CHARACTERIZING THE HEMOLYTIC ACTIVITY OF STAPHYLOCOCCUS AUREUS CLINICAL ISOLATES

By

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Thesis

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Table of Contents

Abstract of Characterizing Staphylococcus aureus clinical isolates, by Olivia Carneiro, ScM, Brown University, May 2023.

Staphylococcus aureus is a gram-positive antibiotic-resistant pathogen that poses a growing global health threat. Bacteria produce virulence factors, including hemolysins, and can form biofilm matrices that render first-line treatments ineffective. As available treatments have the limitation of causing host cell damage despite reducing bacterial burden, there is a need to establish a translatable infection model *in vitro* to bolster the development of a therapeutic. The goal of this thesis is to explore the hemolytic activity of 10 clinical isolate strains, both in planktonic and biofilm states, in various growth conditions to observe the relationship of hemolysis and growth media. These experiments consisted of quantitative hemolysis assays in which planktonic and biofilm strains were grown in various media environments, and the hemolytic activity of these bacterial supernatants was observed in 5% (v/v) rabbit red blood cells. Our findings reveal that bacterial biofilms have greater hemolytic activity per 1 log colony forming unit per milliliter (CFU/mL) of bacteria than their planktonic counterparts across all media tested. These results suggest that the hemolytic activity of bacterial biofilms is not dependent on the media in which they are cultured. In contrast, planktonic bacteria hemolytic activity data suggest a dependence on the media environment. The 9/10 of the planktonic strains tested had the greatest hemolytic activity when grown in BHI compared to other growth media. Future studies should investigate the relationship of other virulence factors produced by *S. aureus* and media environments to better understand the circumstances of bacterial pathogenicity *in vitro*.

Chapter 1: Introduction

1.1: Antibiotic Resistance: A Growing Public Health Threat

Antibiotic resistance is a health threat that is on the rise globally. *Staphylococcus aureus*, a Gram-positive bacterium, commonly found on the skin or in the noses of healthy individuals [1] [2]. As an opportunistic pathogen, *S. aureus* exhibits pathogenicity upon encountering environmental stimuli and develop resistance to first-line antibiotics, making them difficult to treat [3]One of the most serious of these resistant strains is methicillin-resistant *S. aureus* (MRSA). The2019 Centers for Disease Control and Prevention (CDC) Antibiotic Resistance Threats Report revealed the substantial impact of MRSA infections, with approximately 300,000 hospitalizations, 10,000 deaths, and 1.7 billion dollars in healthcare costs in the US in 2017 [4]. These statistics emphasize the critical need to address MRSA-related challenges and develop novel therapeutic approaches.

As illustrated in **Figure 1**, MRSA develops its resistance through several mechanisms that include bacteria developing impermeable cell walls, activating antibiotic efflux pumps, and releasing enzymes. These processes hinder the effects of antibiotics and promote the pathogenicity of bacteria [5]. The mecA gene regulates the primary oxacillin/methicillin resistance mechanism in *S. aureus*, inducing the production of an altered penicillin-binding protein known as PBP2a [5]. This mutant PBP2a has a lower binding capacity to beta-lactam antibiotics, which are the first-line antibiotic treatment. This results in resistance to this class of antibiotics.

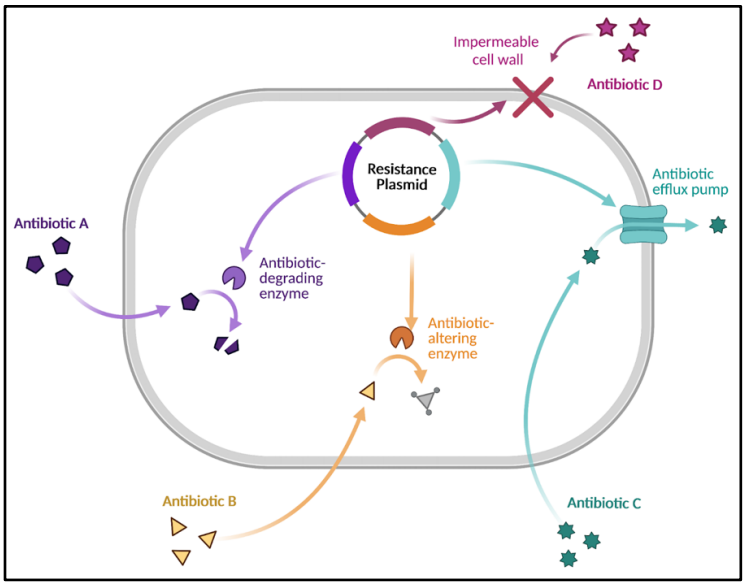

Figure 1: Resistance mechanisms of *S. aureus* include the production of antibiotic altering or degrading enzymes, activation of antibiotic efflux pumps, and development of an impermeable cell wall. Figure made through BioRender.

1.2: Biofilms

To make matters worse, bacteria can form biofilms. Biofilms are the build-up of planktonic bacteria on a surface that is responsible for most chronic infections. They are difficult to treat because these bacteria form extracellular polymeric substances (EPS) around them which is a slime-like barrier that through charge and the physical barrier itself prevents therapeutics from penetrating them [6]. The biofilm formation process, depicted in **Figure 2**, begins with attachment, then advances to matrix development and concludes with the nutrient limitation phase of maturation, which leads to the release of bacteria to start this cycle over again.

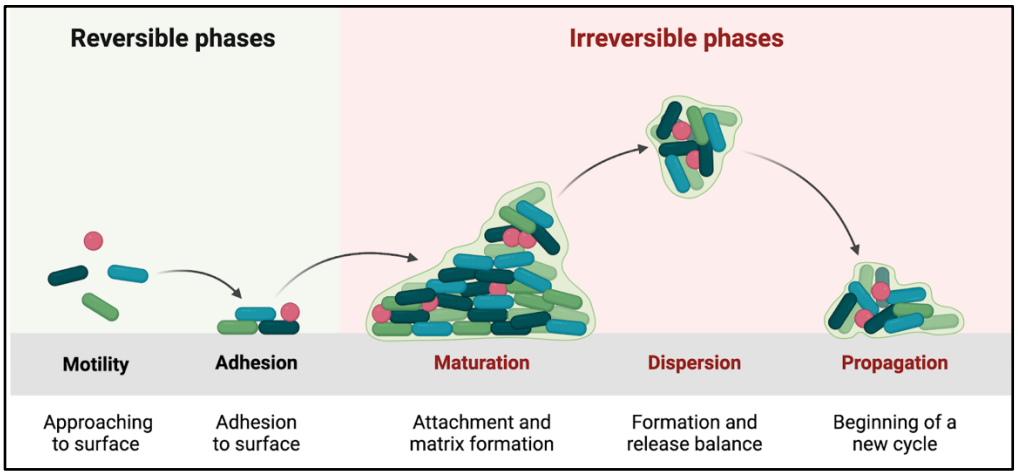

Figure 2: Biofilms form on the surfaces of host cells to develop an impenetrable matrix called the EPS, which render antibiotics ineffective in targeting these infections. This process is cyclic in nature as the biofilms disperse causing the cycle to continue. Figure made through BioRender.

1.3: Toxin Production

Bacteria release enzymes and toxins that contribute to their own pathogenicity [7]. Bacteria can release two classes of toxins: endotoxins and exotoxins. The former is a membrane protein that elicits an inflammatory response in the host, while the latter can act locally or at a distance to elicit the same response at the site of bacterial colonization [8]. Endotoxins are found in the membranes of Gram-negative strains of bacteria while exotoxins can be released by both Gram-negative and Gram-positive bacteria. Exotoxins are soluble bacterial secreted proteins that enter host cells to alter host cell physiology by catalyzing the covalent modification of a host's cellular component. Due to their potency, they are known to be more harmful in targeting host cells than endotoxins [8].

S. aureus specifically produces exotoxins that contribute to the colonization of bacteria. In vivo, they are able to inhibit the host cell's immune response lead to T-cell exhaustion [9]. As seen in

Figure 3a, toxins that are produced by *S aureus* cause virulence through the 5-Lox pathway which is when 5-Lox is catalyzed to activate a pro-inflammatory response. This causes the release of inflammatory cytokines called leukotrienes [10].

Of the exotoxins produced by *S. aureus*, hemolysins are the most widely produced. The most common class of these hemolysins are alpha hemolysins. Alpha hemolysins are pore-forming toxins, which means they can penetrate a host cell membrane. α-hemolysin is secreted as a monomer, and acts through the ADAM10 receptor, as seen in **Figure 3b** [11]. This toxin associates as a homo-heptamer, thus creating a pore within the cellular membrane. The other exotoxins that are produced by s aureus like gamma and beta-hemolysin are also pore-forming toxins, but they act through G protein-coupled receptors (GPCRs) and form hetero-oligomeric pores to penetrate the host. The host cell of interest for these toxins are red blood cells. When these toxins form pores in the cell membranes of red blood cells, it causes lysis which leads to the release of hemoglobin. This eventually results in the decrease of red blood cell production triggered by the immune system and the decreased amount of hemoglobin in the body because of heme oxygenase-1 converting heme to iron, which could induce sepsis [12]. The production of this virulence factor is not well-understood in the context of modeling disease.

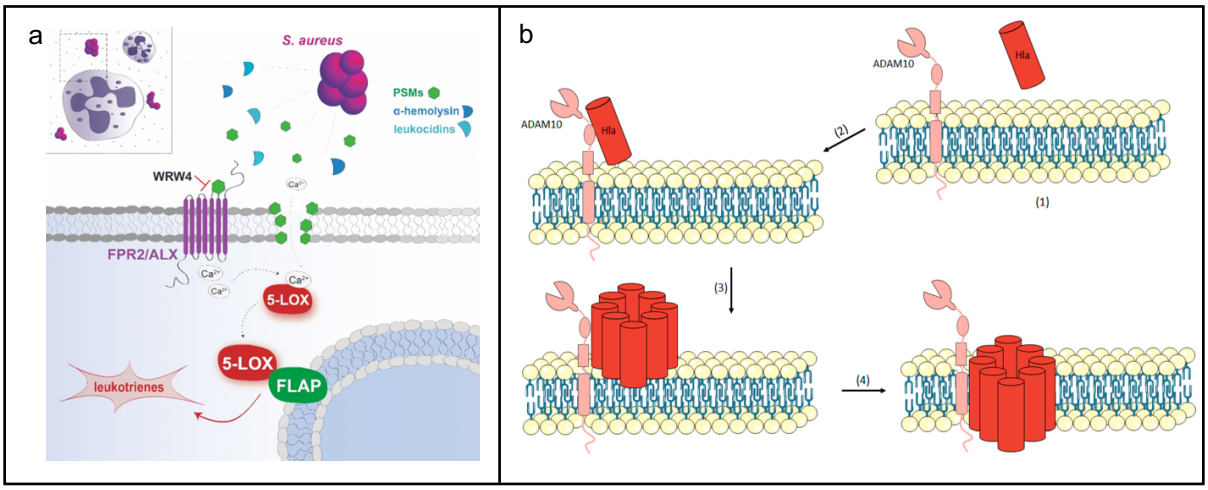

Figure 3: Alpha-hemolysin produced by *S. aureus* is activated through the 5-Lox pro-inflammatory pathway [10]. Upon activation, alpha-hemolysins self-assemble into a homo-heptamer when bound to the ADAM-10 receptor [11]. This protein then penetrates the host cell's membrane, which in this case is erythrocytes. Figures compiled from Romp et. al. and Oliveira et. al..

1.4: Regulation of Virulence Factor Production

Pathogenesis and virulence are often used interchangeably, referring to the mechanism of infection and the mechanism by which the disease develops [13]. The production of virulence factors by bacteria are controlled by the expression of regulatory genes. Among strains of *S. aureus*, *agr* is known to mediate the regulation of hemolysin production [14]. In a study by Pader et. al., they report that during infection, *S. aureus* mutates, and some important pathway regulation genes may lose their expression. They theorize that the inactivation of these genes disrupts the electron transport chain, which caused a loss of *agr* activity, hindering the production of hemolysin [14]. This study highlights the importance of the electron transport chain in the hemolytic activity of bacteria, regulated by *agr*. Additionally, the supplementation of culture media has been reported to increase *agr* expression [14].

1.5: Virulence and Microbial Growth State

It is unclear as to why *S. aureus* detects and decides to contribute energy toward growth or virulence factor production [15]. Moreover, the relationship of virulence factor production and microbial growth state is rather controversial in the field. A comparative proteome analysis by Resch et. al. described that in fully mature *Staphylococci* biofilms, virulence factor production is generally downregulated while stress response genes are upregulated, classifying planktonic bacteria as more virulent [15]. However, a more recent proteomics analysis by Graf et. al. revealed that *S. aureus* biofilms had significantly higher expression of virulence factors, including hemolysins, than planktonic strains [16]. This study revealed that virulence factor production contributed to the increased pathogenesis of *S. aureus* biofilms.

1.6: Motivation

MRSA has become resistant to many first-line antibiotics, mainly due to the misuse/overuse of antibiotics. Thus, there is an urgent need for new drug delivery technologies to minimize the overuse of antibiotics. Various nanoparticles and biomaterials have been recently developed to deliver antimicrobial agents while limiting off-target drug effects. However, there are few delivery systems that can successfully target the site of infection while evading host immune response.

Infection modeling poses challenges in vitro, as there are many variables that can alter the growth of bacteria. One of the largest influences on bacterial growth, adhesion, and biofilm formation is the media environment that is used in bacterial incubation. There is currently a gap in knowledge in the scientific community regarding how the environment of a culture impacts its growth as well as the differences in pathogenicity of strains from the same species in different

media. The relationship between virulence and bacterial state also is controversial in the field. Through this thesis, we seek to answer these questions to bridge this gap. Additionally, we aim to provide insight to assist in the development of a hemolysin-responsive biomaterial.

Chapter 2: Materials and Methods

2.1: *S. aureus* clinical isolate strains

The lab obtained *S. aureus* clinical isolates from Rhode Island Hospital. They were each isolated from a different location of the body in patients. Half of these strains were resistant to oxacillin, while the other half were susceptible, as indicated in Table 1. These tests were conducted by the Rhode Island Hospital. They were tested against oxacillin instead of methicillin is that methicillin is no longer available in the US and oxacillin is commonly used to identify MRSA. The strains were diluted 25% v/v in glycerol (Fisher Scientific, Waltham, MA) and were stored as stocks at -80°C until use.

Media Type	Nutrients					
1×BHI	5 g/L beef heart	12.5 g/L calf brains	2.5 g/L disodium hydrogen phosphate	2 g/L D(+) glucose	10 g/L peptone	5 g/L sodium chloride
1×TSB	17 g/L tryptone (pancreatic digest of casein)	3 g/L soytone (peptic digest of soybean)	2.5 g/L D(+) glucose	5 g/L sodium chloride	2.5 g/L dipotassium phosphate	
1×CMHB	3 g/L beef extract	17.5 g/L acid hydrolysate of casein	1.5 g/L starch			
1×LB	10 g/L tryptone	5 g/L yeast extract	10 g/L sodium chloride			
1×SSM9PR	36 g/L magnesium sulfate	1.8 g/L calcium chloride	10 g/L D (+) glucose	18 g/L thiamine hydrochloric acid	0.9 g/L nicotinamide	

Table 1: List of media components in BHI, TSB, CMHB, LB, and SSM9PR. The above five medias were used in these studies included 1 x BHI, 1 x TSB, 1 x CMHB, 1 x LB, and 1 x SSM9PR. Each media contained different nutrients to promote the growth of bacteria, as listed above.

Growth Curves:

2.2: Media Growth Test:

Clinically isolated strains were inoculated each strain in these four different media types:
Brain Heart Infusion Broth (BHI) (Sigma-Aldrich, St. Louis, MO), Tryptic Soy Broth or TSB (BD, Franklin Lakes, NJ), Luria Broth or LB (BD, Franklin Lakes, NJ), and Cation-adjusted Mueller Hinton Broth or CMHB (BD, Franklin Lakes, NJ). Bacterial overnight culture was diluted in their respective media to 1:500 bacteria to media. Then, the optical density at 600 nm (OD600) was read in the plate reader (Biotek, Cytation3 Plate Reader) every hour or every 30 minutes during the logarithmic (log) phase of growth (which was between 3 and 6 hours). The most optimal media was identified by which media reached the log phase first with the greatest slope, indicating more rapid bacterial growth. All samples were run in triplicate, and the average values were reported.

Bacterial overnight culture was prepared by inoculate bacterial glycerol stocks in BHI and incubated at 37°C for 16-18 hours with orbital shaking at 100 rpm. After incubation, the sub-cultures were prepared with a 1:500 dilution the overnight culture in 1 x BHI. The cultures were incubated at 37°C with shaking at 100 rpm, and samples were taken at regular intervals for cell forming units (CFU) counting and growth curve analysis. For growth curve analysis, OD_{600} values were read every hour or every 30 minutes during log phase with a spectrophotometer (Thermo Scientific, Spectronic 200 Spectrophotometer). For CFU analysis, serial dilutions of each culture were plated on XX plates and incubated at 37°C for 24 hours. The number of colonies was counted, and the cell forming unit per mL of culture (CFU/mL) was calculated. The OD_{600} values were then plotted against the CFU/mL to determine the linear regression model for data analysis.

2.3: Clinical isolate growth curves to determine bacterial concentrations for future studies

Bacterial stocks were inoculated in 1 x BHI and were incubated at 37°C for 16-18 hours. After incubation, the cultures were diluted 1:500 in 1 x BHI. Using a spectrophotometer, the OD600 values were read every hour or every 30 minutes during log phase. At each of these time points, each of these strains were diluted in 1 x PBS from GE Healthcare Life Sciences (Marlborough, MA) at concentrations ranging from $1x10^1$ to $1x10^8$. 5 µL of each solution was then streaked on 1 x BHI agar plates in triplicate. The agar plates were incubated at 37°C for 16-18 hours and colonies were counted thereafter. The OD600 values were then plotted against the colony forming unit per milliliter (CFU/mL) data to determine the linear regression model.

2.4: Qualitative hemolysis test on blood agar plates

Hemolytic activity was monitored via the blood agar method as described by American Society for Microbiology [17]. The bacterial clinical isolate stocks were inoculated in 1xBHI at 37°C for 16-18 hours with shaking. The overnight cultures were mixed thoroughly and 5 µL of each solution was added in triplicate to a 5% Sheep Blood Agar plate from BD (Franklin Lakes, NJ). Once added, the bacteria were spread with a cotton swab.

2.5: Quantitative Hemolysis Assay

The following assay was followed/adapted from Rock et. al., Ghirmai et. al., and an internal protocol from lab alumnus Nisha Hollingsworth created in 2016 [20] [21]. The bacterial frozen stocks were inoculated in 5 mL of 1xBHI, 1xLB (5g/L NaCl), 1xCMHB, 1xTSB, 1 x SSM9PR. The culture tubes were incubated with shaking at 37°C for 16-18 hours. After the incubation period, the bacterial overnight inoculates were diluted in their respective media at a 1:500 dilution

(bacteria to media) and incubated with shaking at 37°C for 4 hours. 1.5 mL of bacterial solution was transferred to a 2mL eppendorf tube and the solution was centrifuged at 8000g for 5 minutes at room temperature. The bacterial supernatant was sterile filtered with a syringe and 0.2 µm filter and collected in a 2 mL eppendorf tube. These bacterial supernatants can be stored at 4°C for up to 1 month.

Rabbit whole red blood cells from Rockland (Limerick, Pennsylvania) were diluted to be 5% packed (v/v) in 1×PBS. The positive control red blood cell solution was prepared with 49% 5% RBCs, 49% 1xPBS, and 2% Triton X-100 (20%) (v/v), so that the final concentration in each well is 1%Triton X-100 (20%). 100 µL of the positive control red blood cell solution was added to 100 µL of the respective media. The negative control was a 1:1 ratio of culture media and 5% RBCs (100 µL respectively). Each sample well included a 1:1 ratio of 5% RBCs to bacterial supernatant (100 µL respectively). The solutions were added to a 96-well v-plate and incubated at 37°C with shaking at 110rpm for 1 hour. The plate was then centrifuged 1000 rpm for 5 minutes. 100 µL of each supernatant was added into each well of a UV-spectral 96 well plate and measure the absorbance from the range 400-550 nm. The ratio of % hemolysis to log CFU/mL was calculated using **Equations A** and **B**.

Equation A

$$\frac{Sample\ peak\ OD\ -\ Negative\ control\ peak\ OD}{Positive\ control\ peak\ OD\ -\ Negative\ control\ peak\ OD} = Percent\ (\%)\ Hemolysis$$

Equation B $\frac{Percent\ (\%)\ Hemolysis}{Log\ (CFU/mL)} = Normalized\ Percent\ (\%)\ Hemolysis\ per\ 1\ Log\ CFU/m$

Chapter 3: Results

Isolate #	SA01	SA02	SA03	SA04	SA05	SA06	SA07	SA08	SA09	SA010
Site of Isolation	Blood	Body Fluid	Body Fluid	Tissue	Body Fluid	Blood	Blood	Body Fluid	Tissue	Tissue
Resistance to Oxacillin (R/S)	R	R	R	R	R	S	S	S	S	S

Table 2: List of S. aureus clinical isolates and their site of isolation. The ten clinical isolates strains were obtained from the Rhode Island Hospital. Information regarding the infection sites of isolation and susceptibility status were provided by the Rhode Island Hospital.

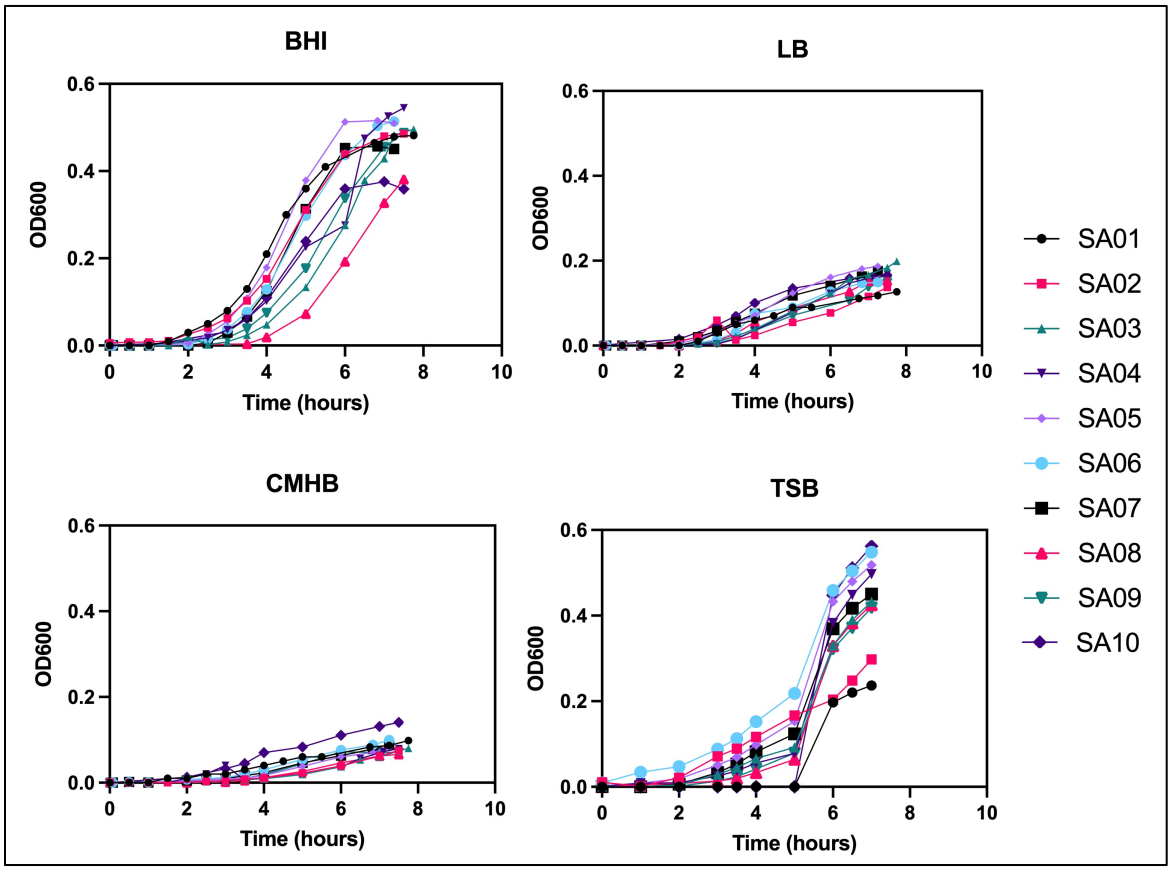

Figure 4: Growth curve analysis of S. aureus clinical isolates. Time in hours was graphed against their corresponding OD_{600} absorbance reading. The stages of growth include lag, log, and stationary phase. Between 0-7 hours of growth, it can be assumed that a higher OD_{600} value represents greater number of bacterial cells.

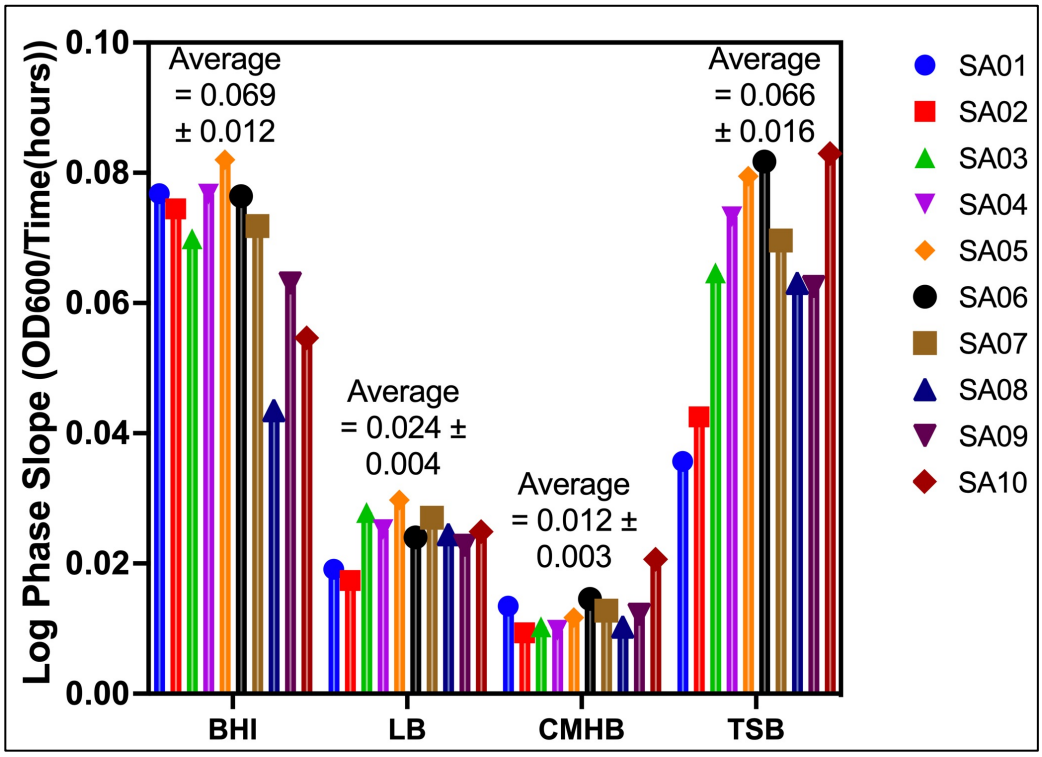

Figure 5: Growth curve log phase slopes. The average slopes of bacterial growth during log phase were graphed for each clinical isolate in 4 different media.

Media Growth Test:

Among the 4 different media conditions, the clinical isolates reached log phase faster when grown in BHI and TSB versus LB and CMHB. Additionally, the rate at which the bacteria replicated during the log phase is exemplified by the slope between the 4 and 6 hour time points. As shown in **Figures 4 and 5**, these average slopes across all 10 strains were 0.069 ± 0.012 for 1×BHI, 0.024 ± 0.004 for 1×LB, 0.012 ± 0.003 for 1×CMHB, and 0.066 ± 0.016 for 1×TSB. Overall, 1×BHI

was identified as the nutrient environment where the strains grew at the highest rate during their log phase. Although some bacterial strains grew well in 1×TSB, 3 strains, SA01, SA02, and SA10, did not grow as successfully in this media. Additionally, it is important to note that the bacterial strains grown in 1×TSB took slightly longer than bacterial strains grown in 1×BHI to reach the highest slope in the log phase.

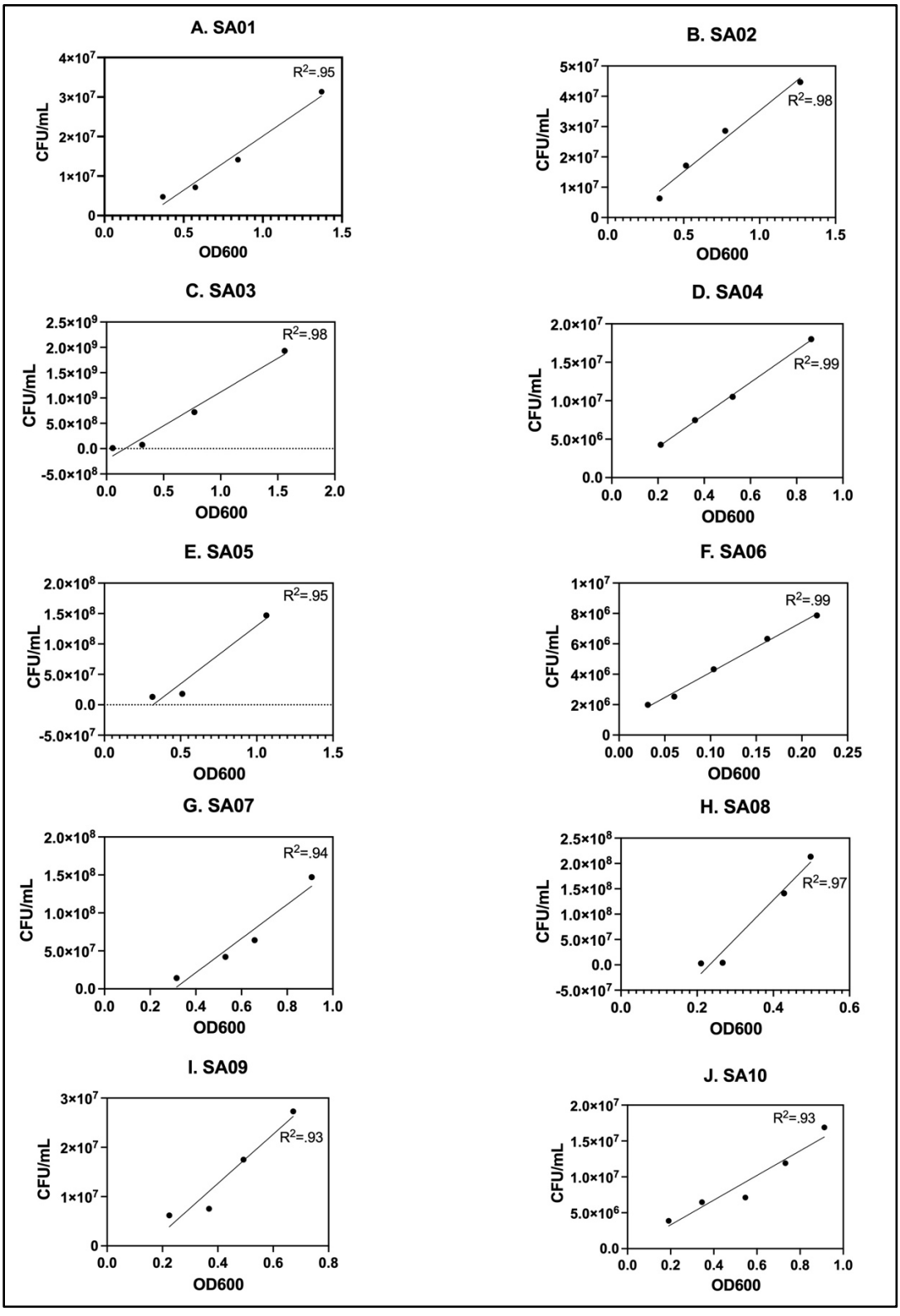
A. SA01
CFU/mL
OD600
R2=.95
B. SA02
R2=.98
C. SA03
R2=.98
D. SA04
R2=.99
E. SA05
R2=.95
F. SA06
R2=.99
G. SA07
R2=.94
H. SA08
R2=.97
I. SA09
R2=.93
J. SA10
R2=.93

Figure 6: Linear regression of clinical isolates during log phase of growth. A-J represent SA01-SA10. The OD600 absorbance readings were plotted against the colony enumeration data normalized to CFU/mL to obtain the linear regression of the data.

Growth curves:

In **Figure 6**, the log phases of growth for each clinical isolate strain fit within their respective linear regression models. The R^2 values for each plot fell between the range of 0.93 and 0.99, indicating that the OD600 and CFU/mL data were linear. The clinical isolates' respective linear equations could be utilized to determine the bacterial concentration from an OD600 absorbance reading by using **Equation C**, where C_1, the value we are solving for, is the initial concentration, V_1 is the initial volume, C_2 is the final concentration, and V_2 is the final volume.

Equation C

$$\boldsymbol{C_1 \times V_1 = C_2 \times V_2}$$

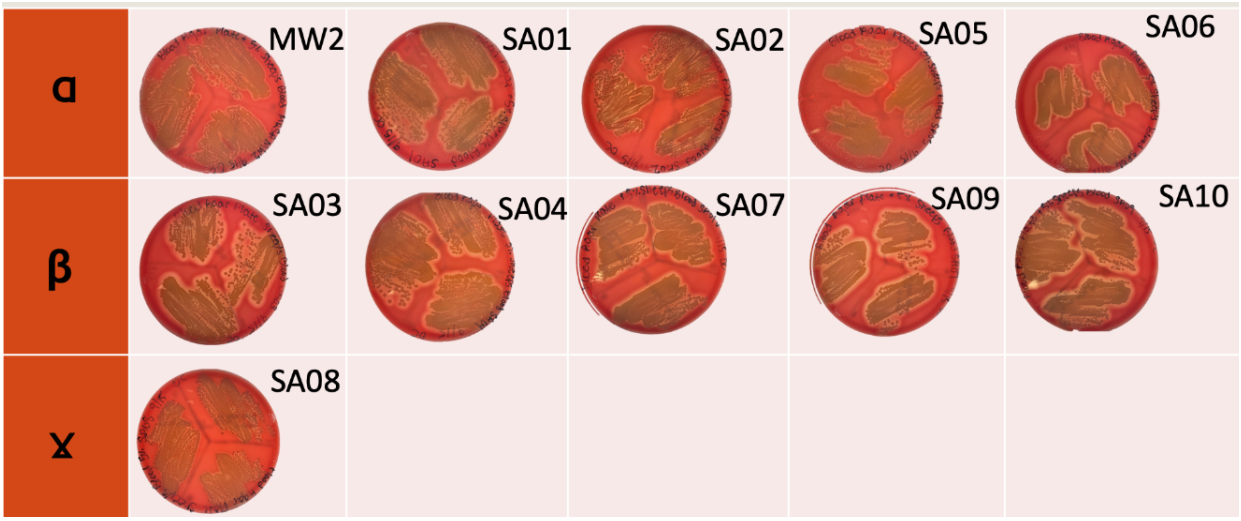

Table 3: Qualitative hemolysis test. Bacterial overnight inoculates were streaked in triplicate on 5% sheep blood agar plates. The highlighted plate in the first row was the reference strain for this test, MRSA MW2, while the other plates include the clinical isolates. The bacterial strains were classified by their class of hemolysin, alpha, beta, and gamma.

Qualitative hemolysis test on blood agar plates:

Different classes of hemolysins have different lytic capabilities. Alpha hemolysins cause partial lysis of red blood cells, beta hemolysins cause complete destruction of red blood cells, and gamma hemolysins do not cause lysis of red blood cells. Beta hemolysis can be identified by a bright yellow zone of red blood cell inhibition, while in alpha hemolysis, red blood cells are visible between the streaked overnight inoculum, meaning that not all of the red blood cells were lysed. Gamma hemolysis is identified by no color change, indicating no red blood cell lysis. As shown in Table 3, The reference strain used (MRSA MW2) produced alpha hemolysin, along with 4 isolates. In 5 isolates, complete red blood cell lysis was observed, while in 1 strain no hemolysis was observed. This was an interesting finding because although these isolates were cultured in the same media and grown on the same plates, different classes of hemolysins were present.

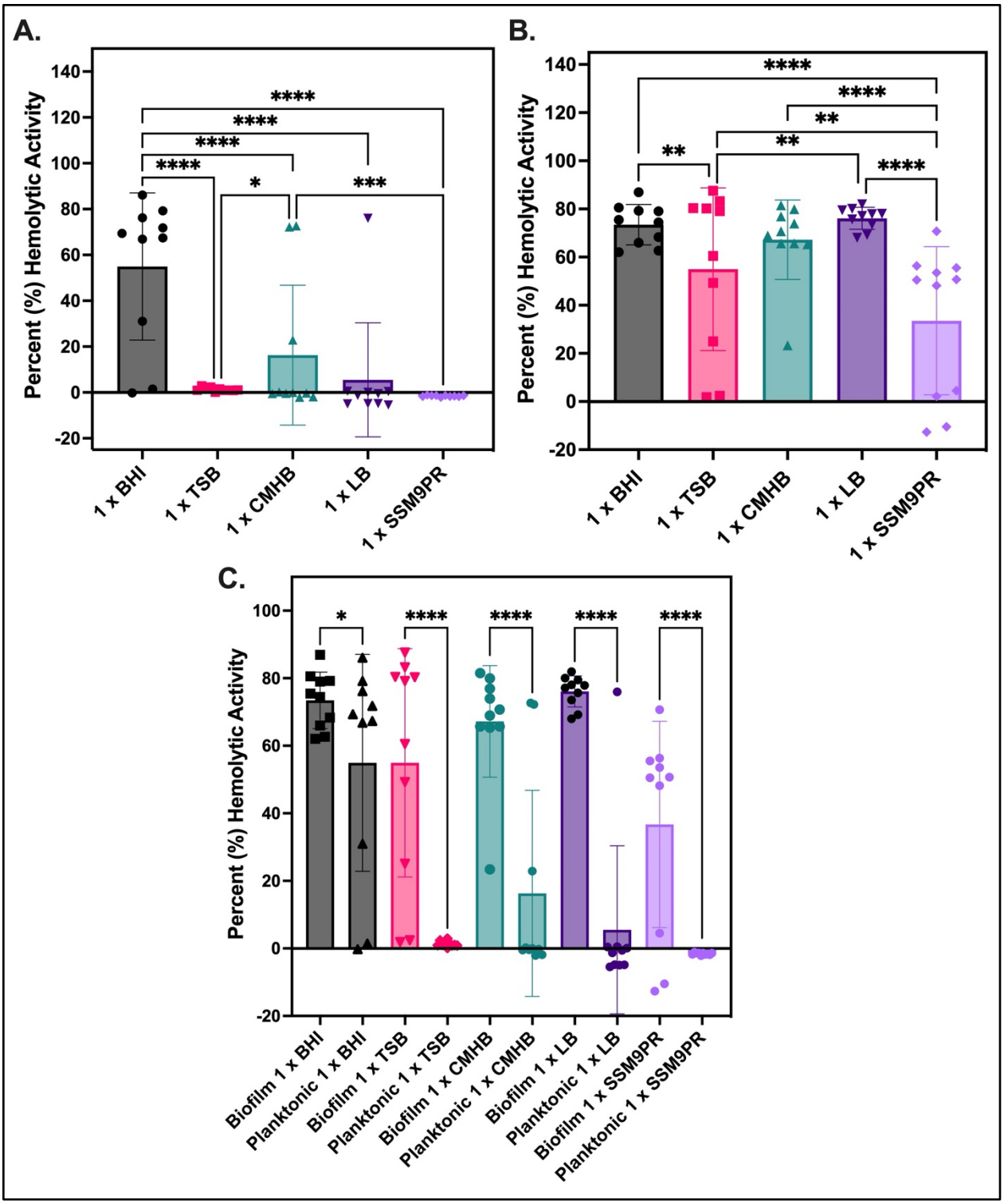

Figure 7: Percent hemolytic activity of clinical isolates grown in BHI, TSB, CMHB, LB, and SSM9PR media in planktonic and biofilm conditions. A. Depicts the percent hemolytic activity in planktonic clinical isolate cultures, B. shows the percent hemolytic activity in biofilm clinical isolate cultures, and C. illustrates both sets of data.

Quantitative Hemolysis Assay:

Statistical analyses were conducted using either one-way or two-way analysis of variance (ANOVA) with Tukey post hoc analysis using GraphPad Prism. As illustrated in **Figure 7A**, planktonic clinical isolate strains that were grown in BHI had 54.9% hemolytic activity, TSB had 2.37% hemolytic activity, CMHB had 16.3% hemolytic activity, LB had 5.51% hemolytic activity, and SSM9PR had -1.57% hemolytic activity, less than the negative control of just SSM9PR media. Planktonic clinical isolate strains grown in BHI on average had significantly greater hemolytic activity than when grown in TSB, CMHB, LB, and SSM9PR ($p \leq 0.0001$). Additionally, clinical isolates grown in CMHB on average had significantly greater hemolytic activity when compared to when grown in TSB ($p \leq 0.05$) and 1 x SSM9PR ($p \leq 0.001$). **In Figure 7B**, biofilm clinical isolate strains on average had 73.5% hemolytic activity when grown in BHI, 54.9% hemolytic activity when grown in TSB, 67.2% hemolytic activity when grown in CMHB, 76.1% hemolytic activity when grown in LB, and 33.6% hemolytic activity when grown in SSM9PR. Notably, strains grown in SSM9PR on average had significantly lower hemolytic activity than other nutrient environments ($p \leq 0.01$). Additionally, strains grown in LB and BHI on average had significantly greater hemolytic activity than those grown in TSB on average ($p \leq 0.01$). **Figure 7C** shows the biofilm and planktonic data grouped together. Across all media types, clinical isolates in a biofilm state had significantly greater hemolytic activity than their planktonic counterparts. For strains grown in BHI, biofilms had significantly greater hemolytic activity than planktonic bacteria ($p \leq 0.05$). In all other nutrient conditions, biofilms also had significantly greater hemolytic activity than planktonic strains ($p \leq 0.0001$).

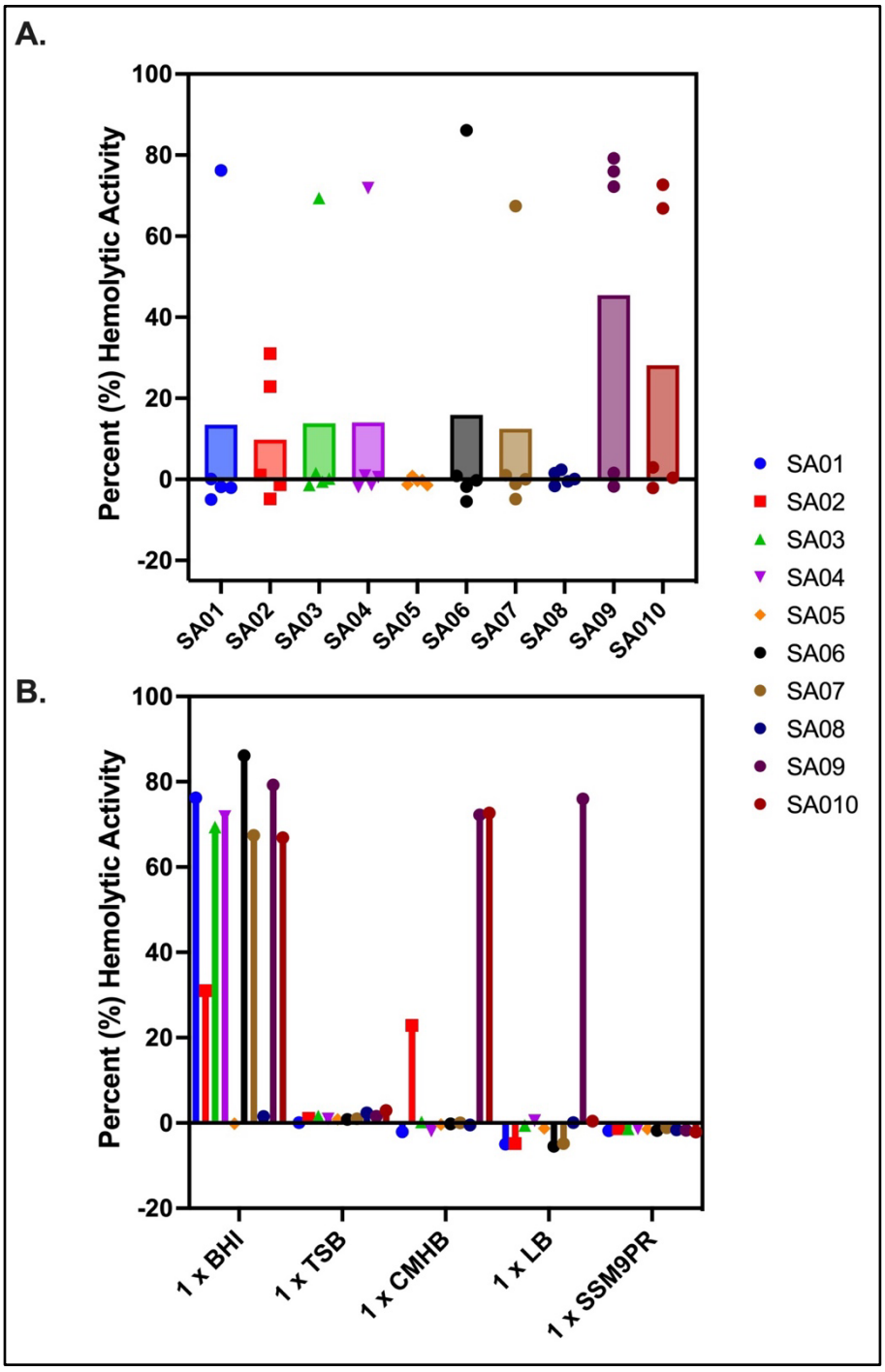

Figure 8: Planktonic hemolytic activity grouped by media and strain. 8A depicts the hemolytic activity that each planktonic strain exhibited across all media tested. 8B shows the hemolytic activity of each planktonic strain in the context of each media condition.

Figure 8A shows the distribution of percent hemolytic activity in each planktonic strain across all media conditions. There is a large standard deviation with these values, highlighting how important media conditions are when testing hemolytic activity. **Figure 8B** shows the percent hemolytic activity of each planktonic strain according to the media conditions. 9/10 of these planktonic strains exhibited the greatest hemolytic activity when grown in BHI while 1/10 planktonic strains, SA10, had slightly greater hemolytic activity when grown in CMHB.

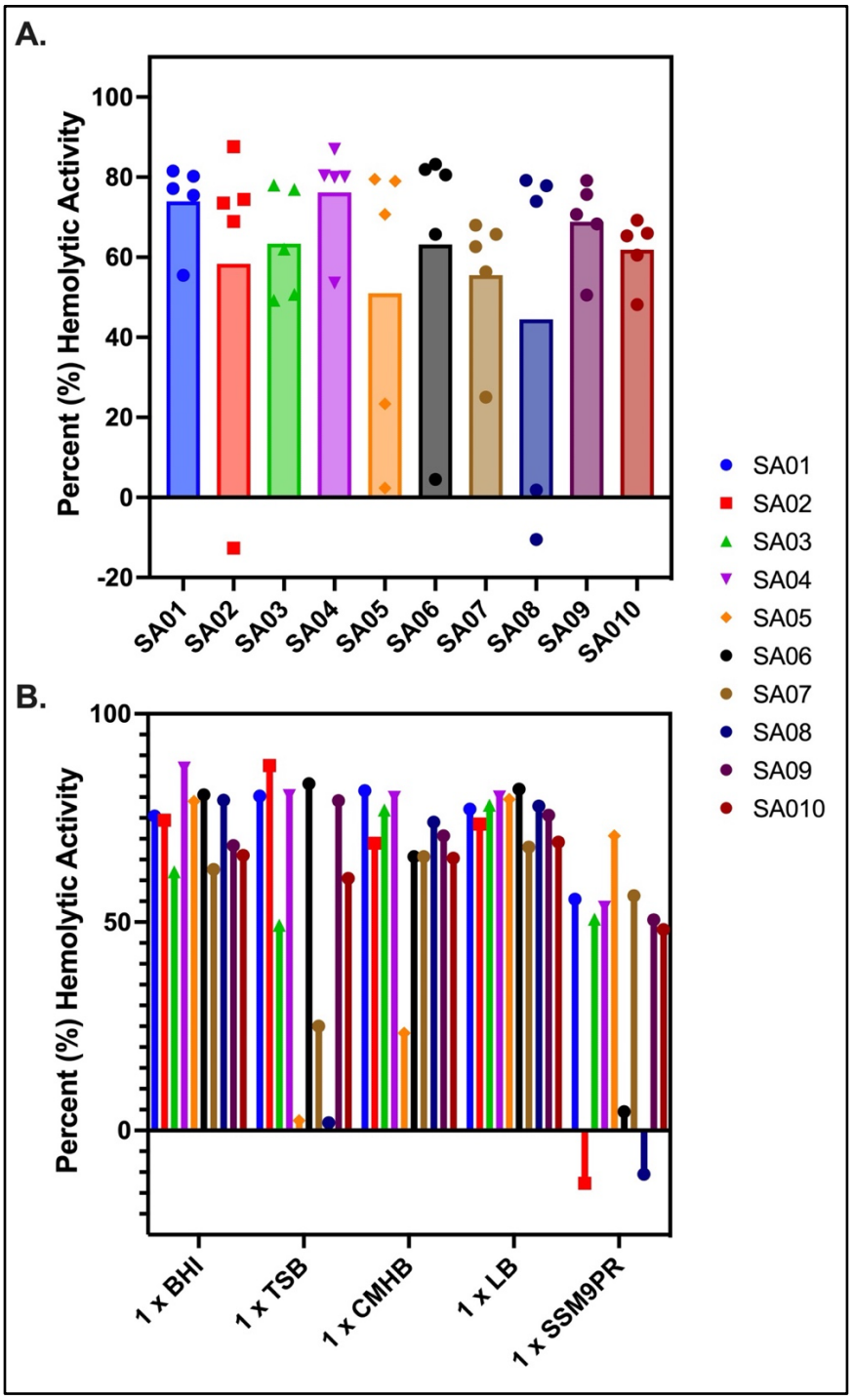

Figure 9: Biofilm hemolytic activity grouped by media and strain. Like **Figure 8**, 9A depicts the hemolytic activity that each strain exhibited across all media tested but under biofilm conditions. 9B shows the hemolytic activity of each biofilm in the context of each media condition. Averages and standard deviations were calculated in GraphPad Prism.

(9A: N=5 media conditions per strain, 9B: N=10 strains per media condition, Technical replicates: N ≥ 3 for each strain and media combination)

Figure 9A shows the range of percent hemolytic activity in each biofilm strain across all media conditions. As also seen in **Figure 8A**, there is a high amount of variation in most strains due to the impact of the environment that the biofilms are grown. As opposed to **Figure 8B**, in **Figure 9B** there is less distinction between the greatest amount of hemolytic activity. When grown in 1 x BHI, SA04 and SA08 had the greatest hemolytic activity. When grown in 1 x TSB, SA02, SA06, and SA09 had the greatest hemolytic activity. When grown in 1 x CMHB, SA01 had the greatest hemolytic activity. When grown in 1 x LB, SA03, SA05, SA07, and SA10 had the greatest hemolytic activity.

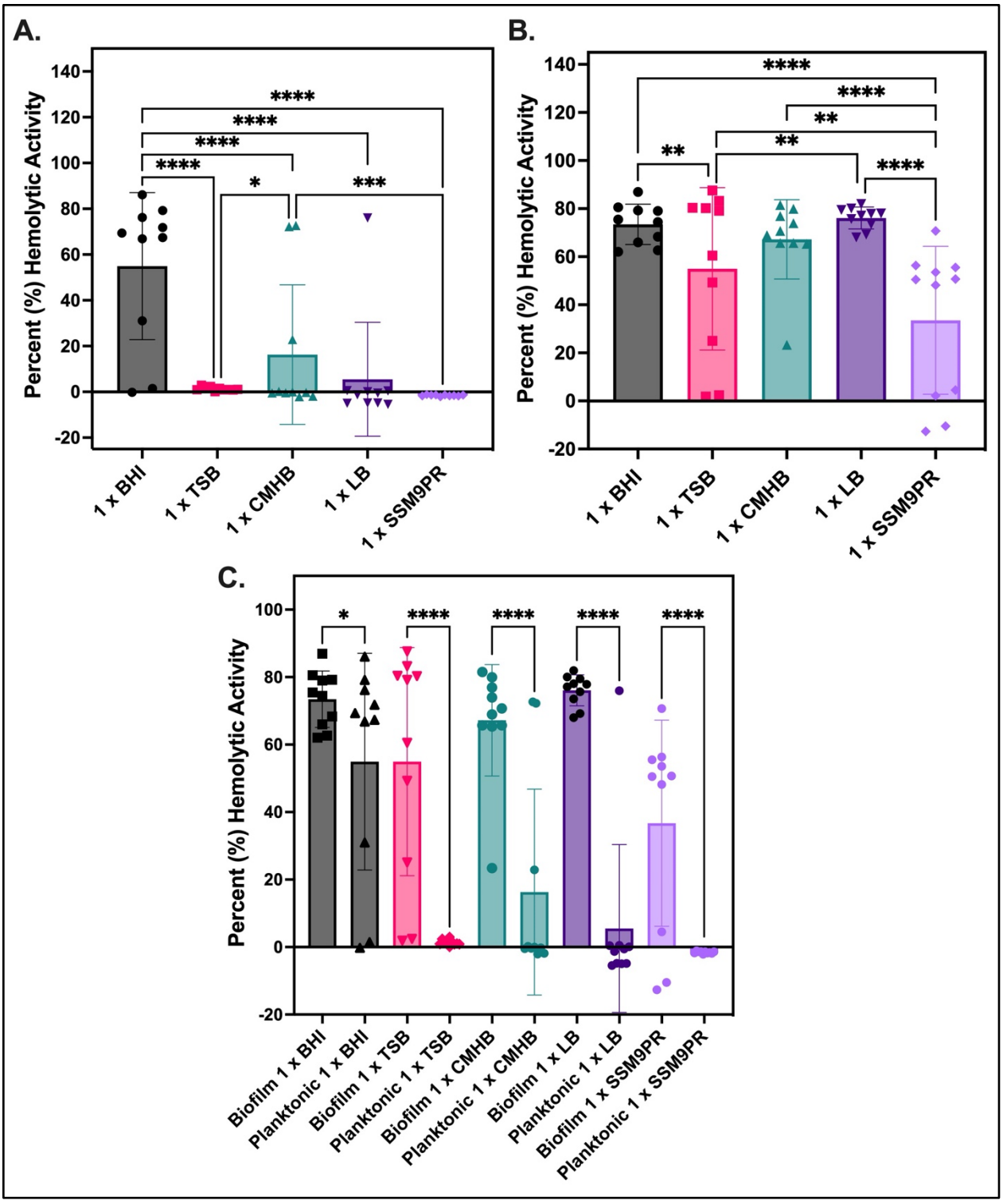

Figure 10: Normalized percent hemolytic activity of clinical isolates grown in BHI, TSB, CMHB, LB, and SSM9PR media in planktonic and biofilm conditions. These graphs are depicting the percent hemolytic activity of planktonic (10A), biofilm (10B), and both (10C). However, these data are normalized per 1 log CFU/mL of bacteria

by calculating the ratio of percent hemolysis to log CFU/mL. Two-way ANOVA with Tukey post hoc analysis in GraphPad Prism revealed significance between groups. (*=$p \leq 0.05$, **=$p \leq 0.01$, ***=$p \leq 0.001$,****=$p \leq 0.0001$) (Biological replicates: N=10 strains for each media, Technical replicates: $N \geq 3$ for each strain and media combination)

Figure 10 depicted the percent hemolytic activity of each strain normalized to 1 log CFU/mL of bacteria. Most of the same trends exist here as did in **Figure 7**, however, in **Figure 10**, there is no significant difference in hemolytic activity between biofilm and planktonic state per 1 log CFU/mL of bacteria when grown in 1 x BHI. This highlights that in 1 x BHI, both biofilm and planktonic bacteria exhibit high hemolytic activity.

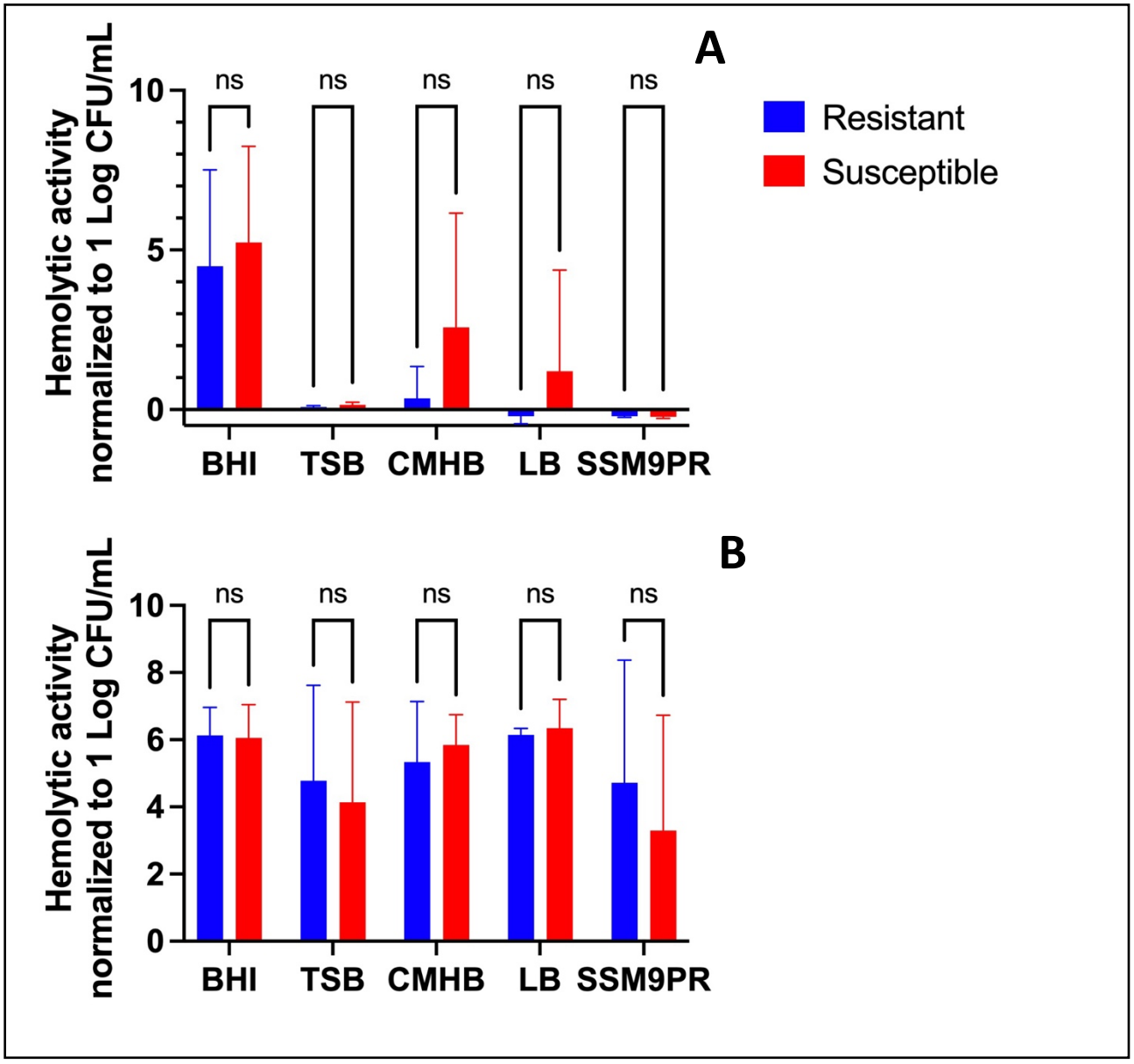

Figure 11: Resistant versus susceptible clinical isolate hemolytic activity. 11A shows the normalized percent hemolytic activity among planktonic cultures in resistant versus susceptible strains, while 11B shows the normalized percent hemolytic activity observes among biofilm cultures in resistant versus susceptible strains.

As **Figure 11A** shows, no significant difference in percent hemolytic activity per 1 log CFU/mL of bacteria between oxacillin-resistant and oxacillin-susceptible planktonic clinical strains. **Figure**

11B shows a similar trend, in that there was no significant difference observed in percent hemolytic activity per 1 log CFU/mL of bacteria between oxacillin-resistant and oxacillin-susceptible biofilm clinical strains.

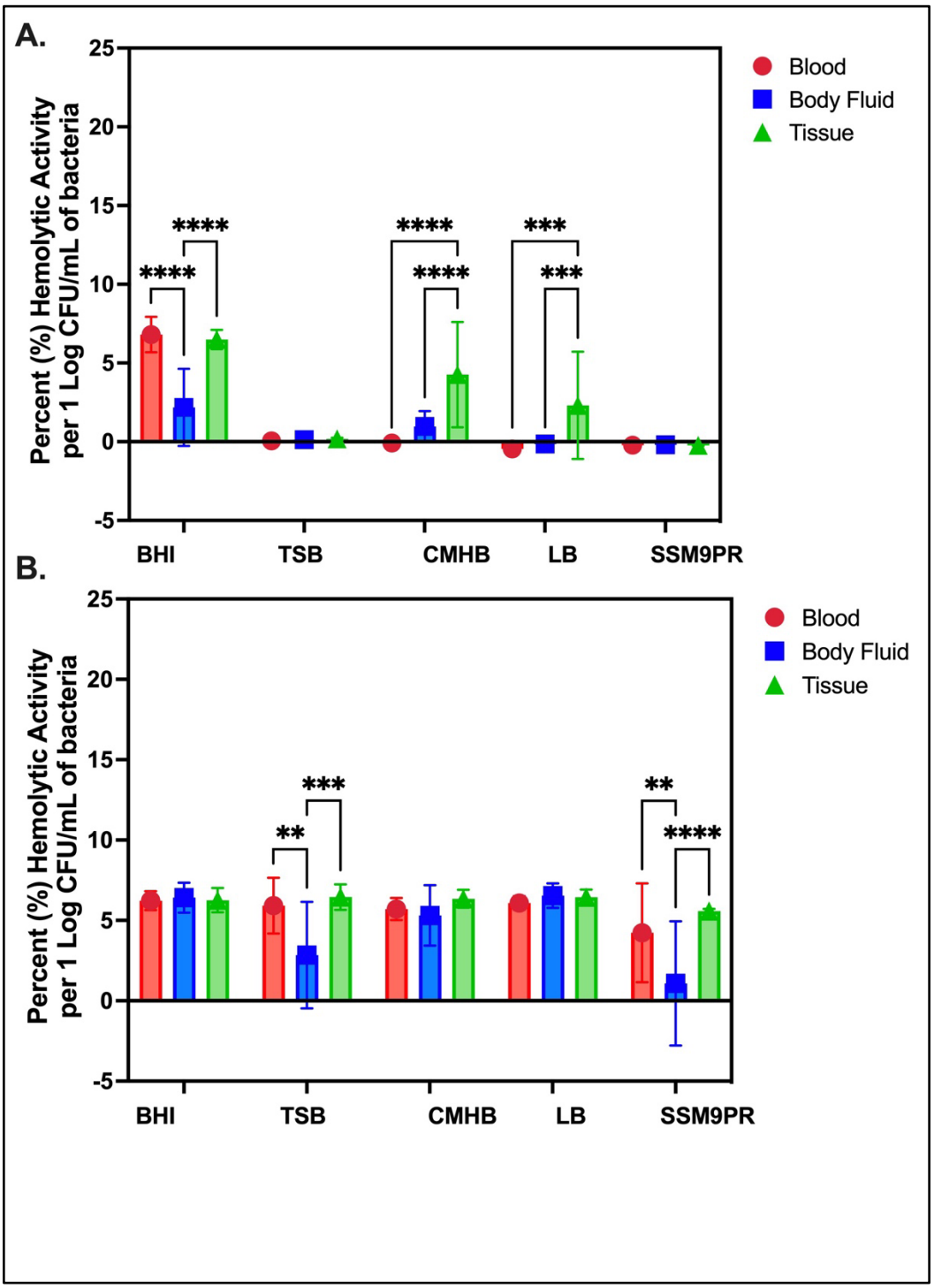

Figure 12: Site of Isolation and Hemolytic Activity. 12A exhibits the normalized hemolytic activity among planktonic cultures grouped by growth media to compare strains isolated from different sites in patients, including blood, body fluid, and tissue. 12B includes data grouped the same way as previously described but with biofilm cultures. . Two-way ANOVA with Tukey post hoc analysis in GraphPad Prism revealed significance between groups. (*=p≤0.05, **=p≤0.01, ***=p≤0.001,****=p≤0.0001) (Biological replicates: N=3 (blood and tissue), N=4 (body fluid), Technical replicates: N = 3 for each strain and media combination)

In **Figure 12A**, the strains are organized by their site of isolation from patients. When planktonic bacteria were grown in 1 x BHI, strains isolated from body fluid had significantly less hemolytic activity per 1 log CFU/mL than those isolated from blood or tissue (p≤0.0001). However, when planktonic bacteria were grown in 1 x CMHB and 1 x LB, strains isolated from tissue had significantly greater hemolytic activity per 1 log CFU/mL than those isolated from blood or body fluid (p≤0.001). **Figure 12B** includes the bacterial biofilm data. Interestingly, among biofilm strains grown in 1 x TSB and 1 x SSM9PR, strains isolated body fluid had significantly less hemolytic activity per 1 log CFU/mL than those isolated from blood or tissue (p≤0.01).

Chapter 4: Discussion and Conclusions

Little is currently known regarding the relationship between growth conditions and pathogenicity of bacteria. These studies uncover the implications of virulence as it pertains to the growth environment of the culture in vitro. It is especially important to explore these fundamental biological questions as it could inform the development of an antimicrobial therapeutic.

Through a preliminary media test in which we examined the growth of 10 clinical isolate strains, we observed that in different media environments the strains grew at different rates, as seen in Figure 1. TSB and BHI were shown to be the most optimal media to grow cultures of *S. aureus*, confirming what is known in literature [18]. However, we were especially interested in the specific nutrient components that contribute to high replication rates of bacterial cells in log phase. As listed in table, each of the media conditions that were utilized have a variety of components. The 1 x BHI and 1 x TSB both contained glucose and hydrolyzed proteins: peptones in 1 x BHI and tryptones and soytones in 1 x TSB. Glucose serves as source of energy for bacterial growth, as the sugars are broken down to provide the energy to the microbes [19]. Additionally, glucose has been reported to promote biofilm formation [20]. These hydrolyzed proteins also aid in fast microbial growth and serve as a source of nitrogen [21]. Identifying the importance of each component in these growth media begs the question: what other factors of microbial activity are dependent on their environment? This question motivated us to explore the relationship between different media and other characteristics such as virulence.

The hemolytic activity quantitative tests confirmed that 1 x BHI media caused planktonic clinical isolates to exhibit the greatest amount of hemolytic activity. When considering the components of

the media that may be inducing the release of these virulence factors, two notable components of 1 x BHI media that were not shared with other media components are beef heart and calf brains. These ingredients have been reported to provide sources of carbon, nitrogen, growth factors, amino acids, and vitamins [22]. We hypothesize that in the presence of cardiac and neural proteins from cows, mammalian cell proteins were recognized, and the 5-Lox pathway was triggered to release hemolysin into the culture.

Interestingly, there was a significant difference between the hemolytic activity of planktonic and biofilm cultures. These results indicate that in the biofilm state, bacteria release more hemolysin into their environment. This finding highlights the importance of developing an effective therapeutic to eradicate biofilm infections and minimize host cell damage. This finding also informs us that *in vitro* biofilm modeling may not necessarily be dependent on the growth media that is utilized. Moreover, the resilience of biofilm hemolytic activity independent of environmental components further emphasizes the severity of this issue.

When comparing the percent hemolytic activity per log CFU/mL of planktonic and biofilm cultures across all media conditions, we observed no significant difference in the resistant and susceptible clinical isolate strains. While this analysis indicates that strain susceptibility is not a confounding variable of hemolytic activity, further testing is needed with a greater strain sample size ($N > 5$) to confirm this finding. While no significant difference between hemolytic activity and site of isolation was observed, a larger sample size of strains from each site ($N > 3$) is necessary to draw any conclusions about the differences in pathogenicity of strains based on their original location in a patient.

There is currently a gap in knowledge in the community pertaining to the relationship between media condition and virulence as well as bacteria state and virulence. This thesis seeks to bridge the gap of knowledge. Most notably, quantitative hemolytic data revealed that biofilms cause more hemolysis than their planktonic counterparts, and the biofilms were able to sustain high hemolytic activity independent of growth media. Future studies could explore other the relationship of other virulence factors to growth media, state of bacteria, site of isolation, or susceptibility status. Additionally, the development of a hemolysin-responsive biomaterial could serve as a biomimetic platform in targeting the infection while mitigating host cell toxicity. Antimicrobial resistance is a growing global-health threat, and the virulence of both planktonic and biofilm infections is cause for concern. More exploration into these challenging fundamental questions brings us steps closer to a solution.

References

[1] T. Taylor and C. Unakal, "Staphylococcus Aureus," *StatPearls,* January 2022.

[2] "MRSA and the Workplace | NIOSH," August 2015. [Online].

[3] G. Silva-Santana and A. L. Mattos-Guaraldi, "Staphylococcus aureus biofilms: an opportunistic pathogen with multidrug resistance," *Reviews and Research in Medical Microbiology,* vol. 32, no. 1, pp. 12-21, January 2021.

[4] Antibiotic Resistance in the United States, CDC, 2019.

[5] H. L. a. J. S. Kim, "Bacterial Targets of Antibiotics in MRSA," *Antibiotics,* vol. 10, no. 4, p. 398, 2021.

[6] D. Sharma, L. Misba and A. Khan, "Antibiotics versus biofilm: an emerging battleground in microbial communities," *Antimicrobial Resistance & Infection Control,* vol. 8, no. 76, 2019.

[7] E. Green and J. Mecsas, "Bacterial Secretion Systems – An overview," *Microbiol Spectr.,* vol. 4, no. 1, 2016.

[8] M. Popoff, "“Bacterial Toxins” Section in the Journal Toxins: A Fantastic Multidisciplinary Interplay between Bacterial Pathogenicity Mechanisms, Physiological Processes, Genomic Evolution, and Subsequent Development of

Identification Methods, Efficient Treatment, and," *Toxins (Basel),* vol. 10, no. 1, p. 44, 2018.

[9] J. T. Barbieri, "Exotoxins," *Encyclopedia of Microbiology,* vol. 3, 2009.

[10] E. Romp, V. Arakandy, J. Fischer and C. Wolz, "Exotoxins from Staphylococcus aureus activate 5-lipoxygenase and induce leukotriene biosynthesis," *77,* vol. 19, 2020.

[11] D. Oliveira, "Staphylococcus aureus Toxins and Their Molecular Activity in Infectious Diseases," *Toxins, Bassel,* vol. 10, no. 6, p. 252, 2018.

[12] F. e. al., "Heme Oxygenase-1: A Critical Link between Iron Metabolism, Erythropoiesis, and Development," *Adv Hematol,* vol. 473709, p. 2011, 2011.

[13] J. W. Peterson, "Bacterial Pathogenesis," in *Medical Microbiology 4th edition*, Galveston, TX, University of Texas Medical Branch at Galveston, 1996.

[14] V. P. e. al., "The Agr Quorum-Sensing System Regulates Fibronectin Binding," *Infection and Immunity,* vol. 82, no. 10, pp. 4337-4347, 2014.

[15] A. R. e. al., "Comparative proteome analysis of Staphylococcus aureus biofilm and planktonic cells and correlation with transcriptome profiling," *Proteomics,* vol. 6, no. 6, pp. 1867-1877, 2006.

[16] A. C. G. e. al., "Virulence Factors Produced by Staphylococcus aureus Biofilms," *Molecular and Cellular Proteomics,* vol. 18, no. 6, pp. 1036-1053, 2019.

[17] A. S. f. Microbiology, "Blood Agar Plates and Hemolysis," 2016.

[18] D. M. &. O. Schneewind, "Growth and Laboratory Maintenance of Staphylococcus aureus," in *Curr Protoc MIcrobiol*, 2013, p. Chapter 9 Unit 9C.

[19] S. Reischke, "The effects of glucose loading rates on bacterial and fungal growth in soil," *Section of Microbial Biology,* vol. 70, pp. 88-95, 2013.

[20] K. S. S. e. al., "Role of Glucose-6-Phosphate in Metabolic Adaptation of Staphylococcus aureus in Diabetes," vol. 9, no. 2, 2021.

[21] H. L. e. al., "Engineering Prospectives in Biotechnology," vol. 3, 2011.

[22] G. Wijesinghe, "Influence of Laboratory Culture Media on in vitro Growth, Adhesion, and Biofilm Formation of Pseudomonas aeruginosa and Staphylococcus aureus," vol. 28, no. 1, 2019.

[23] J. Lei, L. Sun and Q. He, "The antimicrobial peptides and their potential clinical applications," *Am J Transl Res,* vol. 11, no. 7, pp. 3919-3931, 2019.

[24] H. Lade and J.-S. Kim, "Bacterial Targets of Antibiotics in Methicillin-Resistant Staphylococcus aureus," *Antibiotics,* vol. 10, no. 4, p. 398, 2021.