Determining the impact of host-synovial fluid factors on *Staphylococcus aureus* aggregation

Research Thesis

Presented in partial fulfillment of the requirements for graduation *with research distinction* in Microbiology in the undergraduate colleges of The Ohio State University.

by

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Abstract

Chronic periprosthetic joint infections (PJIs) are serious complications of joint-replacement surgeries. These often require subsequent corrective surgeries, as well as many rounds of antibiotics, to correct. Over the past few years, staphylococci have been identified as one of the most common organisms to cause PJIs. Capable of forming biofilms, bacterial communities encased in an extracellular polymeric slime matrix, staphylococci infections can easily become very difficult to clear. This can be due to increased antimicrobial resistance conferred by the biofilm as well as the generation of persister cells[1]. Current hypotheses suggest, prior to the formation of biofilms within PJIs, *Staphylococcus aureus* will form cellular aggregates[2]. To investigate the development of *S. aureus* aggregates we will utilize fluorescence-activated cell sorting (FACS) and light microscopy to differentiate and analyze aggregated and non-aggregated populations. Formation of aggregates has been reported in the presence of numerous factors present in the fluid present in joints, known as synovial fluid. These factors include fibrin[3], fibronectin[3], and hyaluronic acid[4]. Our preliminary data has shown that extracellular DNA (eDNA) might play a role in aggregation and it has been observed that eDNA is essential in the formation of biofilms[5]. We sought to determine the contribution of these factors in the formation of *S. aureus* aggregates. Finally, while there are a number of formulations for artificial synovial fluid (ASF) based on matching viscoelastic parameters for *in vitro* wear testing, there is not a model designed with microbiological testing in mind. Herein, we developed an ASF based on the previously reported concentrations of proteins, lipids, extracellular DNA, as well as hyaluronic acid.

Acknowledgements

First and foremost, I would like to thank Dr. Paul Stoodley for welcoming me into his lab, sharing ongoing projects, as well as trusting me and giving me the freedom to take responsibility and work on my own part of this project. His support of my scientific interests and endeavors has been paramount in my growth as a scientific researcher and has helped to invigorate me in the upcoming pursuit of my doctorate.

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I would like to thank Dr. Madhura Pradhan for graciously agreeing to be a part of this thesis committee. I would like to thank the Ohio State Wexner Medical Center Department of Microbial Infection and Immunity, the Ohio State University College of Arts and Sciences, and the Department of Microbiology. I would also like to thank the National Institute of Health for the financial support of this thesis.

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1. Introduction

Bacterial biofilms constitute a major problem within the US healthcare industry. Biofilms are bacterial communities formed when planktonic cells adhere to a surface and encase themselves in an extracellular matrix composed of various extracellular polymeric substances. These substances include polysaccharides, proteins, and DNA[5]. Prior research has observed increased antibiotic tolerance of the bacterial communities encased within biofilms[5]. With the rise of infection rates also comes a rise in antibiotic usage, thus leading to populations of antibiotic-resistant microorganisms. Problems facing infection are not uncommon in the instance of joint arthroplasties, with infection rates occurring in up to 2% of total knee arthroplasties and reoccurring in up to 10% of revisions[6]. PJIs and complications associated with PJIs are associated with high rates of morbidity and a growing body of data suggests that biofilms formed within the wound are responsible[7]. More severe consequences of infection include increased risk of death within one year[8]. In addition to the high morbidity and mortality associated with PJI, the costs of treatment make this research increasingly relevant, as the instance of PJIs nationally will cost the US healthcare system upwards of $1.6 billion by the year 2020[9]. Two bacterial pathogens in particular, *Staphylococcus aureus* and *Staphylococcus epidermidis*, are among the most prevalent transgressors and are responsible for around 45% of infections[10]. Furthermore, *S. aureus* is the most common agent in the instance of septic arthritis in many countries[11].

Prior studies suggest orthopedic joint infection occurs as a multi-step process (Fig. 1)[2]. Bacterial cells enter the wound, form aggregates, and then the collective phenotype of the population shifts toward a biofilm phenotype, leading to secretion of extracellular

matrix components and subsequent attachment to the artificial joint[2]. While *S. aureus* biofilms are beginning to be understood in the instance of orthopedic joint infection, not much is known about the interaction between single cells and synovial fluid present in joints during aggregate formation prior to biofilm formation and attachment (Fig. 1). Synovial fluid is responsible for lubricating components within joints. Prior studies have linked the formation of aggregates to compounds within synovial fluid such as hyaluronic acid[4], fibrin[3], and fibronectin[3]. Our preliminary data also suggests that extracellular DNA might play a role in aggregation within synovial fluid. Interestingly, fibrin and fibrinogen are not typically found in healthy synovial fluid and are present only in the instance of injury[12] or arthritis[13].

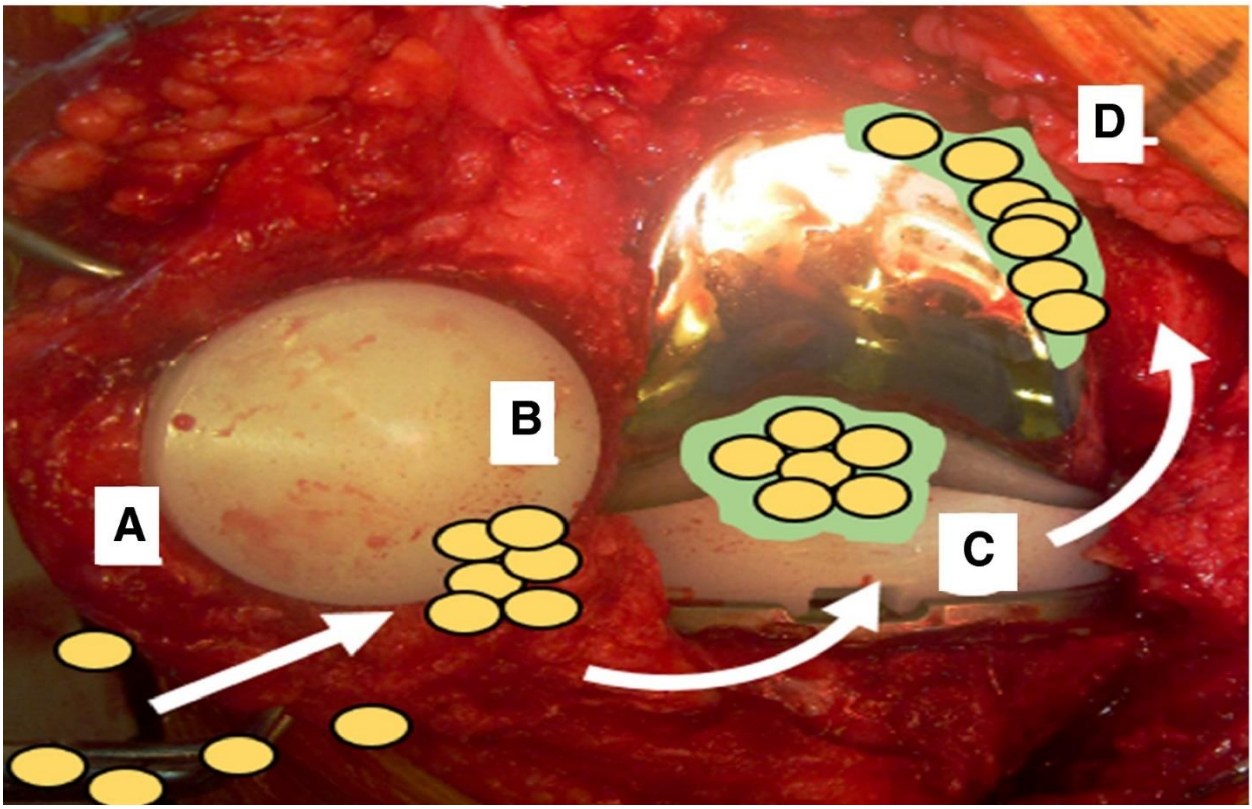

Figure 1[2]: The hypothesized stages of *S. aureus* orthopedic joint infection during knee reconstruction. Single cells of enter the site of infection (A), these cells rapidly aggregate (B), the formed aggregate switches to a biofilm phenotype, producing exopolymeric

substances and (C) subsequently attaches to the surface of the artificial knee and (D) forms a mature biofilm.

It is common practice to use a 10% bovine synovial fluid solution to induce aggregation when examining *S. aureus*[3,4]. However, there are several issues with this method. First, the solution made from synovial fluid is variable in the concentrations of protein (Appendix A). Therefore, it is difficult to directly compare the results from different experiments. General trends can be observed, but direct comparisons are difficult to make. To overcome this, individual components known to be present in synovial fluid are going to be individually examined to determine if the component contributes to *S. aureus* aggregation. After identifying which synovial fluid components induce aggregation, an artificial synovial fluid was developed for standardizing and quantifying bacterial aggregation. This thesis contains two primary aims: to determine the relative contribution of various compounds of synovial fluid and, using this knowledge, to come up with an initial recipe for an artificial synovial fluid. This artificial synovial fluid will then be used to study the kinetics behind *S. aureus* aggregation in future studies and will function as the basis of analyzing aggregation in other staphylococcal species.

2. Materials and Methods

Bacterial Strains and Growth Conditions

In order to investigate varying rates of aggregation as well as breadth of rapid screen application, varying laboratory and clinical *S. aureus* isolates were used (Table 1). To assess the impact of hyaluronic acid on aggregation, a transposon mutant for *hysA*, a gene encoding for a hyaluronidase, from the Network on Antimicrobial Resistance in *Staphylococcus aureus* was used in a similar fashion to what has been previously reported[4]. Strains were incubated in tryptic soy broth overnight at 36°C under shaking conditions. These stationary cultures were then washed and stained with SYTO™ 9 green fluorescent dye, following the manufacturer's protocol, before being used in both the 96 well plate aggregation assay and fluorescent-activated cell sorting assay.

Strain #	Description	Notes
UAMS1	USA300	Osteomyelitis isolate
SAP231	MRSA	Community acquired, lux
WLS4960	USA300 ΔsarA	Low-biofilm forming
BS188		High-biofilm forming, suture isolate
BS187		Mesh isolate
NE334	TN *hysA*	Hyaluronidase precursor mutant

Table 1: Clinical and laboratory strains used in this work. NE 334 mutant acquired from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA).

Aggregation solutions

Solutions used in all methods of quantifying aggregation were made using biological concentrations of each individual compound, based on concentration present in healthy synovial fluid with the exception of fibrinogen. As fibrinogen is not present in

healthy synovial fluid, the concentration found in arthritic patients was used for the purpose of these studies[13]. Concentrations of serum abumin[14] and hyaluronic acid[15] in healthy joints were used for this work. Concentration of DNA was based off preliminary data. 10% bovine synovial fluid in phosphate buffered saline (PBS) or Ringer's Solution (RS) based on previously reported method[3]. There was no significant difference between PBS and RS with regards to aggregation. Artificial synovial fluid was prepared by combining all the synovial fluid components outlined in Table 2 together.

Compound	Concentration
Bovine Serum Albumin (BSA)	19 mg/mL
Salmon Sperm DNA	1 mg/mL
Hyaluronic Acid (HA)	3 mg/mL
Human Derived Fibrinogen (FN)	0.172 mg/mL

Table 2: Component concentration of various synovial fluid components in healthy human adult synovial fluid. All component solutions are based on healthy human synovial fluid except for fibrinogen, which is based on the concentration found in osteoarthritic patients.

SpectraMax 96 Well Plate Reader

A SpectraMax MiniMax 300 Imaging Cytometer (Molecular Devices) was used to both record absorbance (OD_{600}) and fluorescence measurements for SYTO™ 9 stained cells. Microscopic images were also recorded using the device with focus settings at 900 µm and image stitching being performed by the SoftMax Pro software. Images, OD_{600} and fluorescence measurements were taken at intervals of 15 or 30 minutes for up to 3 hours, with intermittent orbital or linear shaking performed between measurements.

Fluorescent-Activated Cell Sorting (FACS) Aggregation Assay

After staining as described above, 250 µL of culture was incubated in 500 µL aggregation solutions (Table 1) for one hour. The bottom 250 µL of the solution was then added to a

FACS tube, diluted, and FITC, forward scatter, and side scatter, was quantified using a BD FACSCanto II. After measurements were taken, FlowJo v9.0 software was utilized to measure bacterial aggregate size and quantity. FITC was used to differentiate between bacterial cells and synovial fluid components. Gates were then used to differentiate between single cell and aggregate events.

Statistical Analysis

One-way analysis of variance (ANOVA) was used to analyze the significance of the various components in addition to a Tukey post-test to compare each aggregation solution to the PBS or RS control.

3. Results

96 Well Plate Screen

Initially, absorbance was tested as a method of quantifying aggregation. In the instance of aggregation, absorbance increased over the course of the experiment. This trend was observed in both high- and low-biofilm forming strains. We hypothesize that increased biofilm forming ability correlates with increased aggregation rate. We observed aggregation in strains WLS4960, BS188, BS187, UAMS1, and SAP231 over the course of 160 minutes (Fig. 2). In these studies, 10% synovial fluid and Ringer's solution served as positive and negative controls respectively.

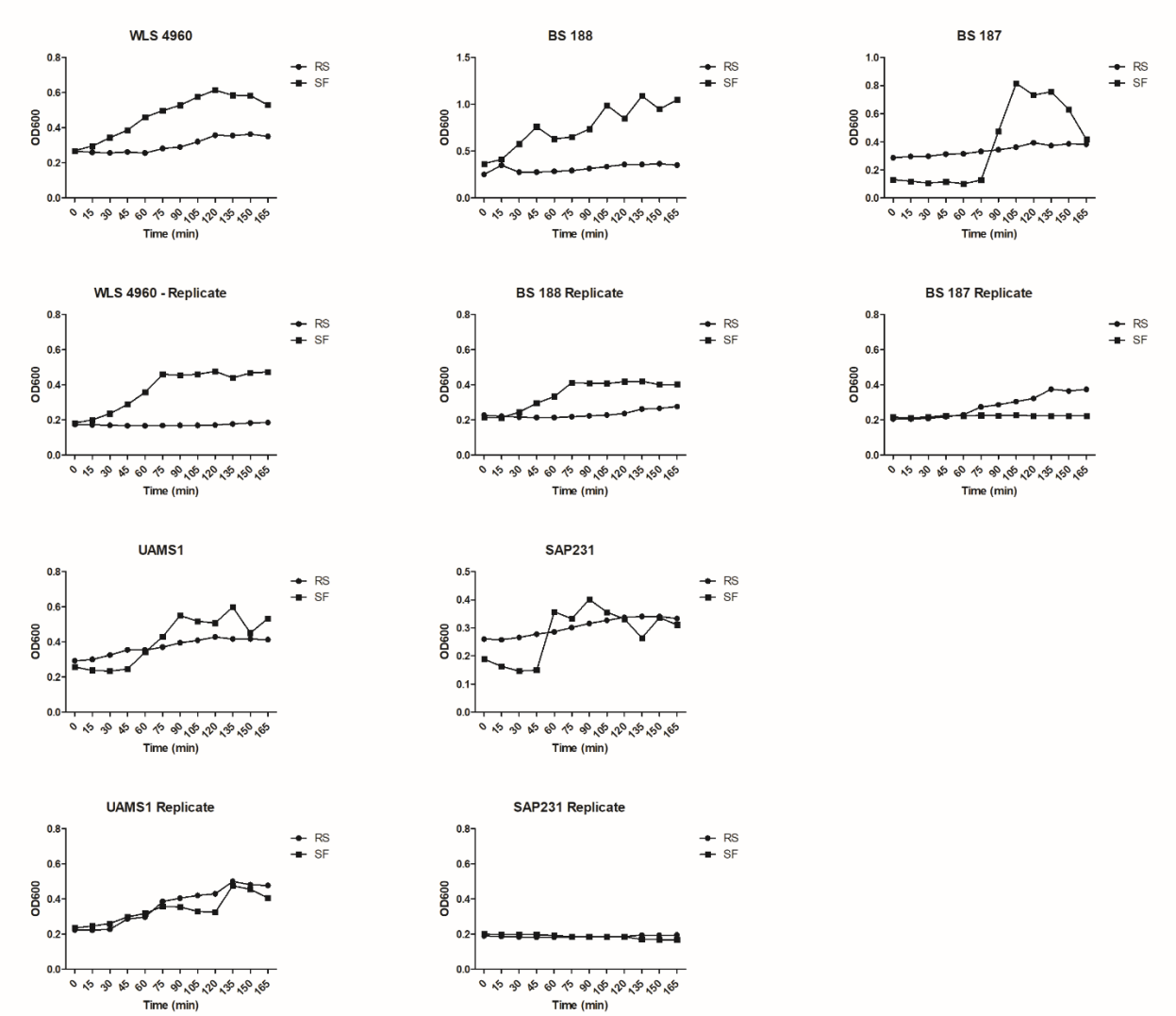

Figure 2: OD_{600} measurements over 165 minutes between various clinical and laboratory strains. Initial studies demonstrating trends for aggregation are shown above replicate values. Data presented as a representation of severity of inconsistency among data collection. Initial studies from the same experiment, replicate studies from the same experiment. SF = synovial fluid. Data presented as a single point, n=1 per experiment.

Following optical density measurements, fluorescently stained cells were then used as another means to quantify aggregation (Fig. 3). Addition of the synovial fluid lead to the formation of dense cell clusters within these strains as observed in the images captured by the SpectriMax imaging after 60 minutes incubation (Fig. 3). Different densities of cells were observed, where BS 188 has been observed to form many smaller

clusters while UAMS1 and WLS 4960 have been observed to form large, dense central aggregates. Fluorescence measurements show negative trends in both the control and 10% synovial fluid treatment groups. The negative trend in the control wells could be due to the typical settling of the cells in the center of the well.

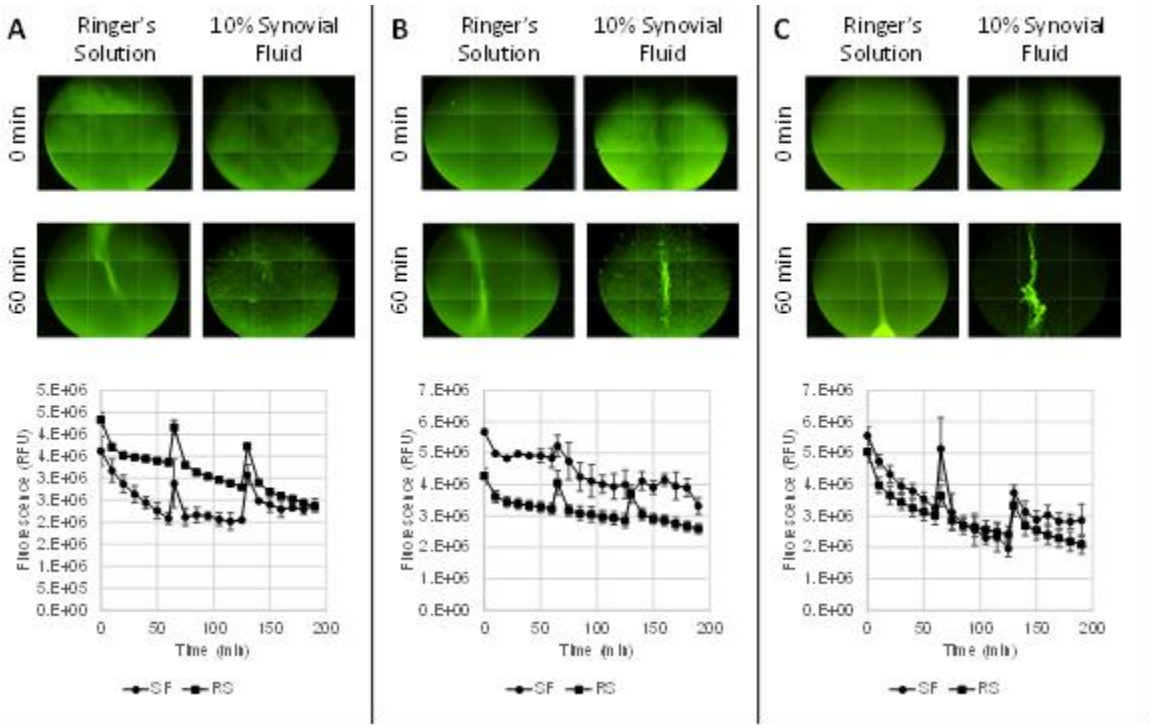

Figure 3: Representative images of the large aggregates formed after 60 minutes exposure to 10% synovial fluid. Ringer's solution control for comparison. Strains shown are A) BS 188, B) UAMS1, and C) WLS 4960. Fluorescence measurements presented as mean ± SD, n=3. SF = synovial fluid

FACS Aggregation Quantification

FACS was used as a second method of quantifying *S. aureus* aggregation. Reproducible trends were observed across different experiments, suggesting that this method would lead to more consistent observations. Additionally, FACS functions allows for the objective measure of a large number of particles in comparison to the limited nature of microscopy. Therefore, FACS was utilized to determine the role of individual synovial

fluid components in aggregation. The first component tested was bovine serum albumin, a protein found in high concentrations of serum and synovial fluid[14]. *S. aureus* showed a minor aggregative response to BSA in two strains, UAMS1 and SAP231 (Fig. 4). Aggregation was not observed when the cells were tested further against extracellular DNA in any of the strains. This result held true for both high biofilm and low biofilm forming strains.

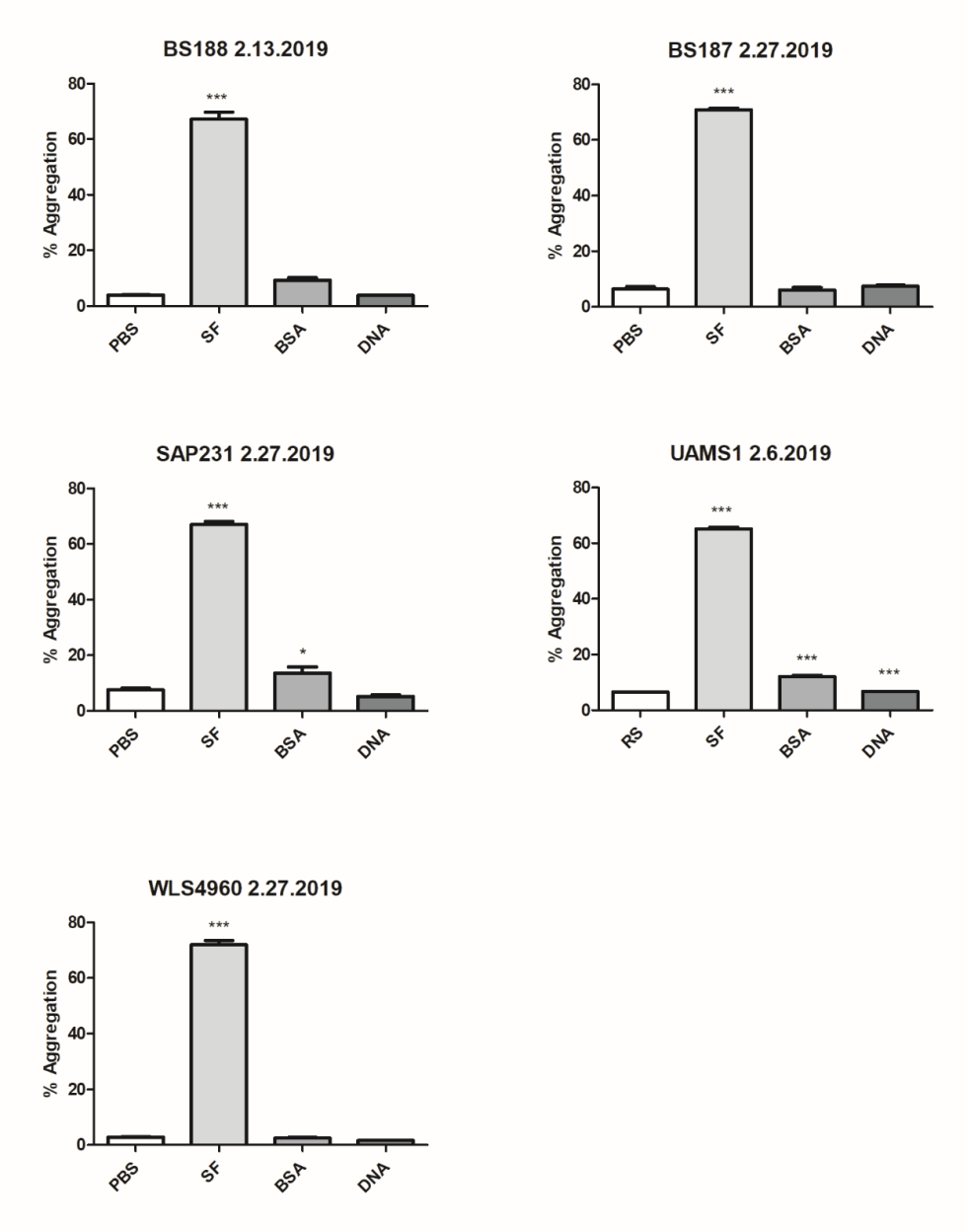

Figure 4: Clinical and laboratory strains tested against bovine serum albumin and eDNA, independently. Data presented as mean ± SEM, n=3. *p<0.05, ***p<0.001.

Aggregation was observed when the cells were tested against the solution containing fibrinogen. This result yielded the highest number of aggregation events when compared to other components. Hyaluronic acid was tested against high- and low-biofilm

forming strains of bacteria in addition to *S. aureus* str. NE334, a strain containing a transposon mutation in *hysA* (Fig. 5). None of the strains demonstrated the ability to aggregate utilizing hyaluronic acid, contradictory to the observations made by Ibberson et al (2016). when analyzing aggregation of the *hysA* mutant within synovial fluid[4]. These results could be attributed to the difference in quantification methods, where the original experiment utilized confocal in comparison to the rapid FACS measurements used in this work.

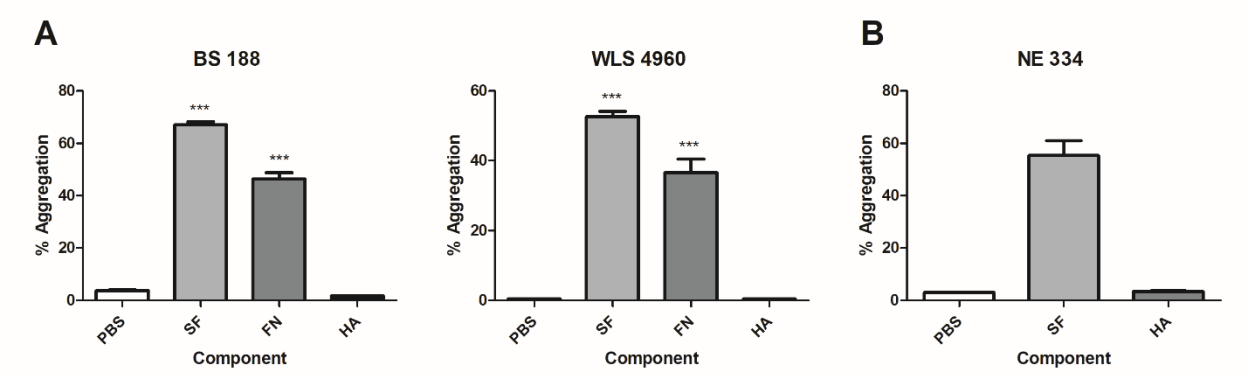

Figure 5: A) Results from testing high- and low-biofilm forming strains against fibrinogen and hyaluronic acid. B) Results of testing Tn-*hysA* mutant against hyaluronic acid, based prior research[4]. Data presented as mean ± SEM, n=3. ***p<0.001.

The next step was to test the different components in combination to determine if interactions between synovial fluid components further promote aggregation compared to individual components. Although aggregation was not observed in eDNA alone interactions between eDNA and BSA could promote aggregation more than BSA alone. Strains were tested against a solution of both serum albumin and eDNA. There was not a significant difference between the solution of serum albumin and eDNA when compared

to the control of serum albumin alone. It was also observed that BSA with eDNA demonstrated similar aggregation trends when compared to BSA alone. There was no significant difference in the percentage of aggregation events measured between these solutions (Fig. 6). This indicates that the addition of eDNA does not significantly impact the aggregation induced by BSA alone.

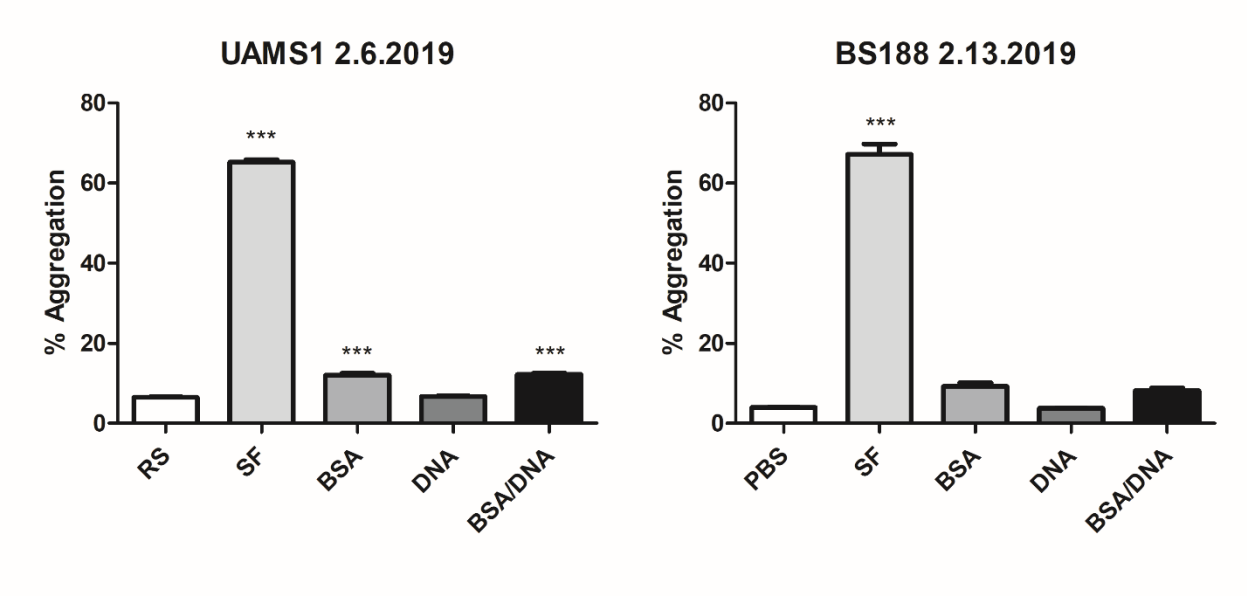

Figure 6: Results of testing *S. aureus* against BSA and eDNA in concert as well as individual solution controls. Data presented as mean ± SEM, n=3. ***p<0.001.

Considering BSA and fibrinogen promote aggregation individually, the next step was to determine if fibrinogen and BSA, together, compounded aggregation when compared to each component individually. BS188 and WLS4960 were tested against a solution containing biologically relevant concentrations of BSA and fibrinogen (Fig. 7). The difference between synovial fluid and the solution of BSA with DNA was still significant indicating that additional synovial fluid compounds may also contribute to aggregation.

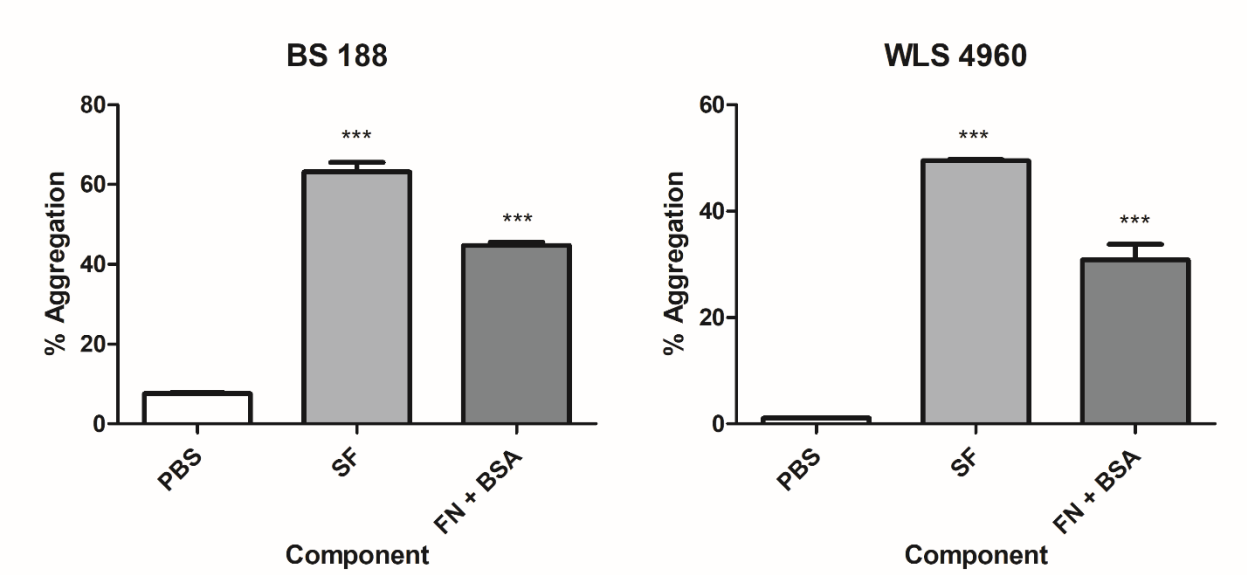

Figure 7: Experimental results for high-biofilm vs low-biofilm forming strains when tested against a solution of both fibrinogen and bovine serum albumin. Data presented as mean ± SEM, n=3. ***p<0.001.

Finally, the next step in the development of the ASF was to add each of the investigated components into one solution to determine if a complex interaction between all four of the tested components was responsible for the aggregation observed in the synovial fluid. The of ASF was significantly different than the 10% synovial fluid control. Compared to fibrinogen alone as well as fibrinogen with BSA, the ASF promoted similar levels of aggregation (Fig. 8).

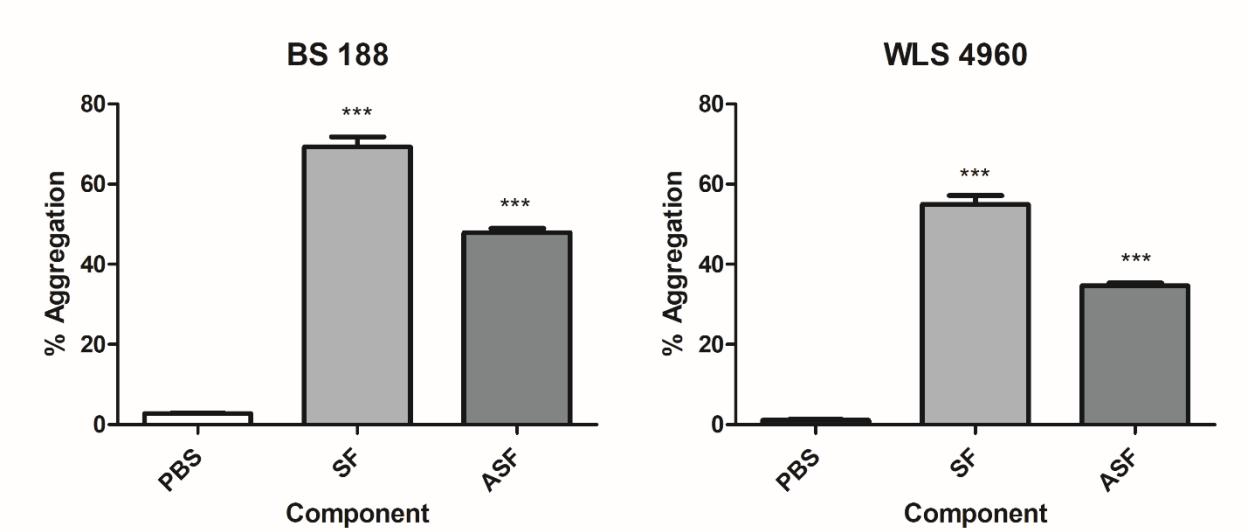

Figure 8: Experimental results of testing high- and low-biofilm forming strains against the proposed artificial synovial fluid. Data presented as mean ± SEM, n=3.

4. Conclusions and Future Directions

Multiple methods were used to measure *S. aureus* aggregation to ensure both accuracy and reproducibility of results. Optical density was useful for establishing early trends. This method needs to be further optimized, as current results yielded high standard deviation among replicates. Although OD_{600} measurements have shown to be limited in reproducibility, initial data shows that measuring fluorescence might be a valid way to measure aggregation (Figure 3). The variation between fluorescence measurements was much lower than OD_{600} measurements and demonstrates a method that, when further optimized, will allow for the rapid analysis of bacterial aggregation. Potential ways to improve this assay include shaking the plates before measuring fluorescence and increasing the concentration of bacterial cells in order to increase aggregate size leading to better imaging. Optimization of this assay would allow for a broad, easily reproducible assay for determining aggregation kinetics. Additionally, fluorescent images captured match the observations made with the FACS aggregative populations. The rapid measurement and visualization of *S. aureus* aggregation can act as a diagnostic tool for determining the presence of biofilm forming bacteria.

Given the limitations in the SpectraMax measurements, flow cytometry was utilized to verify the trends observed. Bacterial aggregation was noted when only some strains were tested against serum albumin. This response to bovine serum albumin could function as a test for classifying aggregation characteristics in future work and allowing for the differentiation between strains. Fibrinogen was observed to promote aggregation in both high- and low-biofilm forming strains, indicating that in the instance of inflammation fibrinogen likely plays a role in *S. aureus* aggregation during infection. The solution of

fibrinogen and serum albumin was still significantly different from the 10% synovial fluid, indicating that a component is still missing from the artificial synovial fluid that would allow for the artificial synovial fluid to match the aggregation caused by the synovial fluid solution. *S. aureus* was not observed to utilize hyaluronic acid in the formation of aggregates. This contradicts data previously reported by Ibberson et al (2016). Finally, eDNA was did not promote aggregation in any of the screens. Interestingly, while eDNA has been shown to be essential in the formation of biofilms[5], it has not been shown to contribute to the formation of aggregates. The ASF was observed to follow similar trends in aggregation to fibrinogen alone, indicating that fibrinogen alone could function as a substitute for synovial fluid.

Future directions will include using fibrinogen as a substitute for synovial fluid in additional strains, as it had the greatest impact on aggregation. This promotes the use of fibrinogen as an artificial synovial fluid, reducing the variability found within using bovine synovial fluid. Additional directions include testing other proteins found within synovial fluid for aggregation promotion. There are proteins upregulated in the instance rheumatoid or osteoarthritis (OA)[16] that could be investigated as the cause for increased aggregation in patients when compared to healthy joints. Analyzing these proteins could lead to the discovery of additional factors present in synovial fluid that lead to aggregation. Synovial fluid could be fractionated via column chromatography and these fractions can then be analyzed and novel compounds stimulating aggregation can be isolated. Future work can also include testing aggregation solutions on *Staphylococcus epidermidis*, another major agent in PJI, to look for differences in aggregation characteristics

compared to *S. aureus* to determine if the findings from this work apply broadly to other staphylococci.

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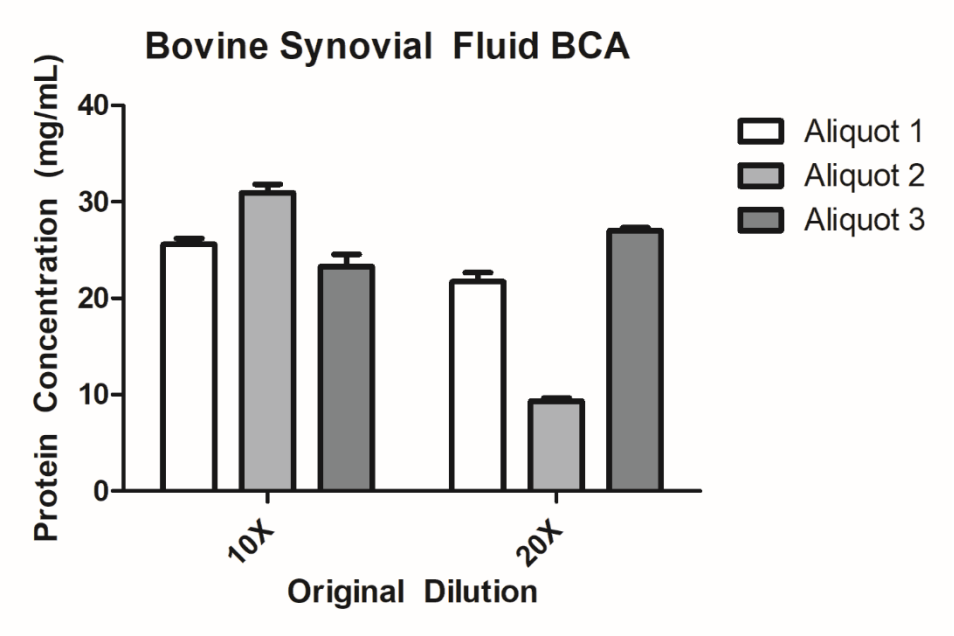
Appendix A: BCA Protein Assay performed on various aliquots of bovine synovial fluid



Bicinchoninic acid assay was performed on three separate aliquots of bovine synovial fluid using the Pierce™ BCA Protein Assay Kit from ThermoFischer Scientific. Dilutions were required due to the high concentration of protein. Total protein concentration within the various synovial fluid aliquots had an average of 22.98 mg/mL with a standard deviation of 7.06 mg/mL. Data does not follow a normal distribution, presented as mean ± SEM, n=3 per aliquot, per dilution.