilm agents treatment under identical conditions and in parallel with a control. Also, this assay provides the researchers with the ability to observe the biofilm development as it grows in real-time.

This microfluidic-based assay can overcome some common disadvantages of other biofilm assays by reducing the reagent consumption and through its parallel chamber linear pathways wherein two samples of the same strain (control vs. treated), (wild-type vs. mutant), or two different strains can be imaged simultaneously under identical experimental conditions. However, one limitation of this assay to study *S. aureus* biofilm formation in these chambers is that the

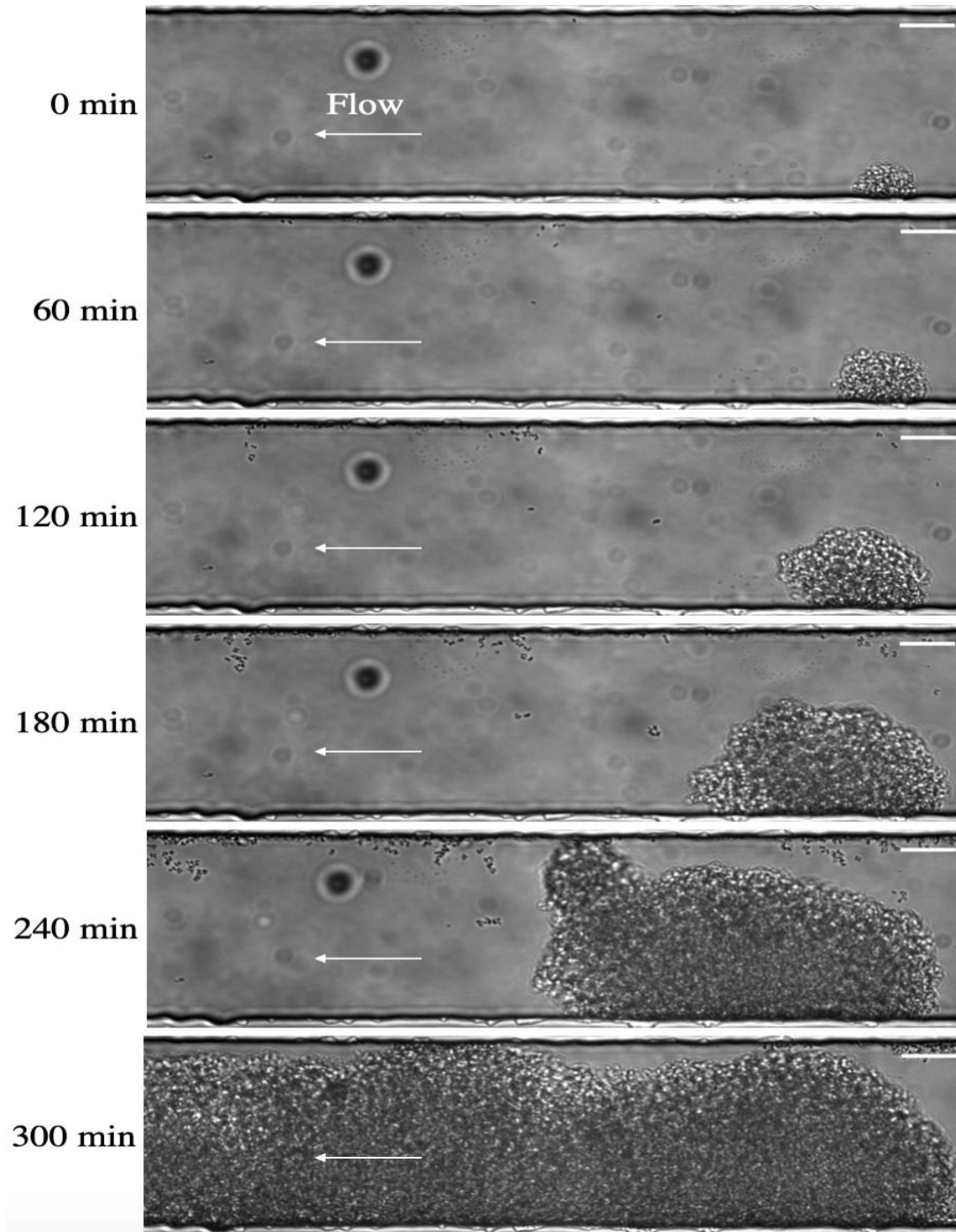


Figure 2.1 The growing of *S. aureus* biofilm under flow. Real time visualization of biofilm formation under constant flow (0.5 $\mu\text{L}/\text{min}$) at ~ 23 $^{\circ}\text{C}$. Representative images are shown at the indicated times for the biofilm formation of MRSA strain USA300. Scale bars are 20 μm in each image. Corresponding time lapse videos of biofilm formation are provided in Video 1.

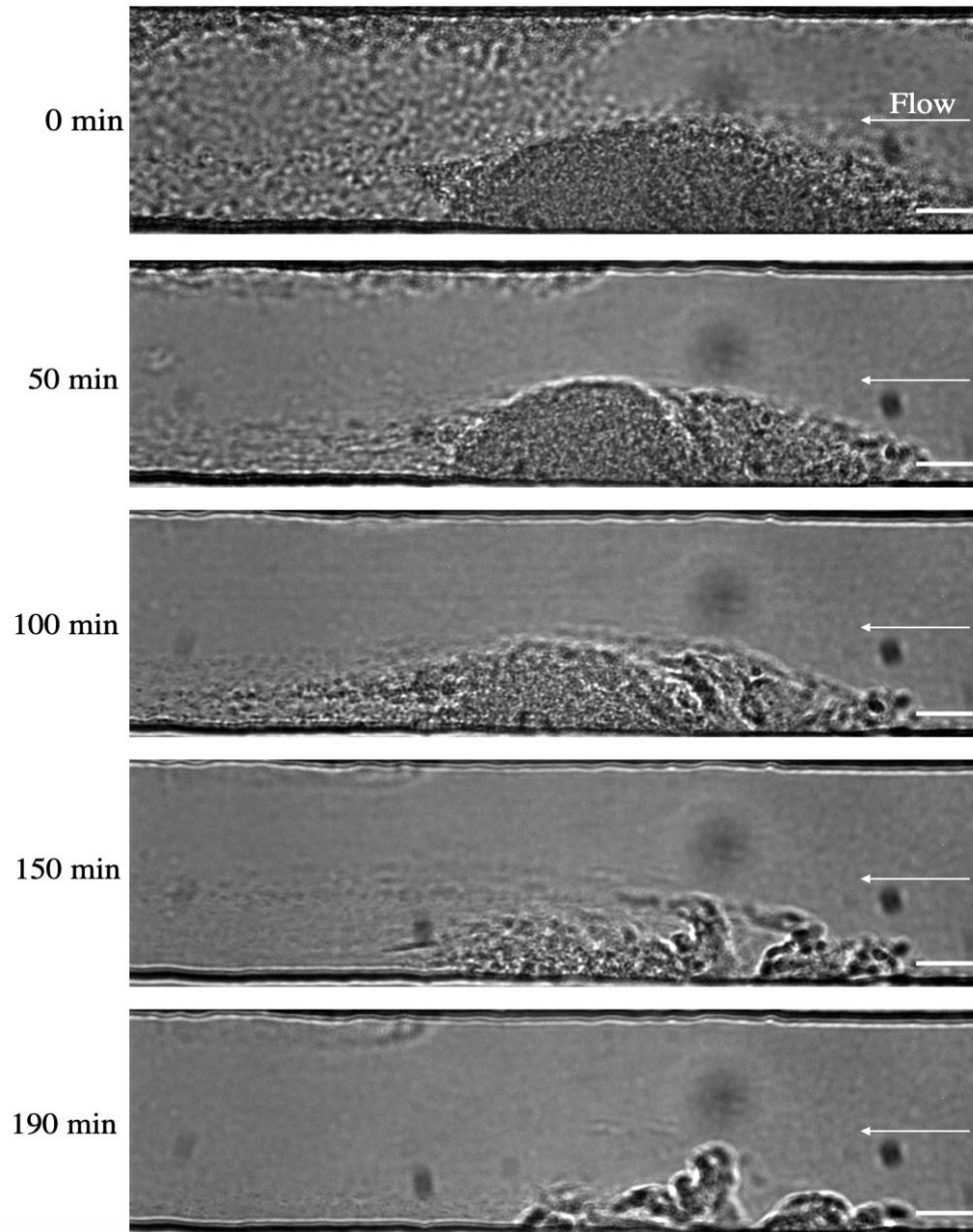


Figure 2.2 Treatment of established biofilms with lysostaphin results in biofilm formation degradation. Real time visualization of degradation of *S. aureus* biofilm in LB growth media that was supplemented with 2 mg/ml of lysozyme plus 100 μ g/ml of lysostaphin under constant flow (0.5 μ L/min) at 23 °C. Representative images are shown at the indicated times for the degradation of established biofilms by *S. aureus* USA300 strain. Scale bars are 20 μ m in each image. Corresponding time lapse videos of biofilm formation are provided in Video 2.

channels may clog causing failure of the chip after ~24 h. Therefore, it is vital to design an experiment to be conducted with 24 h.

Lastly, the described microfluidic-based model is adaptable and flexible and can be changed to evaluate other microbial species, different growth media, flow rates, and incubation times. Additionally, it can be used to assess the biofilm development of mixed-species (two or more), although we only presented the results of single-species biofilm formation. Importantly, this assay provides data of all stages of biofilm formation that include initial adherence, cell proliferation and maturation, and dispersion.

However, few steps are vital in conducting this assay successfully. First of all, it should be known that the plasma bonding step is a time-sensitive process that is required to be performed in a particular time. Otherwise, the sealing of the microfluidic chamber by irreversibly bonding the PDMS and glass slide will not be achieved. Second, air bubbles should be prevented from entering the system since they can damage the adhered cells and formed biofilm. This can be resolved by making sure that the syringes have no air bubbles when they are connected to the microfluidic device. Third, the dilution of bacterial cell culture should take into consideration that the low concentration of bacterial cell culture will result in no cell attachment will be visible, and the high concentration of the bacterial cell culture will clog the channels.

Conclusion:

In summary, we present a microfluidic-based method to study *S. aureus* biofilm formation *in vitro* before conducting *in vivo* studies. Although this model is *in vitro*, it is better than other *in vitro* biofilm models in predicting the results of *in vivo* animal studies since it mimics a more-relevant physiological environment.

Table 2.1 The required materials, equipment, and software for the protocol.

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Blood agar (5% sheep blood in Tryptic Soy Agar (TSA) base) plates 15*100 mm monoplate	VWR	10324-332	Bacterial culture prep
Sterile inoculating loop 1 uL	VWR	89126-870	Bacterial culture prep
Sterile inoculating loop 10 uL	VWR	89126-872	Bacterial culture prep
Parafilm® M sealing film	Heathrow Scientific	HEA234526B	Bacterial culture prep
Luria broth	Research Products inc		Bacterial culture prep
centrifuge tubes 50 mL, Falcon	VWR	734-0448	Bacterial culture prep
Lysostaphin from <i>Staphylococcus staphylolyticus</i>	Sigma-Aldrich	L7386	Bacterial culture prep
Lysozyme from chicken egg white	Sigma-Aldrich	L4919	Bacterial culture prep
Dow SYLGARD™ 184 Silicone Encapsulant Clear 0.5 kg Kit	Ellsworth Adhesives	184 SIL ELAST KIT 0.5KG	Microfluidic prep
Corning® 250 mL Container and Lid	Corning incorporated	430179	Microfluidic prep
Puritan® Wooden Applicator/Stirring Sticks	VWR	10805-018	Microfluidic prep
DESICCATOR W/STOPCOCK 250MM	VWR	24987-004	Microfluidic prep
DESICCATOR PLATE 230MM	VWR	25038-003	Microfluidic prep
2.0 mm biopsy punches Integra Miltex	VWR	21909-132	Microfluidic prep
Scotch Magic Tape , 3/4"	VWR	500026-863	Microfluidic prep
Basic Plasma Cleaner	Harrick Plasma	PDC-32G	Microfluidic prep
Techni-Tool Tweezer, Wafer Handling, 4WF, 4-7/8 in. OAL	Techni-Tool, Inc.	758TW178	Microfluidic prep
glass slides for MC (50*35)	Fisher	12-543-E	Microfluidic prep
Platinum-cured silicone tubing	Cole-parmer	95802-00	Microfluidic prep
tubing for chambers (ETT-24), natural, 1000 ft	Weico	ETT-24	Microfluidic prep
female luer fitting (syringe adaptor)	Nordson	FTL10-6005	Microfluidic prep
5 mL Gastight Syringe Model 1005 TLL, PTFE Luer Lock,	Hamilton	81520	Experiment
10 mL Gastight Syringe Model 1010 TLL, PTFE Luer Lock,	Hamilton	81620	Experiment
1 mL Syringe with BD Luer-Lok™ Tip	VWR	BD-309628	Experiment
5 mL Syringe with BD Luer-Lok™ Tip	VWR	BD309646	Experiment
Nikon inverted microscope (Eclipse Ti)	Nikon	(Eclipse Ti)	Experiment
GenieTouch™ Syringe Pump	Kent Scientific Corporatio	Infusion/Withdrawal Dual Syri	Experiment
NIS-Elements Advanced Research (Software)	Nikon		Experiment
imageJ or Fiji (Software)	NIH	https://imagej.nih.gov/ij/	Experiment

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Chapter 3 Real-time monitoring of the effects of media additives, flow, and potential anti-staphylococcal agents on the *S. aureus* biofilm formation

3.1 Introduction

Infections caused by the Gram-positive bacterium *Staphylococcus aureus* (*S. aureus*) are associated with high mortality and crippling associated healthcare costs (Kyaw et al., 2015). In 2017, nearly 10,600 patients died and 323,700 were hospitalized due to *S. aureus* infections, such as infective endocarditis (IE), osteomyelitis, sepsis, and indwelling medical device infections (CDC, 2019; Gordon & Lowy, 2008). As a consequence, in the United States 2017, about \$1.7B was spent on the management of *S. aureus* infections (CDC, 2019).

S. aureus proliferates on both natural and synthetic materials and often is difficult to control due to the propensity to form a protective layer called a biofilm (Bhattacharya et al., 2015; Otto, 2008). These biofilms are microbial communities encased by an organic polymer matrix, composed of extracellular polymeric substances (EPS), such as polysaccharides, proteins, and DNA (Di Martino, 2018; Hoiby et al., 2011; Sutherland, 2001). Host defenses mechanisms can struggle to eradicate *S. aureus* agglomerations despite their ability to effectively clear their shedded planktonic cells (Costerton et al., 1999; Otto, 2008; Periasamy et al., 2012). Clinically, patients receive long duration, multi-target antibiotic regimens to clear remnant biofilm microbes to prevent infection rebound (Arciola et al., 2012). This process is complicated by the adaptive ability of *S. aureus* to constantly evolve to survive. The best example of this is the formation of small colony variants (SCVs) in Staphylococci for overcoming prolonged vancomycin therapy (Kahl et al., 2016; Mirani et al., 2015). The dynamic and adaptive nature of the staphylococcal communities necessitates increased understanding of factors that are critical to biofilm

development.

One of *S. aureus* pathogenic traits that contribute to develop a biofilm is its ability to form biofilms on damaged host tissues or implantable medical devices, which is mediated by bacteria-host (specific) and bacteria-surface (nonspecific) interactions, respectively (Arciola et al., 2012; Otto, 2013). *S. aureus* is a common part of the normal skin flora in humans and is predominately held at bay by the physical skin barrier and cutaneous defensins and cathelicidin (Hata & Gallo, 2008; Lehrer & Ganz, 1999). Attachment of *S. aureus* in a wound environment is further aided by exposed matrix proteins such as fibronectin, laminin, collagen, vitronectin, and fibrinogen. A class of cell surface proteins on *S. aureus* called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) bind these matrix proteins with a varying degree of affinity and specificity (Otto, 2013; Patti et al., 1994; Patti & Hook, 1994). *S. aureus* attachment to medical devices is mediated by hydrophobic/hydrophilic and electrostatic interactions (Arciola et al., 2012; Periasamy et al., 2012). Bacterial surface autolysins and teichoic acids participate in the medical devices attachment process (Gross et al., 2001; Porayath et al., 2018). Interestingly, devices become covered with host proteins almost immediately after implant (Cheung & Fischetti, 1990; Donlan, 2002; Francois et al., 1996). Therefore, understanding the roles of the most common MSCRAMMs such as clumping factors (ClfA and ClfB) and fibronectin-binding proteins (FnbpA and FnbpB) in the biofilm formation process is crucial (Foster et al., 2014). Dynamic studies under flow conditions that resemble the host environment are needed to recapitulate the stresses and continuous influx of nutrients that the pathogen will encounter (Karimi et al., 2015). The use of microfluidic chambers for the study of staphylococcal biofilm studies is not novel and will likely increase as chambers become easier to fabricate and more cost effective (Persat et al., 2015).

Here, we aim to study the formation of the Staphylococcal biofilms in real-time and

underflow conditions in linear and obstacle-laden microfluidic chambers. Since biofilm development is often a key factor in the development of an infection on a medical device, we sought to initially focus on the ability of *S. aureus* to adhere to native PDMS in a rich carbon source media. As attachment was surprisingly unremarkable, we supplemented the media with human fibrinogen to better mimic the conditions in plasma and to study any fibrinogen-dependent effects that may accelerate or otherwise stabilize the growing staphylococcal biofilm. The surface was not pre-coated with fibrinogen but rather the fibrinogen was added to the secondary media inlet syringe to best simulate medical device insertion. We monitored the biofilm formation in side-by-side microfluidic channels and noted robust biofilm formation and maturation. Using videography and discontinuous assessment of biofilm coverage of the entire linear chambers, we characterized classic shear-force dependent effect on staphylococcal biofilm formation and exopolysaccharide production. Fluorescence microscopy was used to co-localize the elaborated exopolysaccharides with labeled *S. aureus* cells. Obstacle-laden chambers confirmed the formation of biofilm streamers and the presence of exopolysaccharide staining in small clusters of otherwise planktonic cells (Persat et al., 2015). Finally, we employed two novel strategies to combat a mature staphylococcal biofilm. The first treatment was an enzymatic degradation strategy using a mixture of *Staphylococcus simulans* lysostaphin and egg white lysozyme (Schindler & Schuardt, 1964) and the second was a probiotic strategy using a novel bacilli strain, called *Bacillus velezensis* AP183 (*B. velezensis* AP183) (Ravu et al., 2015). Our studies provide a pathway for biofilm studies probing the necessary and sufficient nature of host proteins, characterization of biofilm components in real-time, and novel pre-clinical strategies to combat biofilm integrity.

3.2 Results

3.2.1 Microfluidic chamber adaption for monitoring *Staphylococcus aureus* biofilm formation

Our microfluidic chamber shown in **Figure 3.1** features a dual channel linear design. The channel is optically clear and was visualized by perfusing the chamber with a crystal violet solution (0.3 %w/v). Using this device, positive staphylococcal biofilms were present using a 4 % of overnight culture inoculum, which is equivalent to 5×10^7 and 1.2×10^8 CFU/ mL for MRSA strain USA300 and MSSA strain Tager 104, respectively. To prepare the inoculums bacterial cells overnight cultures of the pathogen were grown in brain-heart media (RPI) and diluted to the indicated percentage with sterile room-temperature media.

The 4 % inoculum aggregated within 8-10 h post-inoculation (pi), at the bacterial inlet of the microchannel. The aggregated cells filled the entire channel by 24-36 h pi. The cell aggregation initiated at the bacterial inlet (lateral) and continued to form abundant biofilms until reaching the other side of microchannels. Some of the adhered biofilms were cleared and followed by a new cell aggregation. This finding led us to use the 4 % inoculum in the subsequent experiments.

3.2.2 Fibrinogen-supplemented media promotes *S. aureus* biofilm formation

Supplementation of nutrient-rich media with extracellular matrix proteins such as fibrinogen (Fbg) was done to mimic the plasma / blood environment. At 6 h pi, the formed biofilms in both the Fbg-supplemented media and control media channels were not statistically significant (**Figure 3.2A**). However, this effect was statistically significant at 12 and 24 h pi, when coverage areas in the Fbg-supplemented media channel were 11% and 14%, respectively. On the other hand, the biofilm formed in the control growth media channel was <5% at both 12 h and 24 h pi (**Figure 3.2B**).

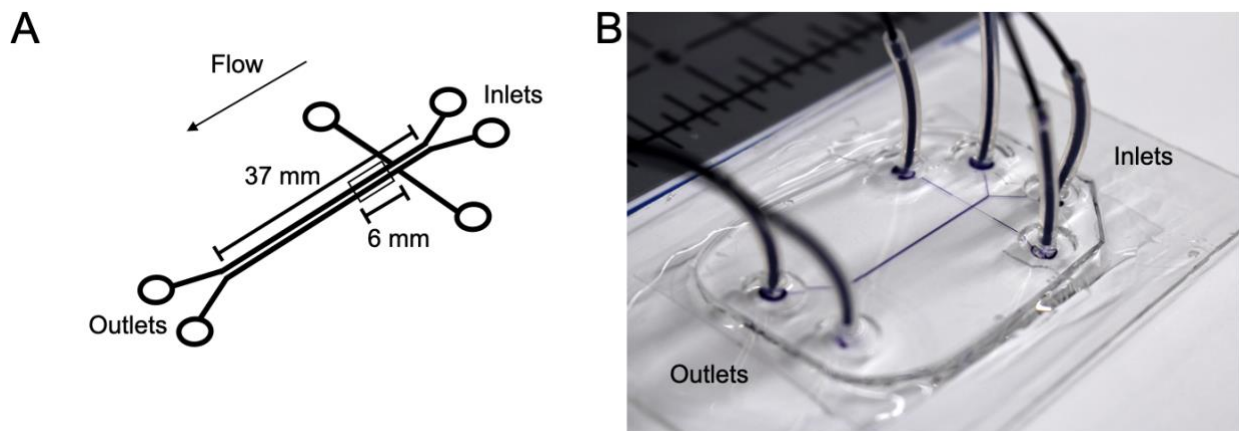


Figure 3.1 Lab-developed microfluidic chamber. (A) A schematic representation of the microfluidic channels with indicated dimensions and designed similar to (Leonardo De La Fuente et al., 2007), and the image window is indicated in relation to the entire chamber. (B) A photograph of our microfluidic device perfused with crystal violet (0.3% w/v) to allow for channel visualization.

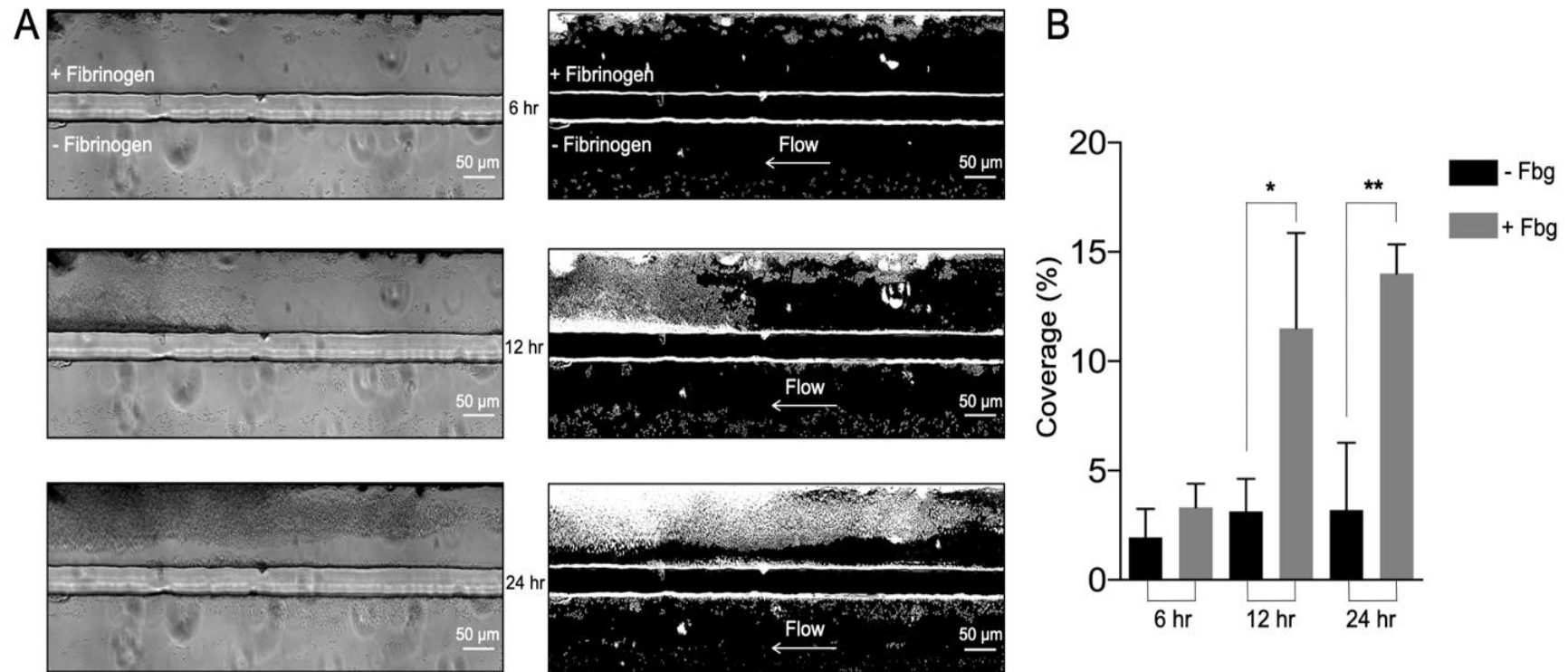


Figure 3.2 Effect of fibrinogen on biofilm formation by *S. aureus* in microfluidic channels. (A) A segment of the bright-field (*left*) and binary image (*right*) taken from the microfluidic channels. Biofilm coverage was monitored along the entire chamber (6mm) at indicated times. (B) Quantification of biofilm coverage taken from a stitched binary image of the indicated channels with and without fibrinogen. The flow rate was 0.5 $\mu\text{L}/\text{min}$. The values from three replicate from independent studies (*:**P value <0.05**, **:**P value <0.01**, ***: **P value < 0.001** ****: **P value < 0.0001**).

3.2.3 Pressure effects *S. aureus* biofilm formation

Laminar flow and pressure alterations were evaluated at flow rates of 0.5, 1.0, 5.0, and 10 $\mu\text{L}/\text{min}$ subsequently exerting chamber pressures of 6.1, 12, 61, and 122 mbars, respectively. The corresponding pressure, resistance, Reynolds number, wall shear rate, flow velocity, and flow regime at each flow rate in the microchannels are listed in **Table 3.1**. The characteristics of the biofilms at each flow rate were interestingly distinct, in terms of coverage and morphology. **Figure 3.3A** shows that the coverage area of formed biofilm at 6.1 mbar was the largest at all time points with a flat shape along the flow direction. At 12.2 mbar, the morphology of the formed biofilm was a circular shape, specifically at 24 h pi, and has a less coverage area compared to 6.1 mbar. However, there was no clear biofilm structure at 61 mbar at 6 h and 12 h pi, but there were few small circular formed biofilms at 24 h pi. At 122 mbar, there few bacterial cells attached to the surface and no clear biofilm structure in the channels throughout the entire experiment.

3.2.4 EPS production in *S. aureus* biofilm can be visualized in real-time under flow

One of the critical components of bacterial biofilm is exopolysaccharides in ECM, such as α -polysaccharides and β -polysaccharides. The production of β -polysaccharides by *S. aureus* (NE1260R) was evaluated in the microfluidic channels, as mentioned in **Figure 1.1**, by the staining of calcofluor white (CFW) fluorochrome. **Figure 3.4A** shows that the aggregated red-labeled-NE1260R bacterial cells produced CFW-stained β -polysaccharides to form a biofilm structure. Moreover, the behavior of the biofilm formation was studied using a new design of microfluidic that has obstacles in the middle of the microchannel, as displayed in **Figure 3.4B**. It was noticed that the bacterial cells aggregated more in the areas that have no flow, which are located between obstacles. After the CFW staining, the CFW-stained exopolysaccharides were colocalized with the outer layers of the biofilms formed in the areas that are under flow, which surround the inner layers

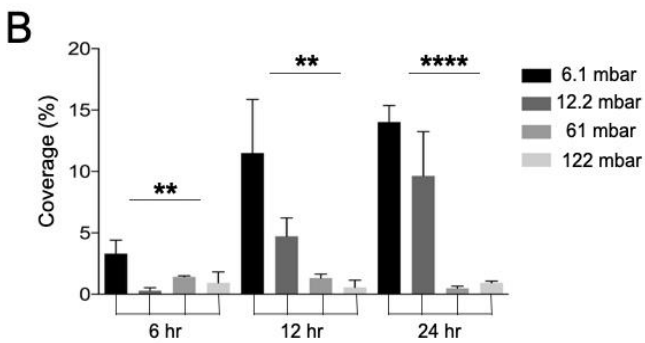
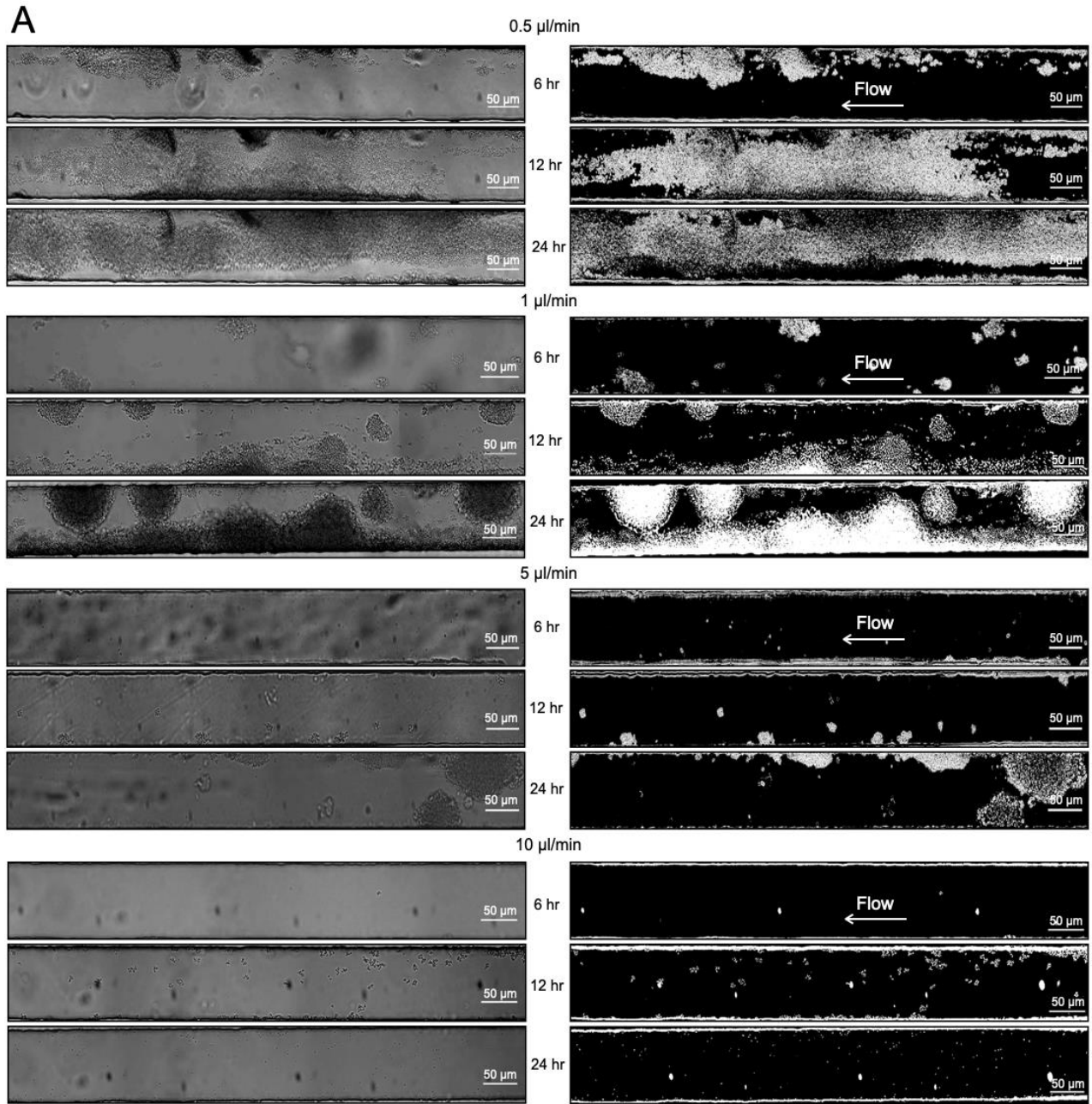


Figure 3.3 Effect of laminar flow rate (pressure) on *S. aureus* biofilm formation (A) Segments

of the bright-field (*left*) and binary (*right*) images taken from the microfluidic channels in which the flow of BHI growth media was set at three different rates: 0.5, 1.0, 5.0, and 10 $\mu\text{L}/\text{min}$. Biofilm coverage was monitored within the indicated image window (6mm) at indicated times. (B) Quantification of biofilm coverage taken from stitched binary images of the microfluidic channels at the indicated flow rates. The values from three technical replicates from the same experiments (*:P value <0.05, **:P value <0.01, ***: P value < 0.001 ****: P value < 0.0001).

Table 3.1 The corresponding characteristics of the tested flow rates.

Flow rate ($\mu\text{L}/\text{min}$)	Pressure (mbar)	Resistance ($\text{mbar}\cdot\text{min}/\mu\text{L}$)	Reynolds number (Re)	Wall Shear stress (dyne/cm^2)	Velocity (mm/s)	Flow regime
0.5	6.1	122.0	0.1	2.5	2.1	(Laminar Flow)
1	12.2	122.0	0.3	5	4.2	(Laminar Flow)
5	61.0	122.0	1.3	25	20.8	(Laminar Flow)
10	122.0	122.0	2.6	50	41.7	(Laminar Flow)

These values were calculated by online tool: <https://darwin-microfluidics.com/blogs/tools/microfluidic-flowrate-and-shear-stress-calculator>.

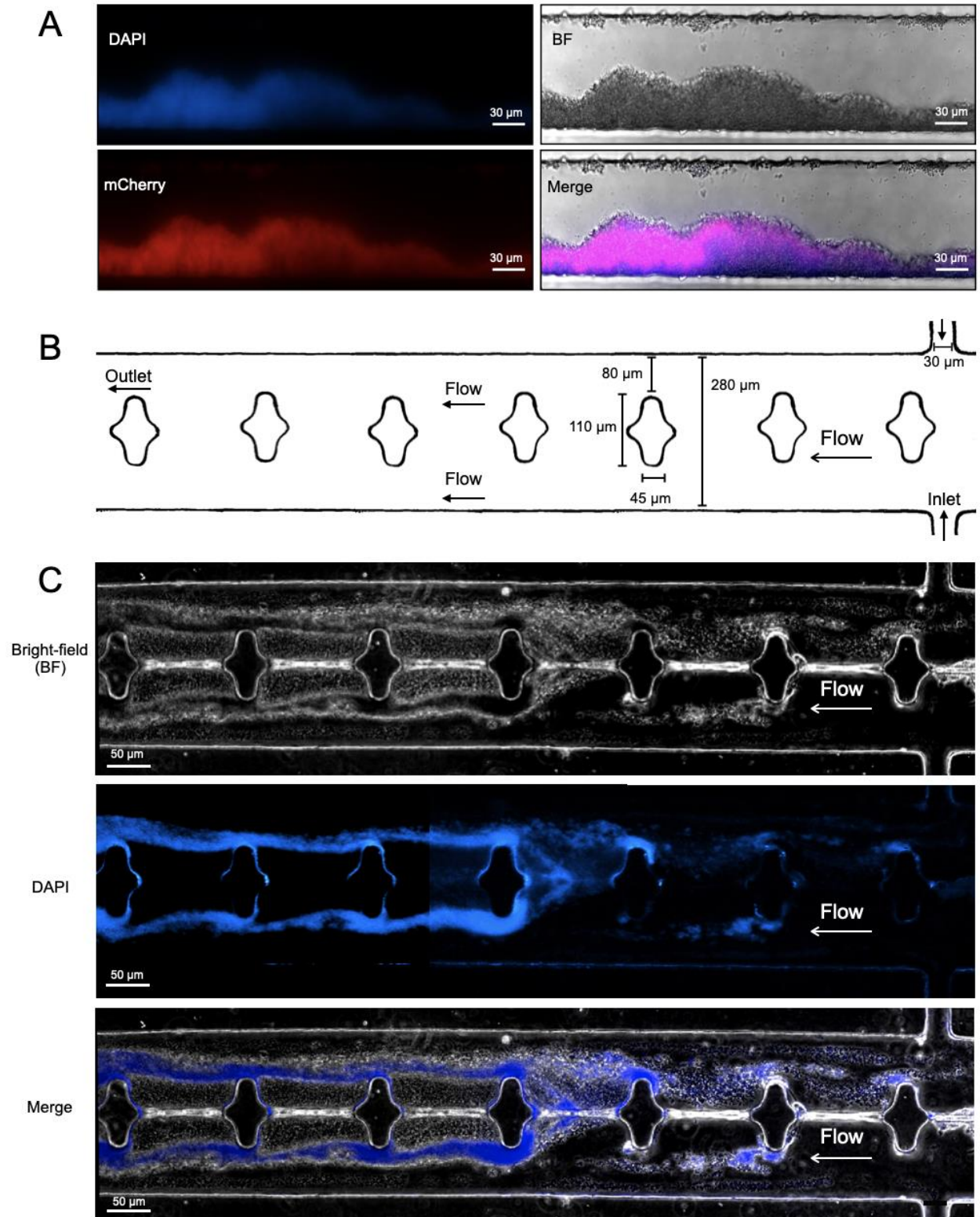


Figure 3.4 Exopolysaccharides-formed monitored in linear and obstacle laden chambers. (A)

Bright-field (BF) image of calcofluor white (CFW)-stained biofilm formed by a fluorescent-

labeled strain of *S. aureus* (USA300-R) in the microfluidic channel shown in **Fig 3.1**, a fluorescent image of the polysaccharides-formed matrix, a fluorescent image of fluorescent-labeled USA300-R cells and a merged image of BF and fluorescent images. (B) Schematic representation of the microfluidic channels with indicated dimensions and designed with obstacles. (C) Bright-field (BF) image of calcofluor white (CFW)-stained biofilm formed by a wild-type strain of *S. aureus* (Tager 104) in a microfluidic channel with obstacles, a fluorescent image of the exopolysaccharides-formed matrix, and a merged image of BF and fluorescent images.

of biofilms in between the obstacles, as shown in **Figure 3.4C**. The fluorescent red-labeled *S. aureus* strain was grown under two different growth media: in the presence of glucose, such as brain heart infusion (BHI) growth media, and in the absence of glucose, such as Luria broth (LB) growth media to evaluate the effect of glucose on the expression of the produced fluorescent signal by labeled strain, as shown in **Figure 3.5**.

3.2.5 Enzymatic and probiotic disruption of *S. aureus* biofilm

The ability of the lysostaphin enzyme to disrupt mature *S. aureus* biofilms (USA300 strain) was assessed in the microfluidic platform. *S. aureus* biofilm of red-labeled-NE1260R was allowed to grow for 24 hr in Fbg-supplemented LB cultural media under continuous-flow. The mature surface-attached biofilms were treated with 150 μ L of a mixture of 2 mg/ml of lysozyme plus 100 μ g/mL of lysostaphin that was mixed with 3 ml of fresh LB media, as shown in **Figure 3.6A**. At 24 hr after lysostaphin inoculation, *S. aureus* biofilm formation was reduced (disrupted) in ~80% in the microchannel at 0 hr (**Figure 3.6B**) in comparison to the untreated channel.

Furthermore, the anti-staphylococcal activity of *B. velezensis* AP183 was evaluated against the *S. aureus* Tager 104 strain under static and dynamic growth conditions. Under the static growth condition, *B. velezensis* AP183 was co-cultured with Tager 104 on TSA agar plates that were incubated overnight at three different temperatures: 25 °C, 30 °C, and 37 °C. **Figure 3.6C** shows that growth inhibition by *B. velezensis* AP183 was clearly seen against Tager 104 at 25 °C, but the zone of clearance was not consistent at 30 °C, and could not be detected at 37 °C (data not shown). The anti-staphylococcal activity of *B. velezensis* AP183 was assessed against mature Tager 104 biofilms that were grown under continuous flow in the microfluidic channels. As shown in **Figure 3.6D**, *B. velezensis* AP183 was bound to several preformed Tager 104 biofilm and formed *B. velezensis* AP183 networks by an unknown mechanism. This binding activity of *B. velezensis*

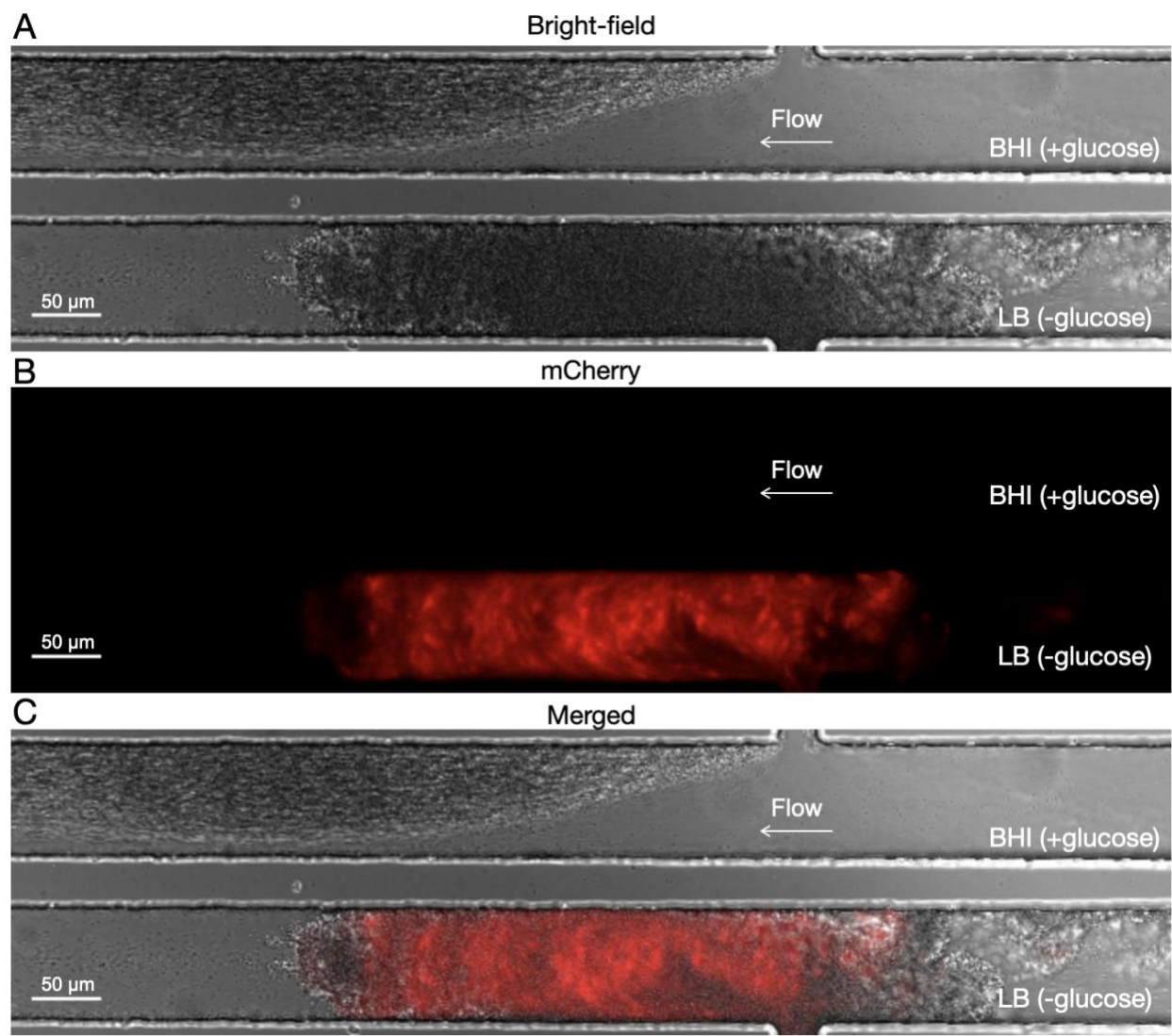


Figure 3.5 The effect of the glucose presence in growth media on the expression of the fluorescent-labeled strain of *S. aureus* (MRSA USA300-R (Bose et al., 2013)) in our microfluidic device. (A) Bright-field (BF) image of microfluidic channels in which the fluorescent-labeled strain of *S. aureus* (USA300-R) grown in BHI growth media (upper channel) and in LB growth media (lower channel) (B) A merged image of microfluidic channels in which the fluorescent-labeled strain of *S. aureus* (USA300-R) grown in BHI growth media (upper channel) and in LB growth media (lower channel).

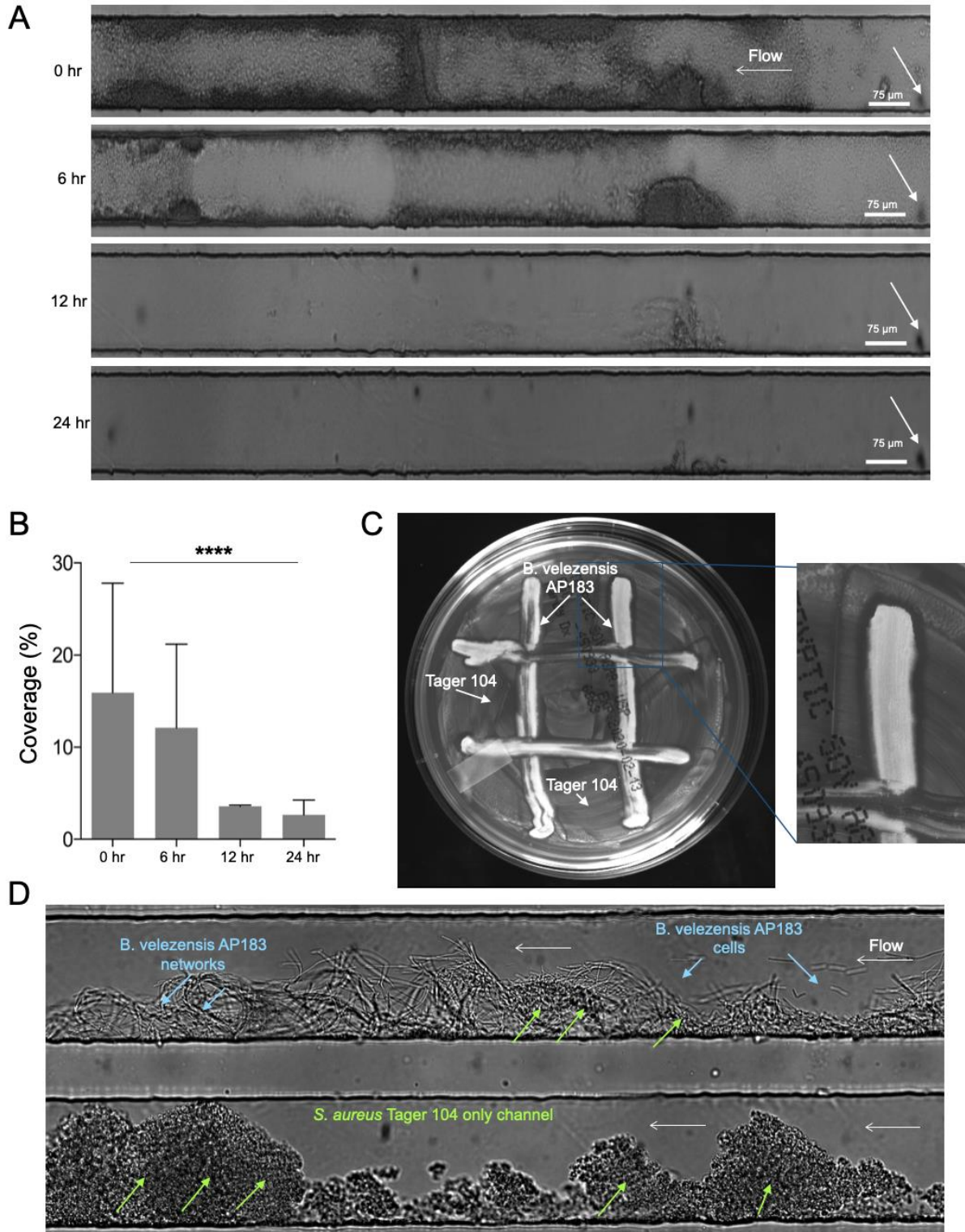


Figure 3.6 Effect of lysostaphin and *B. velezensis* AP183 treatment on biofilm formation. (A) Segments of the bright-field (*left*) taken from the microfluidic channels in which LB growth media

was supplemented with lysostaphin. Biofilm coverage was monitored within the indicated image window (20 mm) at indicated times. (B) Quantification of biofilm coverage taken from stitched binary images of the microfluidic channels at the indicated time points. (C) The zone of clearance of *B. velezensis* AP183 grown on TSA agar plate with *S. aureus* lawn (static condition) grown at room temperature (23 °C). (D) Segments of the bright-field (*left*) taken from the microfluidic channel in which Tager 104 mature biofilms were supplemented with *B. velezensis* AP183. The values from two replicate from independent experiment (*:P value <0.05, **:P value <0.01, ***: P value < 0.001 ****: P value < 0.0001).

AP183 to Tager 104 reduced the coverage of Tager 104 biofilm within the microchannel (upper channel) compared to the coverage of Tager 104 within the microchannel without treatment (lower channel).

3.3 Discussion

Biofilm formation by *S. aureus* is not an uncommon bacterial trait (Neopane et al., 2018). Yet, this ability is complicated by the multipronged arsenal of virulence factors elaborated by *S. aureus* during an infection. The production of exopolysaccharides as the key biofilm component must occur in synergy with both the “catch bond” mediated by clumping factor A and staphylocoagulase-mediated conversion of fibrinogen to insoluble fibrin. Quorum sensing is likely the mediator of this coordinated attack. We previously studied the role of staphylocoagulase at both the molecular and organismal levels providing evidence for its unique mechanism of prothrombin activation and its contribution to staphylococcal pathogenicity during acute bacterial endocarditis (Friedrich et al., 2003; Panizzi, Friedrich, Fuentes-Prior, Kroh, et al., 2006; Panizzi, Friedrich, Fuentes-Prior, Richter, et al., 2006; Panizzi et al., 2011). When it comes to biofilms formation, the production of insoluble fibrin would seemingly be advantageous, leading to improved biofilm stability and resistance to external flow pressures. We sought here to separate the multi-faceted fibrinogen dependence of *S. aureus* to study the contributory function of fibrinogen itself to the biofilm process. To separate the fibrinogen dependent reactions, we used microfluidics wherein we could have dynamic control over chamber flow rates and visualize in real-time the effects that supplements had on overall biofilm maturation.

Prior application of microfluidics toward understanding biofilms have focused on characterizing the quorum sensing systems, the identification of specific matrix components (Fagerlund et al., 2016), or the development of strategies to combat their formation (Gutierrez et

al., 2016). Microtiter plate-based systems that are generally referred to as “closed” systems where biofilms form on the sides and bottom of the plates (Coenye et al., 2007). Visualization is classically achieved through crystal violet staining (Shakeri et al., 2007). The lack of continuous influx of key nutrients causes carbon source depletion and cell debris accumulation (Merritt et al., 2005). So-called “open” or circulating systems provide continuous media turnover and replenishment of these key nutrients. These systems better parallel the *in vivo* situation (Busscher & van der Mei, 2006). Microfluidic-based models are open systems that allow for real-time monitoring of biofilm development (Franklin et al., 2015). In addition, here we have a design that enables the direct comparison of two identically treated samples in a side-by-side manner and under the identical growth conditions (Kim et al., 2012; Lee et al., 2008). Previously microfluidic devices of similar design have been used to study biofilm formation induced by the slower proliferating Gram-negative plant and marine pathogens, *Xylella fastidiosa* and *Flavobacterium columnare*, respectively (L. De La Fuente et al., 2007; Declercq et al., 2019). The design of the microfluidic channels is shown in **(Figure 3.1)**.

Since the initial ‘catch bond’ of *S. aureus* to the damaged endothelium or newly implanted medical devices is critical to establishing an infection (Kwiecinski et al., 2019), we tested initially the ability *S. aureus* to adhere to the naive surface of the microfluidic device. It was evident that both prototypical MRSA and MSSA clinical strains tested (USA300 and Tager 104) did adhere to the device surface but overall attachment was inconsistent. To improve the agglomeration of cells, we supplemented with human fibrinogen to both provide the natural ligand for the bacteria and to determine whether the presence of fibrinogen had an effect on biofilm-genesis. As mentioned, there are multiple *S. aureus* interactions with human fibrinogen that must be considered. Aside from clumping factor A-mediated cell attachment, the presence of

staphylocoagulase as a critical secreted factor for vegetation development during endocarditis. This is owing to the non-proteolytic activation of prothrombin by staphylocoagulase and cleavage of human fibrinogen by the staphylocoagulase-prothrombin complex to insoluble fibrin. Since in our device there will be no prothrombin available, this eliminates insoluble fibrin production allowing cells to flow more freely until attached. Conversely, interactions that clumping factor A-dependent would be engaged and enhanced bacterial aggregation and potentially biofilm development. Our results indicated that biofilm formed in a more uniform manner and with apparent better consistency as assessed by *S. aureus* coverage as quantified by digitally stitching images along the entirety of the channels. Results were compared to the control channel and shown in **Figure 3.2A**.

It is known that *in vivo* biofilm-related infections are subjected to different flow rates and shear stresses of organic fluids such as blood (Isberg & Barnes, 2002; Rupp et al., 2005). Several dynamic systems with large volumes capacity and high shear stresses have been used to evaluate the effect of flow rates on the process of biofilm formation (Cowle et al., 2019; Liu et al., 2017; Ravu et al., 2015; Thomen et al., 2017). Some researchers have published that the biofilm structures were deformed and the biofilm morphology was strongly affected by the flow around biofilm (Liu & Tay, 2002). Shear stress disrupts the availability of the secreted molecules, which lead to limiting growth and decreasing the overall biomass (Kostenko et al., 2010; Liu & Tay, 2002). It was also reported that high flow conditions, such as the flow rate used this study (4.3 ml/min), generated high shear stress that break biofilms and detach bacterial cells from the biofilm community (Purevdorj et al., 2002; Stoodley et al., 1999). In our present study, we have grown *S. aureus* Tager 104 strain in under four flow rates of the growth media: 0.5, 1.0, 5.0, and 10 $\mu\text{L}/\text{min}$. These flow rates, taking into consideration the channel dimensions, are characterized as laminar

flows and exert different pressures (shear stresses) on the attached bacterial cells. We found that the higher the pressure (shear stress), the less adhered bacterial cells and the less formed biofilm within the channels, as shown in **Figure 3.3A**. It was statistically significant that the pressure of 6.1 mbar is the pressure, under which the highest numbers of biofilms have been grown over 24 hours in the presence of Fbg.

Among other biofilm components, the self-produced matrix of biofilm is a crucial component of the bacterial biofilm architecture (Costerton et al., 1987). It consists of extracellular polymeric substances (EPS) such as proteins, extracellular DNA, and polysaccharides, which are varying among the bacterial species (Flemming, 2016). However, it is known that polysaccharides are key components of many bacterial biofilm matrix such as *Pseudomonas aeruginosa*, *Salmonella*, *Escherichia coli*, and *S. aureus*. These bacteria produce polysaccharides to help them in water retention, enzymes binding, and the adherence to the surfaces, or host cells and to overcome several environmental challenges, including the killing by host cells, the onslaught of antimicrobials, competition of other microbes (Flemming, 2016). Moreover, their presence provides access to nutrients and aid in creating specific architectures that are preferable environments for developing persistence phenotype in microbes (Limoli et al., 2015). In this study, we have proven that two *S. aureus* strains (MSSA Tager 104 and MRSA USA300) produce polysaccharides under flow conditions in the microfluidic-based model, as shown in **Figure 3.4**. The polysaccharides were stained with CFW, which is an inexpensive and non-specific dye polysaccharide that has been used in previous studies to stain β -polysaccharides in several bacterial and fungal biofilms (Rodriguez-Lazaro et al., 2018). In our findings under a fluorescence microscope (**Figure 3.4A**), the CFW-stained polysaccharides (DAPI) were visualized and co-localized (a.k.a merged) with the 24 hr-grown biofilms of labeled USA300 strain using the

mCherry filter set. This result verified that the aggregated cells of bacteria are growing in the biofilm mode of growth. Our approach is not the optimal method since it does not distinguish between the live and dead cells population. However, the utilized approach could be improved by using Live/Dead™ BacLight™ bacterial viability kit, which will stain the bacterial cells regarding the integrity of the cell but given the spectral overlap between the filters this was not made a priority (Robertson et al., 2019).

Previous studies have shown that the flow effect on biofilm structures revealed in geometries with corners and bends, and that most bacterial species formed a long, filamentous structure known as a streamer (Rusconi et al., 2010). In this study, a different design of microfluidic channels was used to study the behavior of the biofilm formation of *S. aureus*, as shown in **Figure 3.4B**. This microfluidic layout has a wider channel of 280 μm . In the middle of the channel, there were aligned obstacles posts with dimensions of 110 μm by 45 μm . The presence of obstacles creates pockets with reduced laminar flow and decreased pressure. These areas were settled quickly by the microbe and developed into dense pockets of bacteria and biofilm (**Figure 3.1**). The outer layers of the biofilms bound CFW dye and presented as long and filamentous structures and appeared to be similar in nature to bacterial biofilm streamers reported by (Persat et al., 2015). These streamer-like biofilms were adhered to the side edges of the obstacles, forming a longer layer of biofilms that are connecting by matrix and covering unstained embedded biofilms in the areas between the obstacles. This could be either due to that the CFW dye could not penetrate the outer layer to stain the inner layers of biofilm or due to that the expression of polysaccharides genes are upregulated under the flow, which was found in the outer layer of formed biofilms. In contrast, the inner layers are rich with bacterial cells and other matrix components.

Since biofilm-related infections are not easily treated by conventional anti-microbials

because of the resistance, a therapeutic option with an antimicrobial effect against *S. aureus* biofilm and its extracellular matrix will be a potential candidate to treat staphylococcal biofilm-related infections (Casey et al., 2007). Therefore, we tested a lytic strategy that used lysostaphin enzyme, an enzyme is routinely used for genomic DNA extraction from Gram-positive pathogens due to its ability to disrupt the peptidoglycan layers (Kumar, 2008; Zygmunt et al., 1968). Over the last few years, lysostaphin is regaining interest as an anti-staphylococcal agent that was neglected since the 1970s due to the emergence of anti-microbial-resistant *S. aureus* strains (Harrison & Zygmunt, 1967; Schaffner et al., 1967). Increased popularity is driven by its selectivity, stability, and production cost (Zygmunt & Tavormina, 1972). Previous studies have evaluated the staphylolytic activity of lysostaphin against several clinical strains of *S. aureus* (Climo et al., 1998). Most of the tested strains were susceptible to lysostaphin with rapid elimination of *S. aureus* (Kokai-Kun et al., 2007). Based on our knowledge, none of these studies were performed under continuous-flow conditions environments. In this present study, we tested the anti-staphylococcal activity of lysostaphin against MRSA *S. aureus* strain (USA300) under continuous-flow conditions using the microfluidic-based model. The lysostaphin lysed 24 hr mature MRSA biofilms as assessed by increased cell sluffing and decomposition of the biofilm over time mature. It should be further noted no new bacterial attachment or biofilm was evident over 12 hr following infusion. This enzymatic approach may be an interesting adjunctive therapy to improve antibiotic penetrance, but it is likely too aggressive to be clinically viable at least in the context of mature vegetations in endocarditis as stroke and septic emboli would increase mortality of the patients.

We also investigated the use of a newly identified antibiotic producing soil microbe to combat a mature staphylococcal biofilm. Previous studies have shown that this soil-derived

Bacillus velezensis AP183 has anti-bacterial activity against *S. aureus* among other pathogens (Nasrin et al., 2015; Ravu et al., 2015). *B. velezensis* AP183 produces an antibiotic called Bacillusin A but other tests of its efficacy against have not be reported. Here, we used our *in vitro* microfluidic-based model to evaluate the anti-staphylococcal activity of *B. velezensis* AP183 against MRSA and MSSA clinical strains (USA300 and Tager 104) of *S. aureus*. Under the flow conditions in microfluidic device, *B. velezensis* AP183 was found to bind to preformed *S. aureus* biofilms and disturbed their biofilm structures through three possible mechanisms in order to inhibit the formation of new *S. aureus* biofilms. These possible inhibition mechanisms could be explained by the following: (1) the competition for space and nutrients; (2) the production of Bacillusin by *B. velezensis* AP183, which is a molecule with anti-staphylococcal activity; and (3) cell-cell interaction between *B. velezensis* AP183 and *S. aureus*. This finding was consistent with the result of co-culturing *S. aureus* and *B. velezensis* AP183 on agar plates, which are considered static *in vitro* conditions. Overall, since avoiding the resistance acquisition of the antimicrobial was a priority in our study, the therapeutic options that have been utilized were not classical antimicrobial agents, and their resistance is uncommon or not reported. These results will improve our understanding of this biofilm-related infection and how it can be impaired or prevented.

3.4 Materials and Methods

3.4.1 Bacterial strains and culture conditions

Two *Staphylococcus aureus* (*S. aureus*) strains and one *Bacillus velezensis* AP183 (*B. velezensis* AP183) were used in this present study (Nasrin et al., 2015; Ravu et al., 2015). For media supplementation, flow rate, biofilm formation, and biofilm degradation experiments, we used *S. aureus* Tager 104 strain (Tager 104), which was originally isolated at the New Haven Hospital (New Haven, Connecticut) by Morris Tager *et al.* in 1947 from a patient with a cutaneous

infection. We previously published the closed genome of Tager 104 (Davis et al., 2016). The other strain was a community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA), USA300 strain with a red fluorescent reporter called (NE1260R), which was used in the study of staphylococcal biofilm components experiments (Bose et al., 2013). Tager 104 and NE1260R strains were stored as glycerol stocks in 2X brain heart infusion (BHI) broth or tryptic soy broth (TSB) (Research Products International, Mount Prospect, IL) supplemented with 50% glycerol and kept at -80°C until needed. *S. aureus* was either recovered on sheep blood agar or tryptic soy agar (TSA) plates (Hardy Diagnostics, Santa Maria, CA) before being grown in liquid media under constant shaking at 37 °C for microfluidic chambers inoculation.

3.4.2 Microfluidic chambers fabrication

Microfluidic chambers were made from a silicon wafer master on which channels pattern was designed using photolithography and deep reactive-ion etching followed by replica molding of the wafer surface features with polydimethylsiloxane (PDMS) (Sylgard 184; Dow Corning, Midland, MI) (Xia & Whitesides, 1998). For preparing the microfluidic devices, PDMS was poured over the wafer master and heated at 80°C for 90 minutes (min). PDMS was released from the wafer master, and holes were created at the ends of patterned channels and filled with supply tubing. The cleaned PDMS replica and a glass coverslip (rectangular, no. 12-543E, 50 mm by 35 mm; Fisher Scientific, Bartlesville, OK) were exposed to air plasma at 30 W for about 2 min to facilitate permanent covalent bonding before the assembly of the chamber (Chaudhury & Whitesides, 1991). 51 mm by 76 mm microscope slide previously bored with circular openings was sealed to the underside of the chamber to provide additional stability to the flexible chamber through aligning the holes with outlet and inlet positions. Each hole of the PDMS replica was fitted by silicon rubber tubing (5.1 mm outside diameter, 2.1 mm inside diameter, 0.8 mm wall; Cole-

Parmer, Vernon Hills, IL), and then sealed with unpolymerized PDMS, cured for 20 min. Plastic female luer fitting [syringe adaptor] (Nordson Medical, Loveland, CO) were connected to the extra thin tubing (Weico Wire & Cable Inc., Brentwood, NY) through the barbed ends that were connected to three different types of syringes. Two 5 ml plastic syringes (Becton, Dickinson and Company, Franklin Lakes, NJ) for waste, two 1 ml syringes (Becton, Dickinson and company) for bacterial cells suspension, and two 5 ml gastight syringes (Hamilton Company, Reno, NV) for the growth media (L. De La Fuente et al., 2007).

3.4.3 Microfluidic flow and bacterial biofilm formation

One colony of the studied bacterial strain was scraped from a 1-2 weeks old blood agar plate and inoculated in BHI broth overnight. The overnight culture was diluted to different bacterial concentrations, including 1/10 v/v and 1/25 v/v in fresh BHI growth cultural media to assess the applicability of adapting this microfluidic chamber to monitor biofilm formation of *S. aureus*. To sterilize the chamber, it was infused with ethanol before each experiment. After 10 min, 5 ml syringes containing BHI media and 1 ml syringes containing the diluted culture were connected to the central and outer inlets of the microfluidic chamber, respectively. Two dual automated syringe pumps (GenieTouch™ syringe pump; Kent Scientific Corporation, Torrington, CT) were used to control the flow of growth media and bacterial cells inside the channels of the microfluidic chamber. To stabilize the system and to remove the air bubbles, the flow rate of growth media was initially set at 5 $\mu\text{L}/\text{min}$ for 45 minutes. After that, the flow rates have been maintained to be 0.5 $\mu\text{L}/\text{min}$ for the growth media and 0.1 $\mu\text{L}/\text{min}$ for the bacterial cells. The microfluidic chamber was mounted onto an Eclipse Ti inverted microscope (Nikon, Melville, NY) and was observed with 40x and 10x optics to monitor the biofilm formation. The automated image acquisition was recorded every 1-min by CoolSNAP ES2 monochrome camera (Teledyne

Photometrics, Tucson, AZ) controlled with NIS-Elements Advanced Research (Nikon). The viewing field of the channels was captured at different time points: 6, 12, and 24 hours (hr). All experiments were conducted at room temperature (~23-25 °C).

3.4.4 Study of the media supplementation on the biofilm formation process by *S. aureus*

The effect of fibrinogen (Fbg), plasminogen-Depleted, human plasma purchased from Invitrogen (Paisley, UK) on biofilm formation was done using a microfluidic chamber. In brief, preparing two growth media broths: control media, which had only fresh BHI media broth, and the Fbg-treated media, which was supplemented with human Fbg. Each broth filled a 5 ml gastight syringe that was later connected to the central inlets as earlier mentioned. In this experiment, all settings of the automated syringe pumps for the media and bacterial suspension of *S. aureus* Tager 104 were set to 0.5 $\mu\text{L}/\text{min}$, and 0.1 $\mu\text{L}/\text{min}$, respectively. The formed biofilms in the channels were monitored in two ways: 1. Automated image acquisition every 1-min on a defined spot (near the bacterial cells inlets), 2. Acquiring a large image of the indicated imaging window that covers 6-8 mm at different time points: 6, 12, and 24 hr. To quantify the formed biofilm in the channels, all acquired images at the indicated time points were cropped and converted to binary images (black & white) using the scientific image analysis open platform ImageJ (Schindelin et al., 2015). The binary images were then used to analyze the coverage area (white) in each channel comparing to the background (black). Statistical analysis was done using the student's t-test. The values from three replicates from independent experiments.

3.4.5 Study of the flow rate (pressure) on the biofilm formation process by *S. aureus*

The effect of the flow pressure on biofilm formation was evaluated using a microfluidic chamber. Briefly, the microfluidic chamber was set up to have two replicates in the same experiment under the same growth conditions. The two channels of the microfluidic chamber were

injected with BHI growth media, supplemented with Fbg, through the central inlets and the 1/25 v/v diluted suspension of Tager 104 through the lateral inlets. Four flow rates of the cultural media: 0.5, 1.0, 5.0, and 10 $\mu\text{L}/\text{min}$, were tested in four independent experiments to exert four different pressure inside the channels: 6.1, 12.2, 61, and 122 mbar, respectively, based on an external online pressure calculator. Syringe pumps set the tested flow rate of the growth media throughout the entire experiment. However, the flow rate of the bacterial suspension was maintained constant at 0.1 $\mu\text{L}/\text{min}$ in all experiments. The process of biofilm formation was monitored in two ways: 1. Automated image acquisition every 1-min on a defined spot (near the bacterial cells inlets), 2. Acquiring large images of the indicated imaging window that covers 6-8 mm at different time points: 6, 12, and 24 hr. To quantify the formed biofilm within each channel, all acquired images at the indicated time points were cropped and converted to binary images (black & white) using the scientific image analysis open platform ImageJ (Schindelin et al., 2015). The binary images were utilized to analyze the coverage area (white) in each channel comparing to the background (black). Statistical analysis was performed using one-way ANOVA test. The values from three technical replicates from the same experiments.

3.4.6 Study of the behavior of *S. aureus* biofilm and its components

The visualization of the staphylococcal biofilm components was done as previously described with some modifications (Naranjo et al., 2019). The bacterial cells and the staphylococcal matrix exopolysaccharides (EPS) were detected and colocalized within the channels by initially filling the channels with LB media using the automated syringe pumps. The bacterial suspension of red-labeled NE1260R strain grown in LB was injected. The flow rates of the media and the bacterial suspension were maintained constant at 0.5 and 0.1 $\mu\text{L}/\text{min}$, respectively. After 24 hr of the experiment, the syringe with LB culture media was replaced with

3 ml of LB supplemented with 15 μ L each of potassium hydroxide (KOH) 10% and calcofluor white (CFW). The flow rate of the media was maintained as earlier mentioned for 3-6 hr to allow the mixture flow inside the channels. Image acquisition was performed as described above. The excitation wavelengths of 560 nm (mCherry filter) and 370 nm (DAPI filter) were used to detect both the red-labeled N1260R cells and EPS stained by CFW. To study the behavior of the staphylococcal biofilm formation, a newly designed microfluidic was used. This design has several barriers in the middle of the channels. The bacterial suspension of Tager 104 overnight culture was diluted to 1/25 v/v in fresh BHI broth and injected into the microfluidic with a flow rate of 0.5 μ L/ml. After a period of 24 hr, the BHI culture media syringe was replaced with 3 ml of fresh BHI supplemented with 15 μ L each of KOH 10% and CFW. This flow rate of the media was set for the entire 24 hr was maintained for 3-6 hr to allow the mixture flow inside the channels. The excitation wavelength of 370 nm (DAPI filter) used to detect the CFW-stained staphylococcal matrix EPS. The image acquisition was performed throughout the entire experiment, as described previously.

3.4.7 Study of the biofilm degradation by enzymatic and probiotic treatment

A) The effect of lysostaphin on the biofilm formation by NE1260R strain:

The impact of lysostaphin on the biofilm formation by NE1260R strain was assessed using a microfluidic chamber, as described above. Briefly, the channels were initially filled with LB cultural media by the automated syringe pump. The NE1260R bacterial suspension in LB media was injected into the channels. The flow rates of both media and the bacterial suspension were maintained constant at 0.5 and 0.1 μ L/min, respectively. After observing a critical amount of mature biofilms, the syringe with LB culture media was replaced with 3 ml of LB growth media supplemented with 150 μ L of a mixture of 2 mg/ml of lysozyme plus 100 μ g/ml of lysostaphin. The flow rate of the media was maintained as earlier mentioned for 24 hr to allow the mixture

flows inside the channels (this is time = 0 sec). The lysostaphin effect on the formed biofilm by NE1260R strain in the channels was monitored in three ways: 1. Automated image acquisition every 1-min on a defined spot (near the bacterial cells inlets), 2. Acquiring a large image of the indicated imaging window that covers 6-8 mm at different time points: 0, 6, 12, and 24 hr. 3. Acquiring fluorescent images of the NE1260R bacterial cells using an excitation wavelength of 560 nm (mCherry filter). To quantify the formed biofilm in the channels, all acquired images at the indicated time points were converted to binary images (black & white) using the scientific image analysis open platform ImageJ (Schindelin et al., 2015). The binary images were then used to analyze the coverage area (white) in the channel comparing to the background (black). Statistical analysis was performed using one-way ANOVA test (Brown-Forsythe test). The values from two replicates from independent experiments.

B) The effect of *B. velezensis* AP183 on the biofilm formation by *S. aureus* Tager 104 strain:

The effect of *B. velezensis* AP183 on the biofilm formation by Tager 104 strain was assessed using a microfluidic chamber (dynamic) and co-culturing on an agar plate (static). In the microfluidic chamber, the channels were initially filled with BHI cultural media. The Tager 104 bacterial suspension in BHI media was injected into the channels. Both media and the bacterial suspension flow rates were maintained constant at 0.5 and 0.1 $\mu\text{L}/\text{min}$, respectively, for 24 hr. After observing a critical number of mature biofilms, one of the syringes with Tager 104 bacterial solution was replaced with 1 ml of TSB supplemented with 1/5 v/v of *B. velezensis* AP183 overnight culture. The flow rate of the media was maintained as it is for 24 hr to allow the *B. velezensis* AP183 culture to reach inside the channels. The *B. velezensis* AP183 effect on the formed biofilm by Tager 104 strain was evaluated in two ways: 1. Automated image acquisition every 1-min on a defined spot (near the bacterial cells inlets), 2. Acquiring a large image of the

indicated imaging window that covers 6-8 mm at different time points: 6 hr, 12 hr, and 24 hr. To quantify the formed biofilm in the two channels, all acquired images at the indicated time points were cropped as an individual channel and converted to binary images (black & white) using the scientific image analysis open platform ImageJ (Schindelin et al., 2015). The binary images were used to analyze the coverage area (white) in the channel comparing to the background (black) for each channel. Under static conditions, the anti-staphylococcal activity of *B. velezensis* AP183 was assessed by initially spreading a confluent lawn of Tager 104 on TSA plate and streaking lines of *B. velezensis* AP183 on the same plate. The plate was incubated overnight at 25, 30, and 37 °C. The inhibitory effect was evaluated by zones of clearance in all plates.

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Chapter 4 Conclusion and Future Directions

This dissertation, first, has created a detailed protocol to develop *in vitro* microfluidic-based model to visualize the development of *S. aureus* biofilm, an aspect of the *S. aureus* pathogenesis, and monitor the degradation effect of the lytic agent, lysostaphin against this virulence determinant under dynamic environmental conditions, which are more physiologically relevant to the host environment. Second, the impact of supplementing growth media and increasing flow rates, which increase the internal pressure, on the process of *S. aureus* biofilm formation were assessed using the *in vitro* developed model. An essential component of the *S. aureus* biofilm matrix, exopolysaccharide, also was stained and identified to validate the biofilm growth and matrix production. Additionally, a microfluidic with obstacles was used to study the behavior of the *S. aureus* biofilm formation in the presence of obstacles and sharp edges and corners. Lastly, the treatment of these dangerous biofilms with a potential soil-derived probiotic bacterium and lysostaphin was evaluated in terms of the biofilm coverage area under flow conditions.

Although we were able to obtain findings using this model, there are some limitations, such as the difficulty of extracting surface-attached biofilms from the channels at the end of each experiment to perform further molecular analysis, such as detecting viable bacterial cells or assess upregulation or downregulation of genes. However, a study by (Chen & De La Fuente, 2020) reported that the replacement of growth media with DNA/RNA shield™ (Zymo Research, Irvine, CA, USA) would facilitate the detachment of adhered biofilms within the microfluidic channels and maintain nucleic acids under a flow rate at 20 μ l/min. Since the flow rate is relatively high, there is a risk to increase the internal pressure, which may lead to break the microfluidic chip and

cause leakage and ultimately, contamination occurs. Here, we propose another extraction method by infusing DNA/RNA shield™ in replacement of the growth media and manually controlling the flow inside the channels as the concept of liposomes extrusion technique by mini-extruders (MacDonald et al., 1991). In this technique, a sample is pushed through several membranes back and forth by generating pressure through two glass syringes for nanosizing liposomes. To adapt that in extracting attached biofilms in the microfluidic chamber, the media syringe and the waste-collecting syringe can be manually pushed alternatively to apply moderate pressure on biofilms inside the channels to detach them from the surface and force them to flow to the waste-collecting syringe. We expect that this method will lead to extract most of the formed biofilm with low risk to break the chamber as the applied pressure is low to moderate.

Furthermore, we suggest that the presented model to study the biofilm formation in *S. aureus* can be improved to provide a more physiologically relevant condition for the host environment. The improvement can be achieved by seeding human endothelial cells on the 0.1 % gelatin-pretreated channels before introducing *S. aureus* cells into the channels. A published study has reported that the adherence of bacterial cells to seeded human endothelial cells has increased in the presence of flow in a flow chamber model with a large flow channel compared to the channel of the presented microfluidic chamber (Viegas et al., 2011). Moreover, the small dimensions of channels represent the environment of smaller veins. The improved model will help to predict the findings of *in vivo* studies. Also, other human cell lines can be used in this model to study other disease states under flow conditions. Another strategy that can be used in this system in future studies would be the addition of human plasma or human sera to the growth environment in order to improve it and to become a more relevant to physiological host conditions.

Additionally, the layout and dimensions of the channels' design can be enhanced to mimic *in vivo* microvasculature environment. That can be achieved by creating smaller branching channels, specifically, starting with two channels with 80 μm wide that bifurcate to 4 channels with 60 μm that also branch to the smallest 8 channels with 40 μm wide, which mimic the branching of the smallest vessels of the microvasculature environment in the body, such as arterioles and venules. Also, peristaltic pumping systems can be used, and other designs can be created to model a specific environment to answer a research question. As an example, it has been evidenced that the mitral valve involvement is common in native valve infective endocarditis caused by *S. aureus* (McDonald, 2009). Therefore, the mitral valve model developed by (Lee et al., 2018) can be used as a reference to develop a new model to study the biofilm formation of *S. aureus* within *in vitro* model of the mitral valve.

Also, it is crucial to mention the availability of a collection of sequence-defined transposon (Tn) insertion mutants of *S. aureus* on a library called Nebraska Transposon Mutant Library (Center for Staphylococcal Research [CSR], Bose et al., 2013). The strains in this collection contain mutant derivatives of a parental strain USA300 LAC in which specific genes have been disrupted. Also, these mutants can be constructed with fluorescent reporter plasmids (Bose et al., 2013). The mutants are available for the community of staphylococcal research. Thus, the mutant strains of a gene of interest can be obtained to perform experimental studies. For instance, evaluate the effect of the mutation of a clumping factor gene in the presence of Fbg under flow conditions.

In addition, several researchers have pointed out that biofilms are favorable environments for the formation of persister cells by activating different signals, such as quorum sensing, oxidative stress, and dormancy and the persister cells are major causes of the antibiotic treatment failure within biofilms (Conlon et al., 2015; Lewis, 2010). However, a recent study has

characterized *msaABCR* operon that was found to control virulence and biofilm development in some strains of *S. aureus* and antibiotic exposure increased the number of persister cells in wild type strain, but in *msaABCR* mutant strain, the number of resister cells was reduced (Sahukhal & Elasri, 2014). Thus, more strains of *S. aureus* can be evaluated in order to find if the role of *msaABCR* in persister cell formation is universal in *S. aureus*. Also, the *msaABCR* mutant strains of *S. aureus* formed defective biofilms, which are unfavorable environments for persister cell formation (Sahukhal & Elasri, 2014). These immature biofilms are mediated by the highly produced Atl through the increased production of protease, which caused uncontrolled cell death (Sahukhal et al., 2017). Therefore, it would be valuable to test the hypothesis that excessive production of proteases will effect on the formation of persister cells by the *msaABCR* mutant strains.

On the other hand, the presented microfluidic-based model can be used to evaluate potential newly discovered therapeutic agents, such as antimicrobials, antibiofilm, or currently used agents such as probiotic preparations in order to treat or inhibit the *S. aureus* biofilm. As mentioned previously, some *lactobacillus* (LAB) species showed efficacy against some *S. aureus* strains. However, these LAB species can be evaluated against the *S. aureus* Tager 104 strain under flow condition using the presented model. In addition, other probiotics can be examined against other pathogens such as *P. aeruginosa* under dynamic conditions. Another approach in fighting *S. aureus* biofilm formation that can be tested is treating with quorum sensing inhibitors or specific agr inhibitors. For example, published studies have reported that the antibiotic susceptibility of *S. aureus* biofilms has been increased when treated with Hamamelitannin, quorum sensing inhibitor, specifically, RNAlII-inhibiting peptide (Brackman et al., 2016; Kiran et al., 2008). Although *in vivo* studies have been performed, the *in vitro* model was microtiter plates assay, which is under a

static condition and does not resemble the *in vivo* environment. Also, the *S. aureus* Tager 104 strain was not included in the study. Therefore, further research that will include *in vitro* microfluidic-based model and the *S. aureus* Tager 104 strain would be valuable and supportive.

Another area of research is D-amino acids treatment against *S. aureus* biofilm formation, which has conflicting findings. Some studies have reported that these small molecules inhibited the biofilm formation of *S. aureus* (Hochbaum et al., 2011; Kolodkin-Gal, 2017; Sanchez et al., 2014). However, there is a reported study has published that D-amino acids did not inhibit the biofilm formation of *S. aureus* even at the millimolar concentration (Sarkar & Pires, 2015). Most of these published researches were conducted under static conditions. Thus, it would be valuable to evaluate the effect of these molecules under a more physiologically relevant environment that the microfluidic-based model provides.

Since the composition of biofilm matrix varies between the *S. aureus* strains, the use of the commercially available dyes that stain the components of biofilm would help in characterizing the biofilm matrix composition of the strain of interest. Some examples of these dyes that have been used on different *S. aureus* are the SYTO, SYPRO Ruby, and wheat germ agglutinin (WGA), which stain specifically nucleic acids, proteins, and N-acetyl-D-glucosamine residues (PNAG), respectively (Oniciuc et al., 2016). These dyes can used in the microfluidic-based model and the use of confocal laser scanning microscopy is optional to confirm the results. By identifying the components profile of biofilm matrix in a certain *S. aureus* strain, it will assist in determining the potential agents and strategies that need to be examined in the subsequent experiments against that tested strain.

Moreover, evaluation of targeting the components of biofilm matrix of *S. aureus* Tager 104 strain with degrading enzymes is also an area that needs to be explored. In other words, one of this

dissertation findings visualized the secreted β -polysaccharides, which are component of the biofilm matrix. However, several studies have reported the degradation of the polysaccharides in biofilm matrix with Dispersin B treatment. Thus, treating the biofilm matrix of Tager 104 with Dispersin B with or without antimicrobial agents would be valuable findings to understand this strain better and to investigate the activity of this enzyme against more *S. aureus* strains.

Furthermore, the viability of bacterial cells within biofilms also can be monitored by the LIVE/DEAD BacLight Bacterial Viability kit, which will differentiate the cells based on the integrity of the cell membrane as live or dead cells. The two used stains are SYTO 9 and propidium iodide, which will stain live bacterial cells green and dead cells red, respectively.

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