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A Study of the Polymicrobial Inhibitory Interactions Between Alcaligenes faecalis and Staphylococcus aureus

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By

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ABSTRACT

Members of the *Staphylococcus* genus are found as a part of normal microflora in humans and can commonly be found on the skin or in the nasal cavity. However, these microorganisms can cause serious and life-threatening opportunistic infections when there is a break in the physical barrier of skin. These infections have become difficult to treat as resistant strains emerge, particularly Methicillin Resistant *Staphylococcus aureus (*MRSA). MRSA has become a commonly acquired nosocomial infection which is difficult to treat with conventional antibiotics of the β lactam class. Even Vancomycin, a last resort antibiotic, has been ineffective on some infections. Furthermore, *S. aureus* readily forms biofilms on implanted medical devices which establishes a hardy and difficult to treat infection. These biofilms serve as a point of infection to the bloodstream. Research involving polymicrobial interactions and the inhibitory effects of bacterial-bacterial interactions could be a starting point for the discovery of a new therapeutic treatment for infections. It has been shown in our lab that *Alcaligenes faecalis* has inhibitory effects on *Staphylococcus aureus* planktonic growth. Therefore, in this study, we wanted to examine 1) The mechanism by which *A. faecalis* inhibits *S. aureus* growth and 2) how *A. faecalis* impacts the various phases of *S. aureus* biofilm growth. It was found that *A. faecalis* likely inhibits *S. aureus* using a physical mechanism that requires close contact, rather than using a secreted molecule. However, a Type VI secretion system could also produce similar results. Further research involving the formation of mutants to find the gene allowing *A. faecalis* to inhibit *S. aureus* was started, but no viable mutants were created during the course of this research. *A. faecalis* was found to inhibit the formation of *S. aureus* biofilm growth, but when added to a mature *S. aureus* biofilm, the slow growth rate of *A. faecalis* could not overtake the quickly replicating *S. aureus*. Further research in the polymicrobial interactions between *S. aureus* and *A. faecalis* could lead to a finding of a new therapeutic target for antibiotics or the use of *A. faecalis* in infections.

ACKNOWLEDGEMENTS

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INTRODUCTION

Antibiotics and Antibiotic Resistance

Antibiotics are one of the greatest achievements in medicine of the 20th century. Since the discovery of penicillin in 1928, antibiotics have been used to save countless lives from life threatening infections worldwide [1],[2]. In the United States, antibiotic treatment helped to replace the leading causes of death from infectious diseases to non-communicable diseases, increasing the average life expectancy from 47 to 78 years [2]. Unfortunately, pathogens have developed resistance to these drugs over time, and presently antibiotic resistance is a top concern

to public health [3]. Resistance to antibiotics was first observed in 1940 in the same lab penicillin was discovered, even before the antibiotic was developed for use in medicine [1]. According to the Centers for Disease Control and Prevention (CDC), 2.8 million people are infected with resistant strains of bacteria ultimately causing 35,000 deaths annually in the United States [4]. Antimicrobial resistance is due to a number of mechanisms including beneficial mutations that bacteria accumulate over time as they replicate or acquire antibiotic resistant plasmids due to selective pressures put upon them by the very antibiotics we use to treat infections. Those bacteria that are resistant to antibiotics survive to reproduce generations of resistant bacteria [3].

Bacteria that are resistant to one type of antibiotic are dangerous, but increasingly, bacteria are becoming multidrug resistant, leading to increased mortality. Once a bacterial population has become resistant to one type of drug, alternate drugs that are typically reserved for more serious infections must be used. Due to the misuse of antibiotics, this process occurs even faster, causing what are termed "superbugs" [5]. Superbugs are multidrug resistant bacteria, leaving few or no options for treatment. An example of a bacterial species that has gained resistance is Methicillin Resistance *Staphylococcus aureus* (MRSA). This bacterium can be spread through skin to skin contact [6]. Although it is typically known as a nosocomial infection, or an infection acquired in a hospital, anyone can contract MRSA [6]. Strains of MRSA are becoming better adapted for transmission to other people outside the hospital in a community setting [7]. It is a difficult infection to treat, with vancomycin as the last remaining antibiotic to treat these infections [7]. If left untreated, it can eventually result in life threating infections of the bloodstream and sepsis [6].

The problem does not end with drug resistance. Most modern medical practices and procedures rely on antibiotics to ensure that an infection does not get out of hand. Without them, common medical procedures become much more dangerous. It suddenly becomes very risky to

perform surgery, provide cancer treatments like chemotherapy, or even give birth due to the risk of acquiring an infection that is unable to be treated with antimicrobial drugs [8]. Without these drugs, all recent medical advances are much more difficult to perform. The world would lose years of progress in medicine if bacteria gain resistance to all the antibiotics used to treat them [8]. Alternative methods to treat bacterial infections are needed, and the aim of this research is to provide a possible alternate way to combat infections using the natural inhibition mechanisms microorganisms possess.

Competitive Polymicrobial Interactions

Natural environments contain a variety of diverse microbial species which are continually competing with one another for limited resources using a variety of mechanisms [9]. For example, bacteria produce numerous antimicrobial compounds to target competitors. These antibiotic agents can be either broad spectrum or highly specific, depending on the environment that the bacteria inhabit [9]. *Streptomyces* species inhabiting microbially diverse soil environments produce a wide array of broad acting antimicrobial compounds [9]. On the other hand, some bacterial species produce bacteriocins, a type of antimicrobial peptides, that kill other closely related bacterial species [9],[10].

Another mechanism of competition is the uptake of nutrients to restrict access to these nutrients from other species [9],[11]. An example of this can be seen through the acquisition of iron required for growth [9]. Some bacterial species produce siderophores, which bind ferric iron to capture it from the environment and then import the iron into the bacterial cell [9],[10]. For example, the bacterium *Pseudomonas aeruginosa* uses siderophore production to acquire and deplete iron from the environment as a mechanism of competition [12]. *Pseudomonas aeruginosa* also acquires iron by lysing *Staphylococcus aureus* cells during coinfections of the lungs in cystic fibrosis patients.

Lastly, there are predatory bacteria that can ingest other bacteria in order to kill them [13]. Some bacteria such as *Myxococcus* use a predatory approach to swarm upon target bacteria, while others like *Vampirococcus* act more like vampires as they suck the life out of their prey [14]. Another species, *Bdellovibrio,* bores into its prey and develops in the periplasm of Gram-negative species ultimately causing the target bacteria to lyse [14],[15]. No resistance mechanisms have been found for this type of predation, making it a highly effective way to kill target bacteria [14].

Quorum sensing is an important factor in polymicrobial competition to promote collective behavior within a population [16]. For instance, to be effective, antibiotic agents have to be produced in large amounts [9]. If these compounds are continuously released at sub-inhibitory levels, the target bacteria can become tolerant and the compounds lose effectiveness [9]. One method that bacteria use to combat this is quorum sensing, in which the compounds are not released until sufficient cell numbers are present as indicated through extracellular signals [9]. This ensures that necessary inhibitory levels of the antimicrobial agent for the target bacteria would be produced, minimizing the chance for tolerance towards the compound [9]. Quorum sensing is also a factor in biofilm formation, which is an important biological process that can make infections difficult to treat with antibiotics [16].

Biofilms

Biofilms occur when bacteria and other microbes grow as a community-like film on a surface, encased in a slimy complex [17]. It is understood that microbes primarily exist in this state, rather than in their planktonic, or free-floating, form [18]. Examples of biofilms that form in-vivo are dental plaques, lung biofilm formed by *P. aeruginosa* in cystic fibrosis patients, and biofilms on the surface of implanted devices [17]. Today the use of implanted devices such as catheters or cardiac pacemakers has become a common part of modern medicine [17].

Staphylococcus aureus and *Staphylococcus epidermidis* biofilms from the patient's own normal flora or acquired through a nosocomial infection are both commonly found infecting implanted medical devices that patients depend on [17]. These biofilm infections become much more difficult to treat than the typical planktonic form of the bacteria, as they are more resistant to antibacterial agents [19].

In a laboratory setting, bacteria are in their less hardy form [19]. While in the environment, they must be able to survive harsh or inhospitable environments and have developed protective measures to do this [19]. Biofilm colonies are encapsulated in a carbohydrate polysaccharide capsule to provide protection from the environment [17]. Bacteria encased in the biofilm matrix are protected from phagocytes and other immune defenses, as well as antibiotics [17]. This decreased susceptibility to antibiotics is partly due to the slower growth and metabolic state the bacteria are in when in a biofilm state [16]. It has been shown that the concentration of antibacterial compounds may need to be more than a thousand times the concentration necessary to treat the planktonic forms in order to be effective against biofilms [19].

Oftentimes, it is not a single species in a biofilm. There are many examples of polymicrobial biofilms, the best example being the biofilms that form in the oral cavity [18]. Certain microbes in the oral cavity rely on another species to first form a biofilm and form a "scaffolding" for them to then attach to the oral surfaces. This is called coaggregation, and bacteria may do this in order to maximize space and access to nutrients [18]. Another example is shown in cystic fibrosis, as coinfection with *S. aureus* and *P. aeruginosa* biofilms has negative impacts on treatment options. The biofilm of *P. aeruginosa* releases an exoproduct that causes *S. aureus* biofilm and planktonic cells to become less sensitive to vancomycin, the drug used to treat resistant strains of the bacteria [20]. Cross-kingdom interactions also occur, as shown in the

relationship between *Candida albicans* and *S. aureus* biofilm growth on implanted medical devices. The complex relationship between these two organisms leads to hardier biofilms that are more resistant than pure biofilms of the species and may also impact growth rate within the biofilm [21]. Understanding these polymicrobial interactions within biofilms is important due to the change in the clinical course of a disease and the change in antibiotic sensitivity in the presence of a polymicrobial infection [22].

Staphylococcus **Genus and** ***Staphylococcus aureus***

The genus *Staphylococcus* are Gram-positive, and therefore have a thick layer of peptidoglycan in their cell wall, providing protection from desiccation in dry environments [7]. Cells aggregate in grape-like clusters [7]. There are around 30 known species of *Staphylococcus*, with *S. aureus* as the only primary human pathogen [7]. Other Staphylococcal species such as *S. epidermidis* cause opportunistic infections and infections of implanted medical devices such as a catheter [7]. These bacterial species, particularly *S. aureus*, can commonly be found as a part of the normal microbiota of humans [7]. Around 40% of the general population is colonized with *S. aureus*, where it can be found on the skin, in the throat, nose, and vagina [7]. Therefore, infections of *S. aureus* are oftentimes "autoinfections" where the source of the infection is from a person's own microbiota [7].

The virulence factors of this pathogen include attachment factors such as Protein F on the surface of cells which allows this bacterium to bind to human fibronectin while other surface adhesions allow this species to bind to human collagen and elastin [7]. Clumping factor is a surface adhesin that binds to fibrinogen in blood plasma, helping the bacteria to attach to the endothelial cells of blood vessels [7]. *S. aureus* also has multiple antiphagocytic factors such as a polysaccharide capsule and produces "leukocidin", a toxin that kills white blood cells [7]. An example of this toxin in *Staphylococcus* is the alpha toxin [7]. Still another virulence factor is

Protein A, a surface protein that binds to the Fc region of immunoglobulins to inhibit their opsonization properties [7]. Coagulase activates blood coagulation by converting prothrombin to thrombin. Fibrin may coat the surface of bacteria in order to cloak the bacterium in a human protein to avoid recognition by phagocytes [7]. Other toxins released by *Staphylococcus* include spreading factors such as staphylokinase, DNase, and hyaluronidase enzymes [7]. Enterotoxin causes food poisoning, and exfoliate toxin causes the disease scaled skin syndrome [7]. Lastly, the TSST-1 toxin, causes the deadly toxic shock syndrome [8]. These virulence factors give *S. aureus* the ability to spread easily throughout the body and to evade natural host defenses.

Staphylococci species are receiving increased attention due to their significance in causing morbidity and mortality in hospitalized patients. Currently, *S. aureus* is among the leading pathogens in bloodstream and systemic infections [22]. *S. aureus* is an especially dangerous pathogen due to its ability to resist many antibiotics. Penicillin and many of its derivatives are no longer effective against *S. aureus*. Some strains of *S. aureus* have adapted to have β lactamase activity which cleaves the β lactam ring on penicillin inactivating the compound [23]. Even methicillin, a derivative of penicillin that is modified to be resistant to β lactamase activity, is no longer effective against the strain of *S. aureus* called Methicillin Resistant *Staphylococcus aureus*, or MRSA [23]. There are few antibiotics available today to treat resistant *S. aureus* infections and the remaining few are used sparingly so to avoid future resistant strains. Vancomycin is used as a last resort to combat the resistant strains of *S. aureus*, but there are reports of resistant strains to this antibiotic as well [23].

Alcaligenes faecalis

A. faecalis is a Gram-negative, rod shaped, or bacilli, organism with peritrichous flagella [24]. It is an aerobic organism, nonfermentive, oxidase-positive, urease-negative, and indole-negative organism [24],[25]. The name of the genus, *Alcaligenes*, is due to its ability to produce

an alkaline reaction in certain media [26]. *A. faecalis* inhabits the environment in soil, water, and is sometimes even found in human intestinal flora and hospital environments [26]. *Alcaligenes faecalis* is usually non-pathogenic, but cases of endocarditis, meningitis, peritonitis, and abscesses have been reported [26]. Most infections have been nosocomial, and most commonly occur in those who are immunocompromised as a result of contamination of hospital equipment [26]. If *A. faecalis* enters the bloodstream, it can be a difficult infection to treat due to the large amount of antibiotic resistance genes in its chromosome [25].

The most significant virulence factor of *A. faecalis* are genes for antibiotic resistance, however, there have been other virulence factors discovered such as histamine sensitizing factor, adherence and cytotoxicity, and the extracellular 'o' antigen [25]. Most of the studies involving virulence factors were conducted on avian and mammalian cells to test susceptibility. The same virulence factors could potentially apply to human cells as well [25]. *Alcaligenes* has the potential for widespread use in the environment due to the capability of pollutant bioremediation, for example, with minimal impact on human health [25].

Objectives and Present Work

In the past, diseases were associated with one microorganism. However, as research methods have developed, many diseases are now being classified as polymicrobial infections [18]. Determining what microorganisms are present in the infection can determine how the disease will progress and the predict severity of the disease [18]. Understanding the polymicrobial interactions among these infectious agents is important as the different combinations of microbes can impact how treatment will be responded to. For instance, some bacterial infections that are coinfected with fungal species can affect antibiotic treatment [16]. Antifungals may also be less effective if there is a coinfection with a bacterial species [16]. Knowledge of these interactions is an important area of research as a better understanding can

lead to new therapeutic interventions and an understanding of why previous interventions may not work [16].

Our lab has previously indicated that *Alcaligenes faecalis* has inhibitory effects on *Staphylococcus aureus* planktonic growth. To further understand the extend of the inhibitory effects of *A. faecalis* on *S. aureus* growth, our goal was to research the interactions between the two bacteria in biofilm growth and with varying concentrations of the two organisms. We also explored whether different stages of *S. aureus* biofilm growth were affected by *A. faecalis* inhibitory action. Understanding the inhibitory mechanisms of *A. faecalis* may provide us insight on new interactions between microorganisms, but also provide new targets for potential therapeutics for *S. aureus* infections.

METHODS

Strains, Culture, and Experimental Growth: *A. faecalis* strains and handling. *A. faecalis* strain ATCC 8750 was cultured from freezer stocks onto Luria Broth (LB) agar plates and incubated at 37^{o}C overnight to generate stocks used for the experiments. All subsequent LB liquid cultures were obtained from the colonies formed on these plates. ***S. aureus* strain and handling.** *S. aureus* strain ATCC 25923, and *S. aureus* clinical isolate strain were cultured from freezer stocks onto Mannitol Salt Agar (MSA) plates and incubated at 37^{o}C overnight to generate strains used for the experiments. *S. aureus* strain ATCC 25923 is capable of biofilm formation in vitro. All subsequent LB liquid cultures were obtained from the colonies formed on these plates. For experiments involving the use of *A. faecalis* and *S. aureus* grown together, MSA plates were used to isolate and select for the growth of *S. aureus*. ***E. coli* strains and handling.** *E. coli* strain containing pRL27 was cultured from freezer stocks onto LB agar plates and incubated at 37^{o}C overnight to generate strains used for the experiments. This strain encodes the kanamycin resistant Tn5 transposon plasmid. This was used for transposon mutagenesis to discover mutants

that did not show inhibition of *S. aureus*. LB plates containing the antibiotics Kanamycin (50µg/ml) and Ampicillin (100µg/ml) were used for the isolation of *A. faecalis* mutants.

Agar Spot Test: Overnight cultures, LB broth 37ºC shaking (250rpm), of S. *aureus* were used to spread bacterial lawns on two LB agar plates using a sterile cotton swab dipped into the liquid culture. On the first plate four 20µl drops from an overnight culture of *A. faecalis* were placed on the lawn of *S. aureus*. On the second plate, eight disks each containing *A. faecalis* (20 µL) were placed on the lawn of *S. aureus*. Both plates were incubated at 37ºC for 24 h and were observed the next day to measure zones of inhibition (ZOI) at the *Alcaligenes* inoculation sites.

Cell Dependent Interaction Test: To determine if the presence of *Alcaligenes* cells were necessary to cause inhibition or if there are secreted factors causing the inhibition of *S. aureus*, an overnight *A. faecalis* culture was centrifuged to separate the cells from the liquid culture and passed through a 0.22µm filter. A lawn of *S. aureus* was made on a LB plate and both 20µl of an overnight *A. faecalis* culture and 20µl of the cell-free supernatant *A. faecalis* culture was spotted onto the surface. This plate was incubated for 24 hours at 37ºC and monitored for ZOI. Another variation of this test was done as an agar spot test to test for concentration dependent increases of ZOIs. An overnight culture of *A. faecalis* was added to four different microcentrifuge tubes, centrifuged for 5 min at 10,000 rpm, the liquid was discarded, and the pellet was resuspended in varying amounts of PBS (500 µL, 250 µL, 125 µL, and 60 µL). After a lawn of *S. aureus* was created on a LB plate the varying concentrations of *A. faecalis* (20 µL) were spotted onto the surface, the plate was incubated at 37ºC for 24 h, and ZOI were measured to determine the impact of the differing concentrations.

Biofilm growth studies. Overnight cultures of S. *aureus* and *A. faecalis* were used to seed 6-well cell culture plates. To add the appropriate number of cells to each well, the optical density OD600 of both cultures were determined. *S. aureus* was inoculated to an OD600 reading of 0.01 (~1x10^6 cells/ml) and *A. faecalis* was inoculated to an OD600 reading of 0.1 (~1x10^8 cells/ml). **Attachment phase of biofilm growth.** The well plates were inoculated to contain a *S. aureus* monoculture control and a *S. aureus/A. faecalis* co-culture suspended in 2000µl of LB and incubated for 24 or 48 hours at 37ºC. After the 24 or 48 hours, a pipet was used to carefully draw off the LB broth and loose cells from each well. PBS (1 mL) was gently added to wash off non-adherent cells and discarded. Another 1 mL of PBS was added gently to each well before using the tip of the pipet to vigorously scrape off the biofilm formed on the bottom of each well and suspend in the PBS liquid. These suspensions were serially diluted and plated on MSA, incubated for 24 hours at 37ºC, and CFUs were enumerated. **Maturation phase of biofilm growth.** To see if *A. faecalis* can inhibit already established *S. aureus* biofilms, we followed the above protocol for attachment except *A. faecalis* was not added to the 6-well plate until after the *S. aureus* was allowed to grow 24 hours prior. *A. faecalis* was then added to the experimental wells and incubated for 24 hours. Wells were then washed, scrapped, serially diluted and plated on MSA. **Concentration dependent inhibition.** An overnight culture of *A. faecalis* was added to four different microcentrifuge tubes, centrifuged for 5 min at 10,000 rpm, the liquid was discarded, and the pellet was resuspended in varying amounts of PBS (500 µL, 250 µL, 125 µL, and 60 µL). These different concentrations were then used to inoculate cocultures with S. aureus in the 6-well plates as described in the biofilm attachment protocol. Plates were incubated for 24 h at 37ºC, wells washed with PBS, serially diluted, and plated on MSA to enumerate CFUs. **Biofilm Formation on Coverslips.** To view the amounts of *A. faecalis* and *S. aureus* after a coculture of the two species, coverslips were placed in the bottom of a six-well plate before

adding *A. faecalis* and *S. aureus* to the wells. After incubating for 24 h at 37ºC the coverslips were carefully taken out, washed, and placed on a microscope slide. The coverslips were fixed to the slide, stained using the Gram stain method, and examined under the microscope using the 100X objective to view the differing amounts of *A. faecalis* and *S. aureus*.

Transposon Mutagenesis of *Alcaligenes faecalis*: Overnight cultures of *A. faecalis* (500µl) and *E. coli* pRL27 (500µl) were combined, centrifuged for 5 min at 10,000 rpm, liquid was drawn off, and replaced with 1mL of LB. The pellets were resuspended and four 20 µL drops were placed on an LB plate and allowed to incubate for 24 h at 37ºC. The next day the resulting co-colonies were resuspended in an LB tube and serially diluted. The dilutions were plated on LB plates that contained Kanamycin and Ampicillin and the plates were incubated for 24 h at 37ºC. Kanamycin eliminated any *A. faecalis* that did not integrate the pRL27 transposon and Ampicillin eliminated the *E. coli* that remained, so that the only colonies that would grow were *A. faecalis* cells that had incorporated the transposon into their genome that contained the Kanamycin resistance gene. These colonies were spot replicated onto another LB Kanamycin/Ampicillin plate to confirm the dual resistance. These potential mutants were screened against S. aureus for their potential loss of function phenotype.

RESULTS AND DISCUSSION

A. faecalis shows inhibition of *S. aureus* on solid media.

Lawns of *S. aureus* created on LB plates showed zones of inhibition around spot tests of *A. faecalis* cells (Figure 1). These ZOIs at 24 hours are very robust, large, and clear zones. Upon further incubation, the zones become cloudy with *S. aureus* minimal growth. We believe, that A. faecalis has a much slower generation time than the quickly growing *S. aureus*. Thus, while

initially inhibited at 24 hours, the *S. aureus* eventually overtakes the *A. faecalis* ability to inhibit. Another experiment using disks saturated with *A. faecalis* placed on a lawn of *S. aureus* also showed ZOI (Figure 1). However, this experiment shows slightly smaller zones of inhibition. This experiment displays that *A. faecalis* likely inhibits *S. aureus* using a contact dependent method. The *A. faecalis* cells are embedded in the disks and are not able to diffuse out of the disks to make contact with *S. aureus* as easily. If a molecule was responsible for the inhibition, it would be able to diffuse out of the disk and a larger zone of inhibition would occur around the disks. Ultimately, these experiments exhibit the inhibiting nature of *A. faecalis* on *S. aureus* growth on a solid medium.

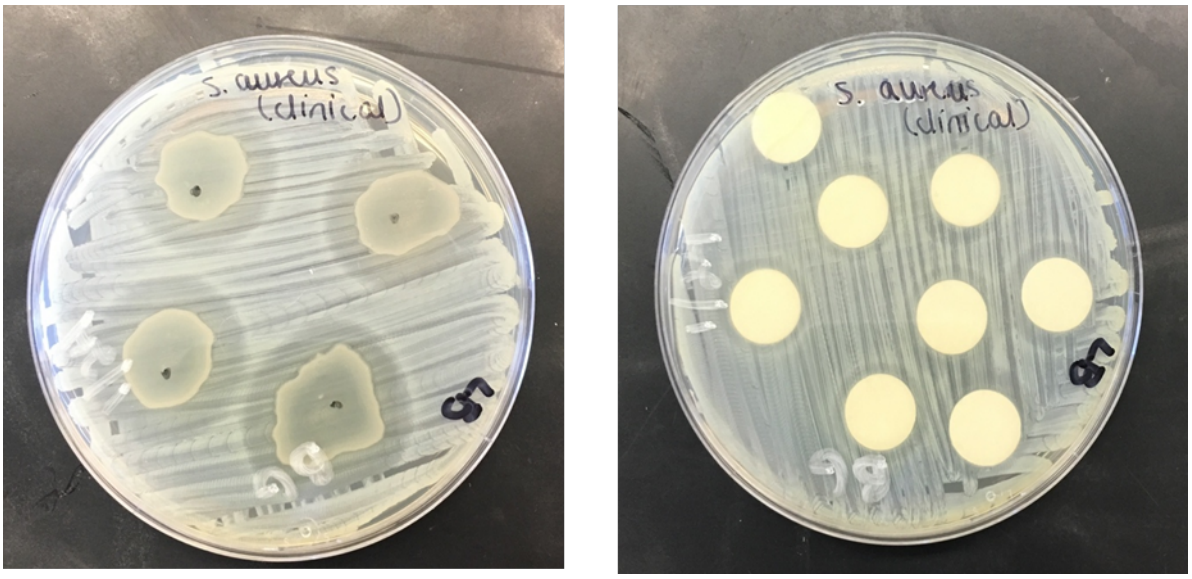


Figure 1: Spot test inhibition of *S. aureus* by *A. faecalis*. (Left) An LB plate with a lawn of *S. aureus* grown with 20µl drops of *A. faecalis* shows inhibition around the drops. **(Right)** An LB plate with a lawn of *S. aureus* grown with 20µl of *A. faecalis* embedded on disks placed on top of the lawn shows inhibition around the discs.

A. faecalis cells must be present to inhibit growth of *S. aureus* on solid media.

To examine the mechanism by which *A. faecalis* inhibits *S. aureus*, *A. faecalis* liquid cultures were centrifuged and the liquid was drawn off and filtered. This was to determine whether *A. faecalis* secreted a molecule as a form of inhibition toward *S. aureus* or whether it

was a physical mechanism by the *A. faecalis* cell itself. The *A. faecalis* drops on a *S. aureus* lawn did not show ZOI when the cells were filtered out while spots that contain the cells show the characteristic ZOI (Figure 2). This indicates that there is a physical mechanism by which *A. faecalis* inhibits *S. aureus* and a secreted molecule is likely not the cause of inhibition.

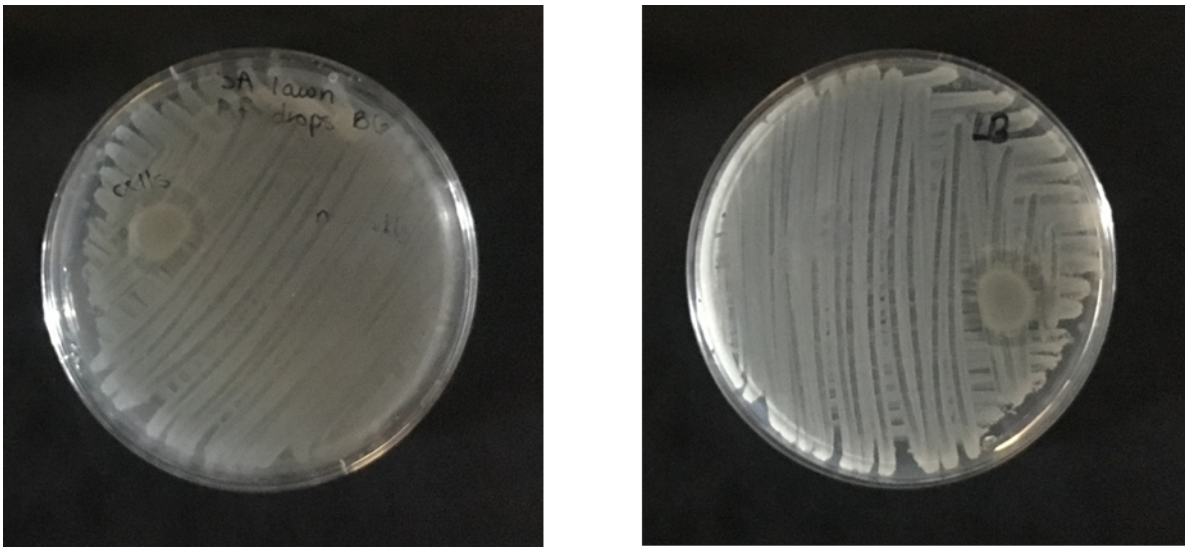


Figure 2: Cell free vs cell inhbition of A. faecalis. (Left) An LB plate with a lawn of *S. aureus* (clinical strain) grown with 20µl drop of *A. faecalis* cells shows inhibition and 20µl of cell free *A. faecalis* growth liquid shows no inhibition around the drops. **(Right)** An LB plate with a lawn of *S. aureus* (laboratory strain) grown with 20µl drop of *A. faecalis* cells shows inhibition and 20µl of cell free *A. faecalis* growth liquid shows no inhibition around the drops.

***A. faecalis* exhibits increased inhibition against *S. aureus* with increasing cell-density.**

The concentration of *Alcaligenes* inoculum was manipulated to examine whether an increasing concentration of *A. faecalis* cells showed an increase in *S. aureus* inhibition. This was performed by using a standard sample of *A. faecalis* along with four varying dilutions of *A. faecalis*, thus each drop had a reduced amount of liquid volume creating 2X, 4X, 8X, and 16X concentrations of *A. faecalis*. These were dropped onto a lawn of *S. aureus* in 20µl amounts and the resulting ZOI were measured. As depicted in Figure 3 and 4, the concentration of *A. faecalis* increased the ZOI on the *S. aureus* lawns.

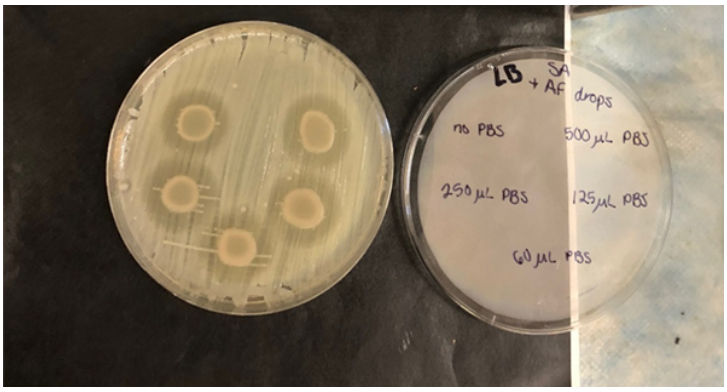


Figure 3: Concentration dependent inhibition of *A. faecalis* on *S. aureus* agar plates. ZOIs slightly increased as the concentration increased of *A. faecalis* drops placed on a lawn of *S. aureus*.

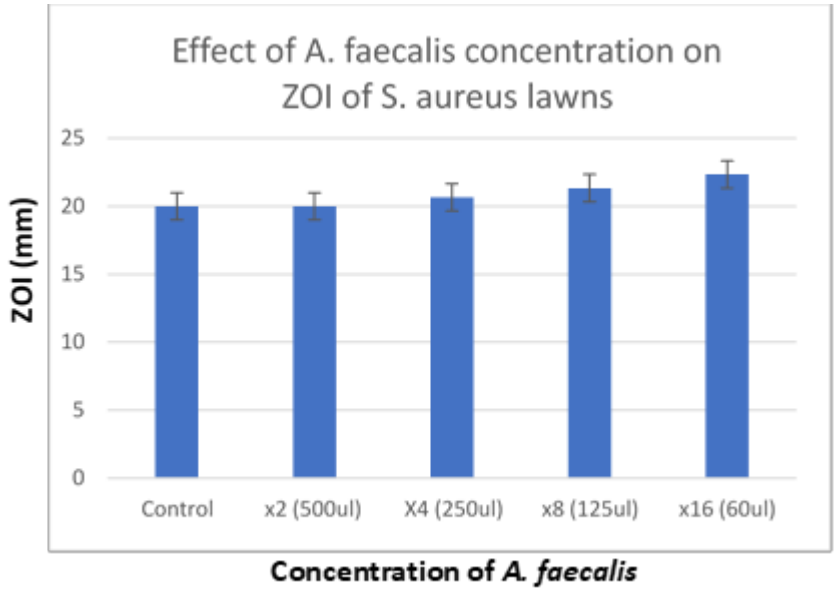


Figure 4: Inhibition by *A. faecalis* increases as the cell density increases. Graphical representation of increasing inhibition of *S. aureus* growth as the concentration of *A. faecalis* was increased.

A. faecalis concentration and inhibition of *S. aureus* biofilm (attachment phase) of growth.

Biofilms develop in discreet stages, with the attachment phase the initial step to biofilm formation. To examine the inhibitory effects of *A. faecalis* on *S. aureus* biofilm attachment growth, cocultures of *A. faecalis* and *S. aureus* were made, inoculated into 6 well plates, and tested under varying time conditions. The first condition was a coculture grown for 24 hours.

The wells were plated on MSA plates to select for *S. aureus* growth following the methods outlined in the serial dilution procedure. The results indicate that there were inhibitory effects on the biofilm attachment growth of *S. aureus* after a coculturing with *A. faecalis* and comparing the colony forming units (CFU) to the control plates (Figures 5 and 6). Microscopic photos of coverslips in the bottom of the 6-well plates show that the monoculture of *S. aureus* produces thick robust biofilms (*S. aureus* Gram stains positive) while *S. aureus* coculture biofilms with *A. faecalis* (*A. faecalis* Gram stains negative) show a dramatic reduction in *S. aureus* biofilm attachment (Figure 7).

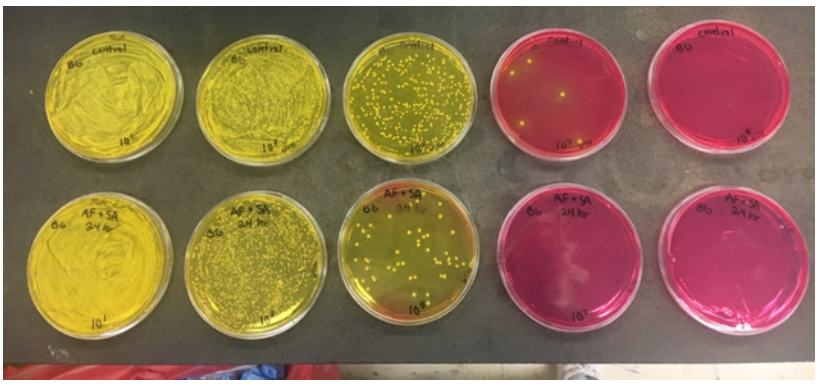


Figure 5: CFUs of mono and coculture biofilms 24h. (Top row) *S. aureus* monoculture biofilm attachment growth without the addition of *A. faecalis*. **(Bottom row)** *S. aureus* coculture biofilm attachment growth with the addition of *A. faecalis*. From left to right shows serial dilutions and CFUs on MSA plates.

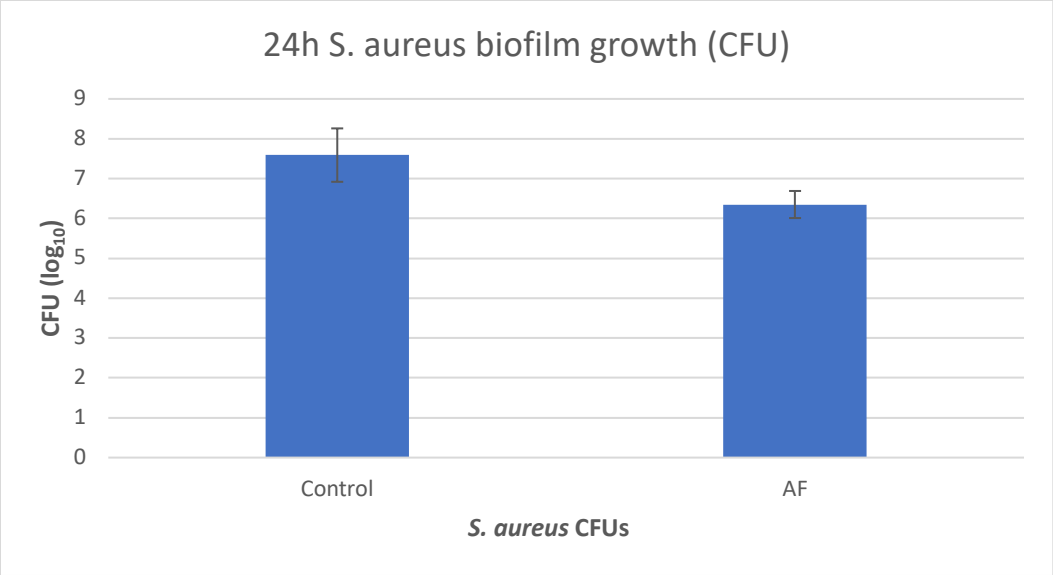
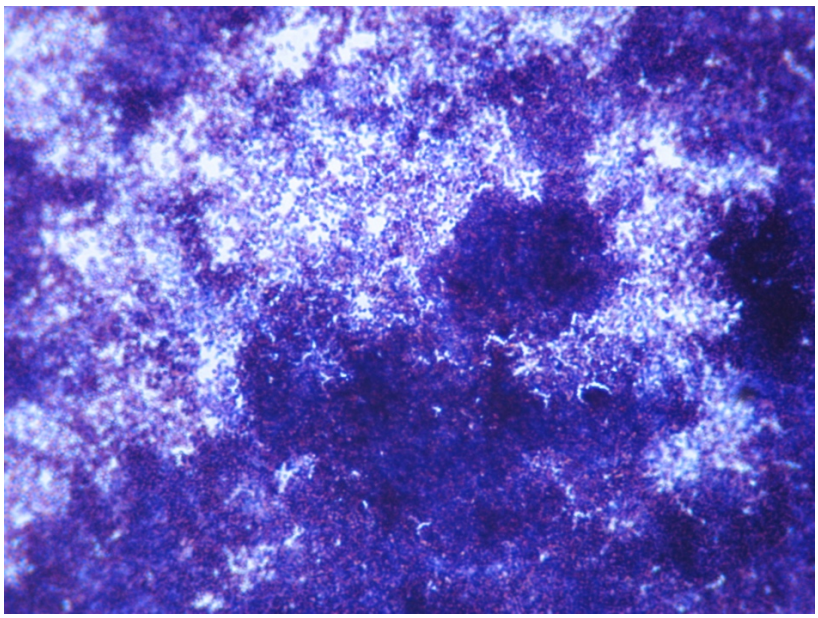


Figure 6: Graphical representation of CFUs from *S. aureus* mono and coculture biofilm attachment growth with *A. faecalis* 24h.



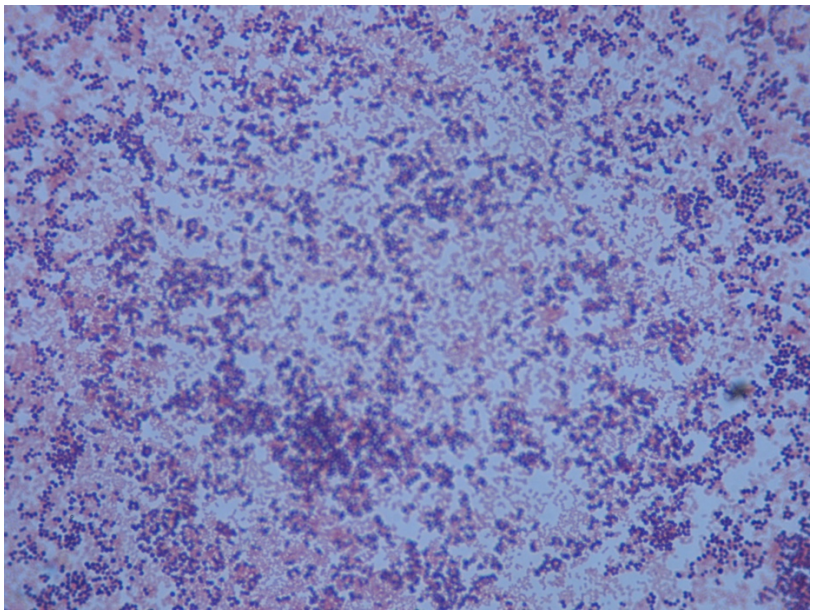


Figure 7: Microscopic images of *S. aureus* mono and coculture biofilm attachment growth with *A. faecalis*. (Top photo) Biofilm attachment growth of *S. aureus* monoculture on a coverslip after 24 h of growth. **(Bottom photo)** Biofilm attachment growth of *S. aureus* coculture on a coverslip after 24 h of growth with *A. faecalis*.

After finding the results above, we wanted to see if this inhibition of biofilm attachment by *A. faecalis* on *S. aureus* would be sustained over a longer period of time or, if like the agar plates experiments, *S. aureus* can overcome the inhibition. Another trial was performed using the same methods as in Figures 5, 6, and 7, but the coculture was grown for an additional 24 hours for a total of 48 hours. These plates also showed inhibition on biofilm growth, but the increased time the coculture was grown did not have a significant impact, less than 10% reduction, on the inhibitory effects of *A. faecalis* on *S. aureus* (Figure 8 & 9). This is likely due to the slower growth rate of *A. faecalis* compared to *S. aureus*. Over time, *S. aureus* growth eventually overtakes *A. faecalis* growth. It initially has a great inhibitory effect on *S. aureus* and it will keep inhibiting *S. aureus,* but it does not overtake it due to the fast growth rate of *S. aureus*.

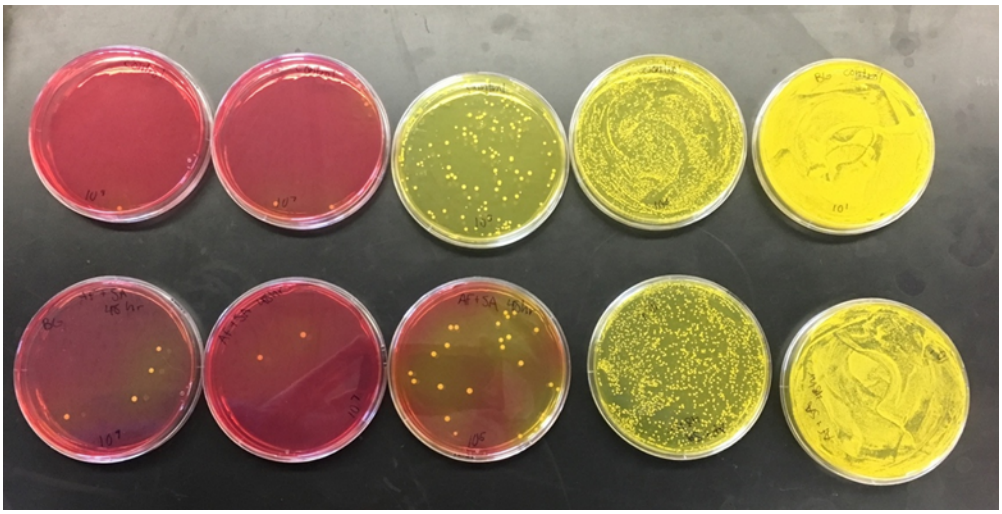


Figure 8: CFUs of mono and coculture biofilms 48h. (Top row) *S. aureus* monoculture biofilm attachment growth without the addition of *A. faecalis*. **(Bottom row)** *S. aureus* coculture biofilm attachment growth with the addition of *A. faecalis*. From right to left shows serial dilutions and CFUs on MSA plates.

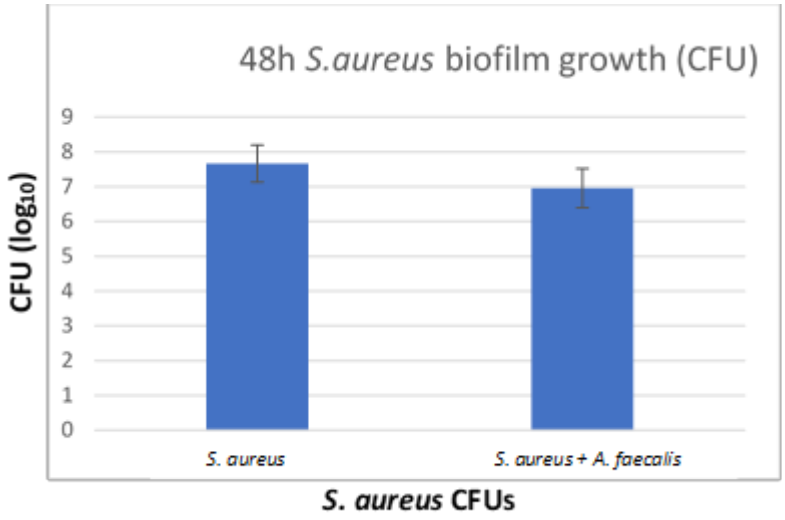


Figure 9: Graphical representation of CFUs from *S. aureus* mono and coculture biofilm attachment growth with *A. faecalis* 48h.

We wanted to examine a later stage of biofilm growth and the inhibitory effects of *A. faecalis* on *S. aureus* on biofilm growth. This phase, the maturation phase of biofilms, considered the effects of adding *A. faecalis* 24 hours after a *S. aureus* biofilm had been allowed to reach a

mature phase. This experiment was performed in order to determine the inhibitory effectiveness of *A. faecalis* on *S. aureus* after a mature biofilm was already established. *A. faecalis* showed slight inhibitory effects on mature *S. aureus* biofilm growth and could have potentially destroyed some of the existing biofilm there were less CFUs on the MSA plates from *S. aureus* grown with *A. faecalis* (Figure 10). However, there was less inhibition exhibited from this experiment than the previous attachment experiments.

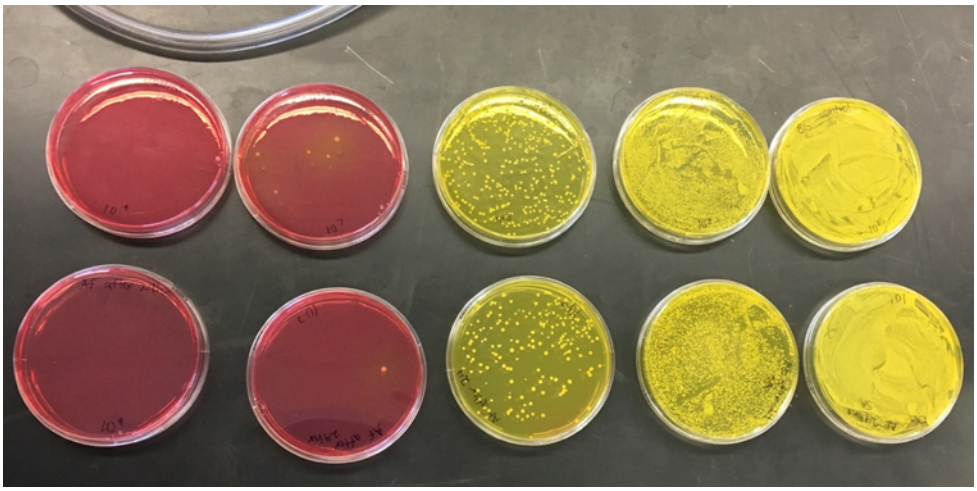


Figure 10: CFUs of mono and coculture biofilms after allowing S. aureus to grow initially for 24 hours. (Top row) *S. aureus* monoculture biofilm attachment growth without the addition of *A. faecalis*. **(Bottom row)** *S. aureus* coculture biofilm attachment growth with the addition of *A. faecalis*. From right to left shows serial dilutions and CFUs on MSA plates.

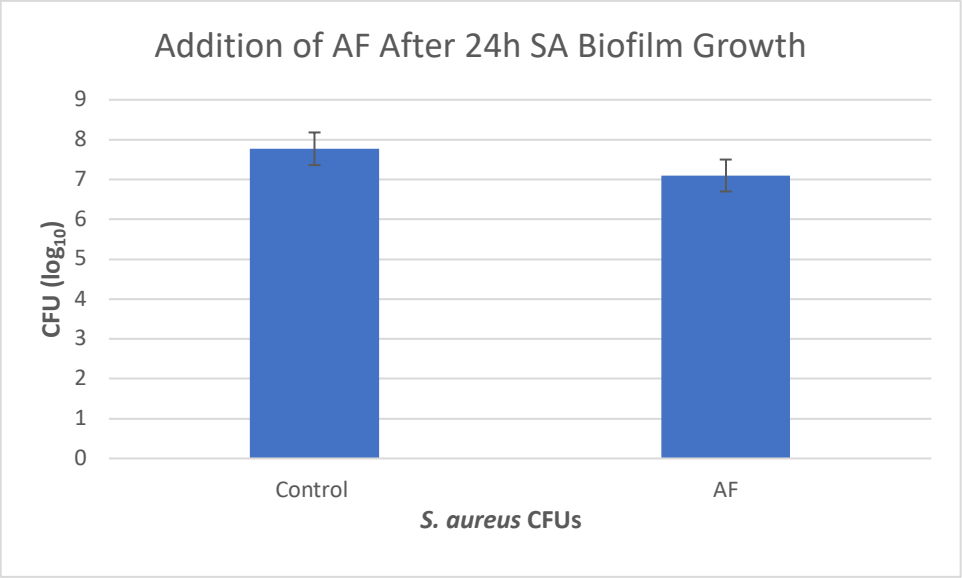


Figure 11: Graphical representation of CFUs from *S. aureus* mono and coculture biofilm during the mature phase of growth with the addition of *A. faecalis* after S. aureus was allowed to grow for 24h.

***A. faecalis* concentration and inhibition of *S. aureus* biofilm growth.**

We found in earlier experiments that the concentration of *A. faecalis* affects agar plate interactions**.** We then wanted to examine if this finding could be translated to biofilm growth of *S. aureus*. There was no noticeable difference when increasing concentrations of *A. faecalis* upon the biofilms of *S. aureus* growth. There was still a significant reduction by *A. faecalis* on biofilm growth of *S. aureus*, but the increasing amounts of *A. faecalis* did not cause a decrease in biofilm growth beyond the 1X concentration (Figures 11 and 12).

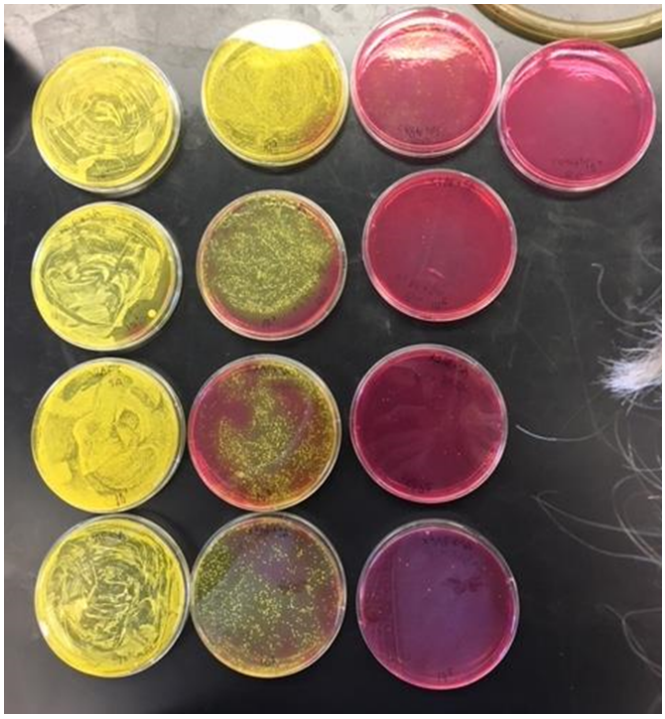


Figure 12: Concentration dependent inhibition of *A. faecalis* on *S. aureus* biofilm growth. CFUs decreased at the same amounts despite the concentration increase of *A. faecalis* cocultured in biofilms of *S. aureus*. **(Top row in descending order)** *S. aureus* monoculture, *S. aureus* with 1X concentration of *A. faecalis*, *S. aureus* with 2X concentration of *A. faecalis*, *S. aureus* with 3X concentration of *A. faecalis.*

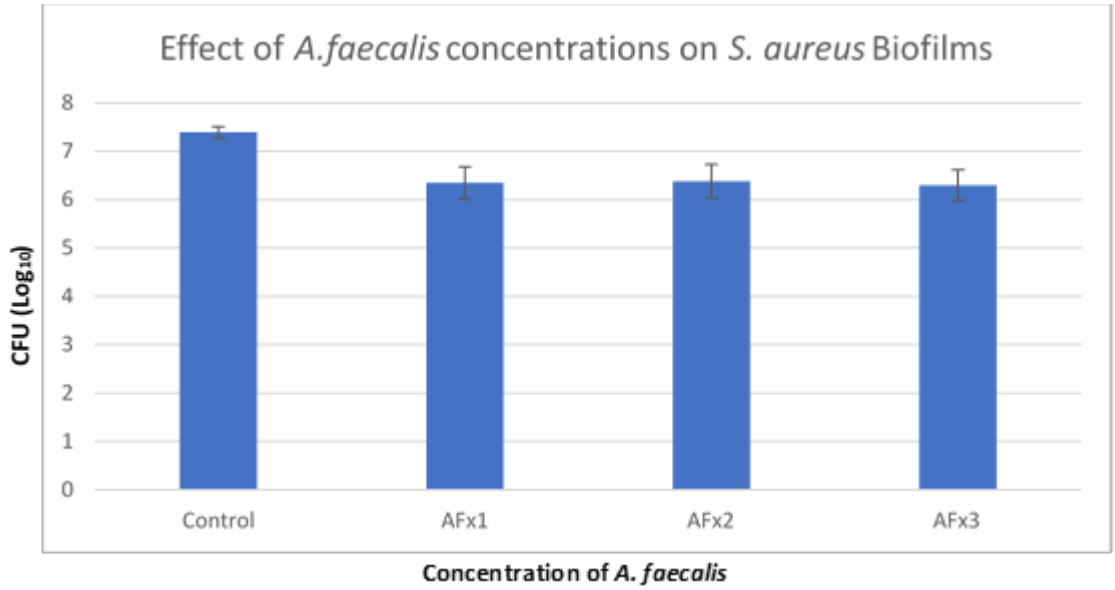


Figure 13: Inhibition by *A. faecalis* various cell densities on *S. aureus* biofilms. Graphical representation of inhibition of *S. aureus* biofilm growth (CFUs) as the concentration of *A. faecalis* was increased.

Transposon mutagenesis to determine genetic elements of *A. faecalis* inhibition.

Currently, the mechanism that *A. faecalis* utilizes to inhibit *S. aureus* is unknown. To begin to identify the genetic elements involved in this interaction, we utilized random transposon mutagenesis to screen A. faecalis for loss-of-function mutations. Using *E. coli* BW20767 that possess the conjugative pRL27, a pir-dependent hyperactive Tn5 transposon system, and confers Kanamycin resistance. Upon conjugation with *A. faecalis*, numerous potential mutants were created and screened on lawns of *S. aureus* to look for *A. faecalis* colonies that lost the ability to create ZOIs. Unfortunately, none of the mutants displayed the particular phenotype. However, a research group using the same transposon system in a different bacterium screen 10,000 mutants to only find seven mutants with the correct phenotype. We plan on continuing the conjugative transposon method to saturate the *A. faecalis* genome.

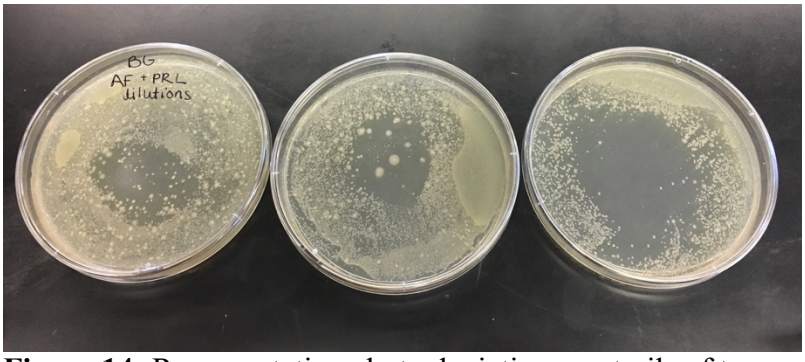


Figure 14: Representative photo depicting one trails of transposon mutagenesis on double antibiotic selection plates.

CONCLUSION

Bacteria and other microbes are becoming increasingly resistant to the antimicrobials currently available for use. As the number of deaths from resistant infections rises, our increased understanding of the countless polymicrobial interactions that are occurring in our environment and inside our bodies is essential in improved treatments for resistant infections. Discovering the variety of mechanisms microorganisms use to compete and interact with each other hold endless potential for the creation of novel antibiotics or antimicrobial treatments. This preliminary research evaluates a physical mechanism that *A. faecalis* employs to inhibit *S. aureus* growth. Of

particular interest is the ability of *A. faecalis* to inhibit *S. aureus* biofilms at both the attachment and the maturation phases of growth. On agar plate growth and in the initial attachment phases of biofilm growth, *A. faecalis* is a potent inhibitor of S. aureus. Additionally, this inhibition is cell density dependent with more concentrated amounts of *A. faecalis* having increased inhibition qualities. However, in mature established *S. aureus* biofilms, the inhibition appears muted and increased concentrations of *A. faecalis* have no change in the amount of inhibition. This appears to be attributed to the slower generation time of *A. faecalis* in respect to the much faster doubling times of *S. aureus*. The mechanism of this inhibition remains elusive at this time as preliminary transposon mutagenesis of *A. faecalis* has not yielded a phenotypically significant mutant. Further research involving the creation of a library of *A. faecalis* mutants to discover the genes that allows the inhibition would be the next step in this project.

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