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The Inhibitory Effects of an Antimicrobial Gel on the Staphylococcus Species

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ABSRACT

The prevalence of antibiotic resistant bacteria has made the choices for topical treatments for patients who experience burns wounds extremely limited. The *Staphylococcus* genus is naturally occurring in and on the human body but can become harmful once it enters the bloodstream. A novel antimicrobial gel has been shown by our laboratory to inhibit both the planktonic growth and biofilm formation of *Staphylococcus aureus* in previous studies. The antimicrobial gel is made of seven natural compounds including antioxidants (vitamin C and E). We wanted to examine the effects of the antimicrobial gel on numerous other Staphylococcal species because it is prevalent on the body and becomes harmful when the immune system is compromised. The species tested were *Staphylococcus capitis, Staphylococcus epidermidis,* and *Staphylococcus saprophyticus*. A planktonic broth challenge test, biofilm attachment test, and biofilm maturation test were all performed in order to test this hypothesis. These tests showed a significant inhibition of the Staphylococcus species as a result of the effects of the antimicrobial gel. The antimicrobial gel inhibited the attachment, maturation, and growth of *Staphylococcus* colonies in a 10% antimicrobial gel solution. The antimicrobial gel shows promise as an option in treating burn patients and should be considered in further testing for its uses in other areas of medicine.

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INTRODUCTION

Staphylococcus species

Staphylococcus is a Gram-positive genus of bacteria, which can become pathogenic to humans and other animals (1). Staphylococcus typically grows in clusters, pairs, or short chains when the Staphylococci divide into two planes (1). This genus is commonly divided into coagulase-positive Staphylococci and coagulase-negative Staphylococci, although this does not distinguish its virulence factor, but can be used as a marker for *Staphylococcus aureus* (*S. aureus)* in most cases (1). Staphylococcus has multifactorial pathogenesis, making it difficult to determine the role of each factor (1). Although, strains from isolated diseases have shown expression in particular factors (1). The Staphylococcus species is the cause of many infections associated with internal medical devices. Staphylococcus adheres to fibrinogen and fibronectin host cells, which become prevalent on internal medical devices after implantation (1). *S. aureus* can also bind to endothelial tissue, but the mechanism of adherence remains unclear. α-toxin, β-toxin, δ-toxin, γ-toxin and leucocidin are all membrane damaging toxins (1). Treatments for Staphylococcal infections include the surgical removal of the medical device and antibiotics if the strain is not resistant (1). Numerous strains of Staphylococcus were shown to develop resistance to methicillin and penicillin in the mid 20th century, making vancomycin the primary form of treatment for methicillin resistant *S. aureus* until Vancomycin resistant strains appeared in the 1980's (2). These antibiotic resistant strains have continued to become more prevalent in hospitals and there appears to be no new antibiotics to combat the quickly adapting species.

Staphylococcus epidermidis

Staphylococcus epidermidis (S. epidermis), while previously viewed as an innocuous microorganism residing on the skin, now presents as an opportunistic pathogen due to the rise in nosocomial infections it causes making it comparable to *S. aureus* (3, 4). Along with nosocomial

infections *S. epidermidis* is recognized as the most common source of infection on internal medical devices due to the nature of insertion of these devices during surgery (4,5). The devices come into contact with the skin and carry *S. epidermidis* with them once implanted. *S. epidermidis* does not appear to be life threatening, but is important due to the frequency and difficulty treating as a result of antibiotic resistance and biofilm formation (4).

S. epidermidis is the most commonly isolated microorganism from the human skin and has the ability to acclimate to extreme salt concentrations as well as to varying osmotic pressures of the environment (4). It is a very diverse coagulase-negative Staphylococcus species with 74 identified, unique deoxyribose nucleic acid (DNA) sequences (6,4). It causes the highest number of infections among coagulase-negative Staphylococci (CoNS) bacteria and accounts for 22% of bloodstream infections in intensive care unit patients in the USA (3,4). It has been shown that *S. epidermidis* was involved in prosthetic joint, vascular graft, surgical site, central nervous system shunt, and cardiac device infections as well as accounting for 13% of prosthetic valve endocarditis infections, according to Chu, cited by Otto. (4,7)

Staphylococcus capitis

Staphylococcus capitis (S. capitis), just like *S. epidermidis*, is an opportunistic human pathogen and a coagulase-negative Staphylococci bacterium (8). The findings by Van Der Zwet and Rasigade cited by Cameron indicate that *S. capitis* causes up to 20% of neonatal sepsis cases in the neonatal ICU (9, 10, 11). It has also contributed to the development of prosthetic valve endocarditis and hospital-acquired meningitis in patients (12,9). It is interesting to note that CoNS bacteria rely on biofilm formation for their virulence (9, 13). Biofilm production has been associated with virulence in the absence of prosthetic material in an animal model (13). This

study also found that gene regulation was involved in bacterial virulence, rather than the ica operon, as previously suspected (13).

Staphylococcus saprophyticus

Staphylococcus saprophyticus (*S. Saprophyticus*) is a CoNS bacterium that is a common cause of uncomplicated urinary tract infections, making up 42% of all infections, especially effecting women ages 16-25 who are sexually active (14). Although more uncommon it is also responsible for acute pyelonephritis, urethritis, epididymitis, and prostatitis (14). Unlike other CoNS bacteria, it is known to be resistant to the antibiotic, Novobiocin, an aminocoumarin. (14). Another study showed that a third of *S. saprophyticus* urinary tract infection cases were resistant to oxacillin, a beta lactam antibiotic. (15) Some strains have the ability to create biofilms to increase their virulence and resistance (14). This bacterium colonizes in the perineum, rectum, urethra, cervix, and gastrointestinal tract (14). There is an increased incidence of *S. saprophyticus* infection in patients with nosocomial urinary tract infections, pregnant patients, and patients who are urinary catheterized (14). Patients who are more susceptible to *S. saprophyticus* include those who are immunocompromised, HIV positive, diabetic, or elderly (14).

Staphylococcus and topical burn wounds

The environment of the skin is ideal for bacteria because of the protein rich environment found in avascular necrotic tissue (16). Also, the avascular nature of the skin inhibits immune cells from entering via the bloodstream. Additionally, the bacterial release of toxins inhibits the local immune response. Taken together, the skin provides an enriching area for bacterial growth (16). While a burn is sterile immediately afterwards, Gram-positive bacteria, including Staphylococcus, residing in sweat glands and hair follicles survive the burn and quickly multiply

(16). Gram-positive bacteria colonize the burn area heavily within the first 48 hours without the application of an antimicrobial agent (16). Biofilms develop within 48-72 hours (16). Within 5 to 7 days the burned area becomes colonized with other Gram-positive and Gram-negative bacteria and fungi (16). *S. aureus* is the most common cause of early burn wound infection and has continued to become challenging to treat due to antibiotic resistance.

Current burn wound treatments

Topical gels decrease morbidity and mortality in patients with burns; however, treatments have become limited due to antibiotic resistance (16). Silver nitrate is a less commonly used drug that is effective against Gram-negative bacteria (16). It is scarcely used treatment due to the potential toxicity, electrolyte imbalances, and discoloration of the wound that may follow treatment (16). The most common treatment for burns is silver sulfadiazine, a combination of silver nitrate and sulfadiazine (16). It is a broad spectrum antibiotic that is especially toxic to Gram-negative bacteria (16). It has been shown to have limited toxicity with once or twice daily repeated application, with the addition of leukopenia, low white blood cell count. (16). There are a few reported cases of antimicrobial resistance in silver sulfadiazine and it is less effective in patients with severe burns because it can only be absorbed within one surface of the epidermal layer (16). Another burn treatment is Mafenide acetate, a topical broad spectrum cream that is effective against Gram-negative bacteria but has very little effect on Gram-positive bacteria, such as the Staphylococcus species (16). This treatment is used sparingly due to its toxicity profile (16). Acticoat A.B. dressing has been shown to be effective against aerobic Gram-positive bacteria, including methicillin resistant *Staphylococcus aureus* (MRSA), and reduces the amount of dressing changes needed after injury (16). It is considered the broadest spectrum bacterial coverage against burn wound pathogens currently (16). Mupirocin, a topical antibiotic,

has been shown to kill MRSA but should be rotated during treatment to prevent resistance (16). Nystatin is an antifungal, topical medication that is used in conjunction with antibiotics, due to its lack of toxicity on bacteria (16). Other non-topical treatments include selective bowel decontamination, which has been shown to reduce burn wound colonization, and immunization of tetanus toxin via intramuscular injection (16).

Antimicrobial Gel

Existing antibiotic resistance and the evolving risk for further resistance illuminates the importance of new treatments to combat newly evolving pathogens of burn wounds. An antimicrobial gel (AMG) made of seven natural components including antioxidants (vitamin C and E) and zinc has previously been shown in our lab to inhibit microorganisms that commonly infect burn wounds. Previous tests have shown that this AMG has the most significant antimicrobial effects against *S. aureus* biofilms and planktonic cultures (17). The AMG, from other lab tests, demonstrate that the effects on pain relief and wound healing are significant (17). Taken together, the information suggests it would be useful and versatile in the healthcare field. Therefore, it is important to examine the properties of the AMG on other members of the Staphylococcus genus (*S. capitis, S. epidermidis*, *S. saprophyticus*). It would be useful in wound care treatments to determine if this predilection for inhibition of *S. aureus* could be applied to other Staphylococcal species or if it is specific to only *S. aureus*.

MATERIALS AND METHODS

Strains and Growth Conditions

Mannitol salt agar (MSA) stock streak plates for isolated colonies were made for *S. aureus* using stock ATCC#25933, *S. epidermidis* (ATCC #), *S. saprophyticus* (ATCC #), and *S. capitis* was (ATCC #) . These plates were incubated at 37°C for twenty-four hours, then sealed with parafilm and placed in a 4°C cooler for storage. This process was repeated every two weeks to obtain fresh colonies for the following experiments. For all experiments, bacteria were propagated in Luria-Bertani (LB) broth and MSA plates. When needed, the AMG was supplemented in LB broth in a weight to volume (w/v) to produce a 10% solution.

Planktonic Broth Challenge Test

Fresh overnight cultures were inoculated for each Staphylococcal species and grown at 37°C for twenty-four hours. An optical density (OD 600) was then obtained. Experimental and control LB tubes were inoculated using the OD600 reading and adjusting mathematically to obtain a final concentration of cells of ~$1x10_6$ cells/ml. The bacterial mixture was added to either a 5 ml LB tube (control) or a 5 ml 10% AMG LB broth. The tubes were placed in a 37°C incubator with shaking (250rpm) for twenty-four hours. Serial dilutions were then performed on each sample, plated onto MSA or LB agar plates, and incubated at 37°C for twenty-four hours. The colonies from each plate were then counted. This series was repeated three times to determine validity.

Biofilms Attachment

The attachment assay was conducted to observe the effects of AMG on the Staphylococcus attachment phase for biofilm formation. A 10% solution of AMG LB broth was added to a 96-well plate with either *S. epidermidis, S saprophyticus, S. capitis,* and *S. aureus* inoculated at an OD600 of 0.01 which is equivalent to $1x10_6$ cells/ml. As a control, *S. epidermidis, S saprophyticus, S. capitis,* and *S. aureus* were also inoculated into LB broth without AMG and added to separate wells. The plate was then covered in parafilm and placed at 37°C for twenty-four hours. The plate was removed, the remaining liquid was drawn off, washed with Phosphate Buffered Saline (PBS) and OD600 of the biofilms were measured. A Crystal Violet assay was then performed by adding 200 ml of 0.001% CV to the wells, incubating for five minutes, drawing the liquid off and rinsing with PBS, then adding 200 ml 33% Glacial Acetic Acid for five minutes. This assay was read at OD595 with shaking. This series was repeated three times to determine validity.

Maturation Assay

A maturation assay was performed by adding *S. saprophyticus, S. epidermidis, S. aureus,* and *S. capitis* LB broth to a 96-well plate to test the effects of AMG on the maturation phase of biofilm growth. Each species was inoculated in LB and grown for twenty-four hours at 37ºC without the addition of AMG. The liquid was then drawn off and 200µl of 10% AMG was added to half of the wells, while 200µl of LB was added to the other half of each species biofilm for comparison. The well plate was then incubated at 37°C for twenty four hours. The plate was then removed and assayed in the same manner as the attachment assay. This series was repeated three times to determine validity.

RESULTS

Planktonic cultures of Staphylococcus species with 10% AMG.

It has been previously demonstrated in our lab that among a panel of burn wound infection microorganisms, that AMG has an increased ability to inhibit *S. aureus*. We therefore set out to determine if this inhibition could be seen in other Staphylococcal species using planktonic cultures. Upon inoculating each strain in LB broth, either with or without 10% AMG, we found that AMG significantly reduces the growth of *S. epidermidis, S. capitis*, and *S. saprophyticus* as demonstrated by colony forming units (CFU). Figure 1 shows the significant reduction in CFUs when all three Staphylococcal species are grown with 10% AMG over a twenty-four-hour period. *S epidermidis* showed an 81% reduction in CFUs, *S. saprophyticus* showed a 100% reduction in CFUs, and *S. capitis* showed a 94% reduction in CFUs when compared to their LB broth only (no AMG) matched controls. This data is comparatively similar to the prior data we obtained when *S. aureus* is inoculated into LB AMG with a robust inhibition of CFUs over a 24-hour period. Taken together, it would appear that the AMG inhibits all members of the Staphylococcal family, at least in planktonic culture, equally and not specific for only *S. aureus* inhibition. Figure 2 and 3 are representative photos of one trial of *S. saprophyticus* planktonic cultures with AMG (Figure 3) or without AMG (Figure 2). As shown in all three trials, addition of AMG to the culture produced no viable colonies of *S. saprophyticus*. Trials with *S. capitis* and *S. epidermidis* produced a similar trend with minimal bacterial growth on all dilution plates of the cultures containing 10% AMG (photos not shown).

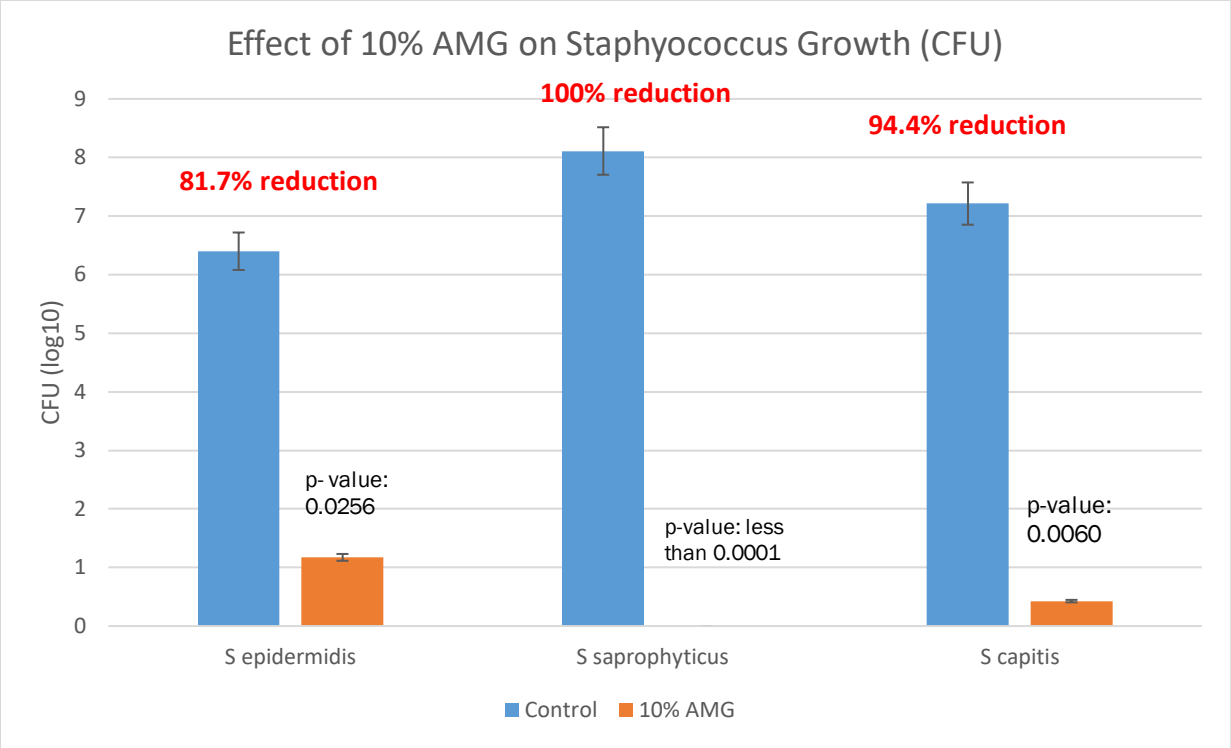

Figure 1: Graphical representation of 24 hour planktonic cultures of Staphylococcal species with or without AMG (three separate trials).

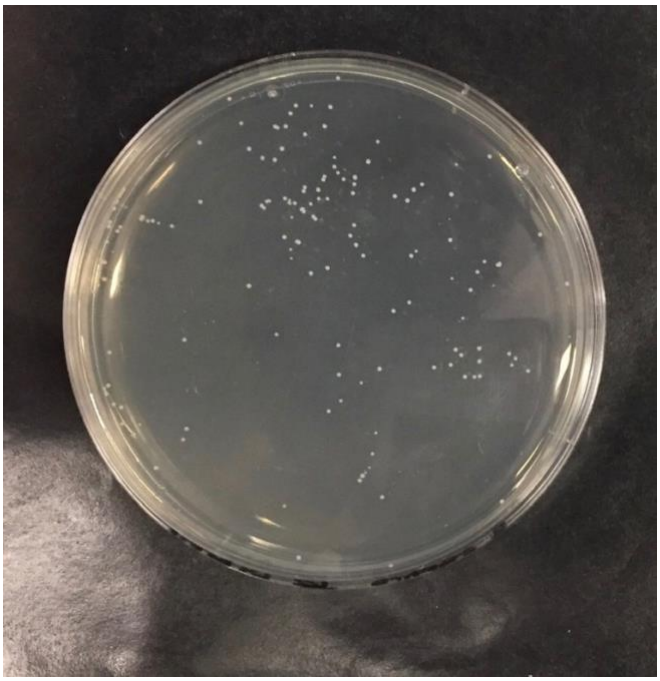

Figure 2: *S. saprophyticus* (without AMG) serially diluted to plate 6 which produced 148 bacterial colonies (one out of three trials)

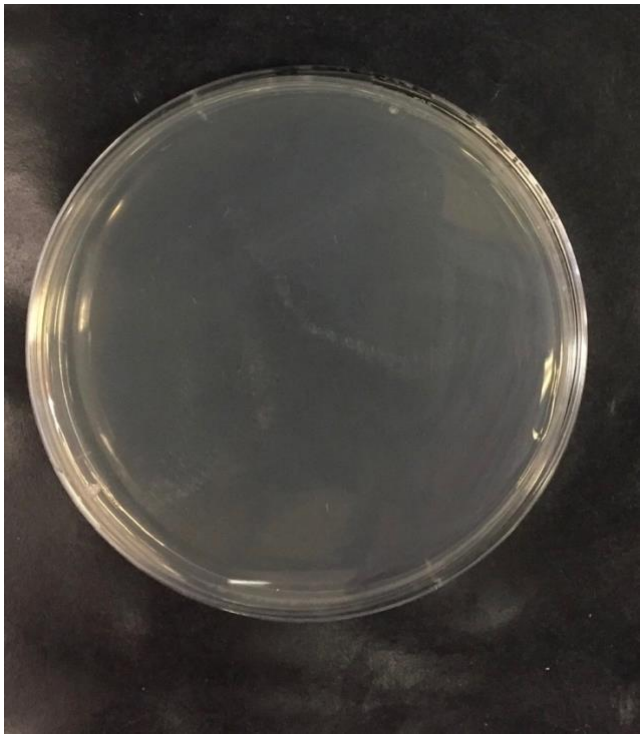

Figure 3: *S. saprophyticus* (with 10% AMG) serially diluted to plate 1 which produced no bacterial colonies (one out of three trials)

Biofilm, attachment phase, of Staphylococcus species with 10% AMG.

After demonstrating that AMG inhibits not only *S. aureus*, but also planktonic cultures of *S. capitis*, *S. epidermidis*, and *S. saprophyticus*, we turned our attention to biofilm formation. Biofilms form in discreet ordered steps beginning with the attachment of planktonic cells to a surface. It was postulated that AMG could inhibit the early stage of bacterial biofilm formation. AMG showed a significant reduction in biofilm attachment among all Staphylococcal species tested. Two different methods were used to quantify biofilm attachment. The first method used optical density as a measure to determine the amount of bacterial density present (Figure 4). *S. capitis* density decreased by 63%, *S. epidermidis* density decreased by 47%, and *S.*

saprophyticus density decreased by 57% with the application of AMG as compared to controls with only LB broth and bacteria. While all three bacterial species were reduced in their ability to attach to the 96-well plates, they did so in different amounts of reduction as compared to the planktonic growth. In planktonic growth, *S. saprophyticus* was completely inhibited followed by *S. capitis*, and then *S. epidermidis*. In the attachment phase, *S. saprophyticus* had the most growth, followed by *S. epidermidis* and *S. capitis*. These differences could be due to the growth characteristics of planktonic verses biofilm growth. In planktonic growth, the cells are continuously bathed in the AMG, whereas in biofilm growth, the bacteria grow in communities and the layers of bacteria can protect the community and keep the AMG from penetrating the biofilm.

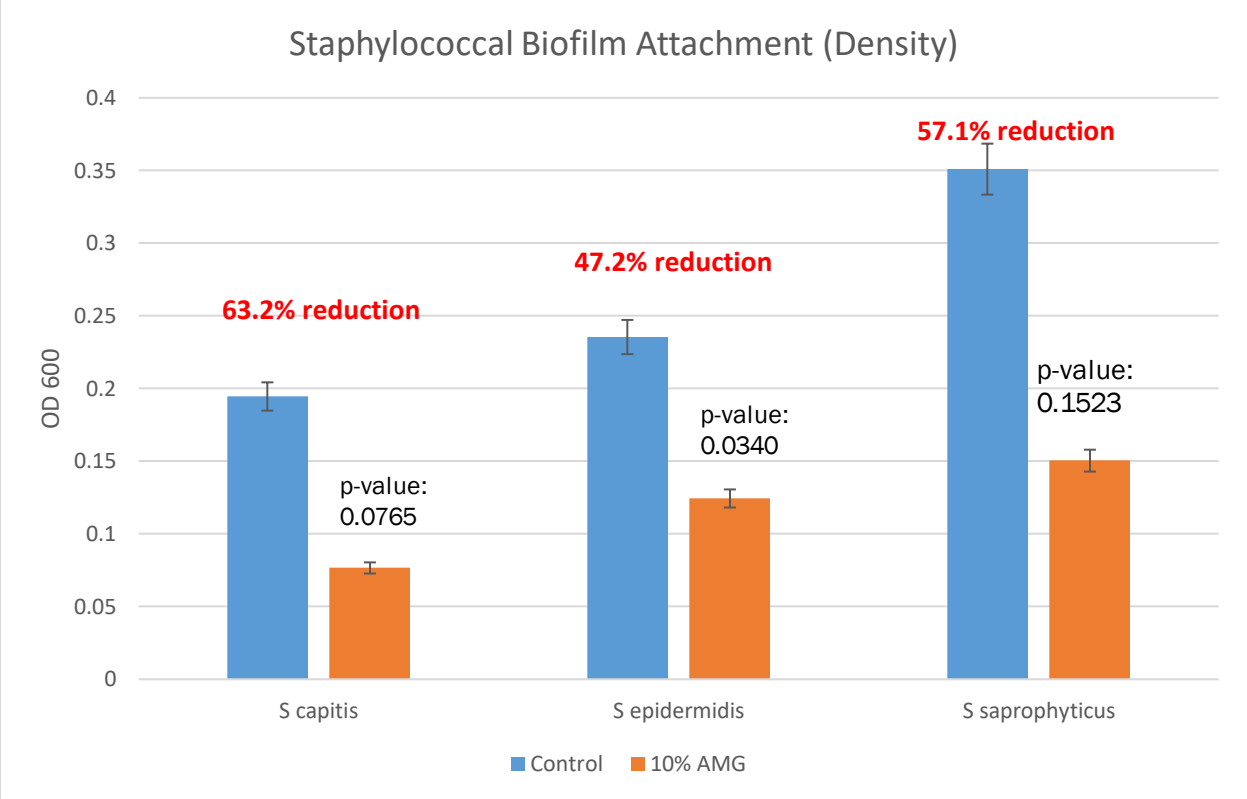

Figure 4: Graphical representation of biofilm growth over a 24 hour period of Staphylococcal species with AMG added at the same time as bacteria – Attachment Phase OD600 test (three separate trials).

The second method used CV staining and OD595 readings to determine the amount of biofilm density present (Figure 5). *S. capitis* density decreased by 19%, *S. epidermidis* density decreased by 39%, and *S. saprophyticus* density decreased by 55% with the application of AMG as compared to controls with only LB broth and bacteria. This data correlated with the optical density data from Figure 4 with all three species being reduced in their biofilm attachment abilities when AMG is added. Again, this inhibition is not as pronounced as the planktonic inhibition due to the different growth characteristics between the two forms. This inhibition does support the findings of the optical density data as they are proportional to the decreases seen from the OD600 readings.

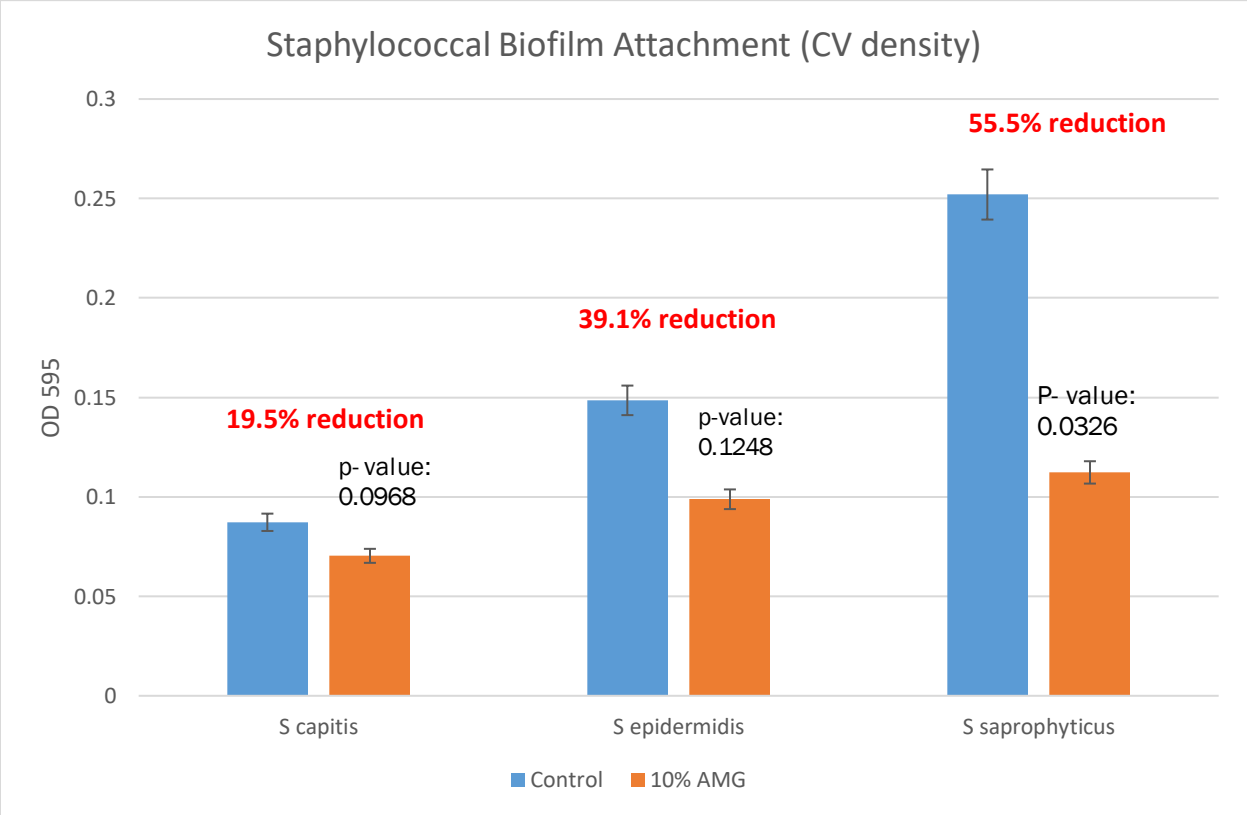

Figure 5: Graphical representation of 24-hour biofilm growth of Staphylococcal species with AMG added at the same time as bacteria – Attachment Phase – CV test (three separate trials).

Biofilm, maturation phase, of Staphylococcus species with 10% AMG.

After demonstrating that AMG inhibits the attachment phase of biofilms formed by *S. capitis*, *S. epidermidis*, and *S. saprophyticus*, the second phase of biofilm growth, mature biofilms, was evaluated. In many health care infections, biofilms are already formed on implanted devices or wounds. Treatments must then be directed at destroying these pre-formed biofilms that can typically be 10 to 1000X more resistant to antibiotics. We therefore wanted to determine if AMG could inhibit a later stage of bacterial biofilm formation i.e. an established biofilm on a surface. For this biofilm evaluation, bacteria were allowed to grow without AMG for 24 hours to create an established bacterial biofilm on the plate surface. After 24 hours, the media was removed, 10% AMG-LB was added and the biofilms were incubated for another 24 hours. The two methods for biofilm quantification were used as previously described. OD600 readings indicated that *S. capitis* density decreased by 39.9%, *S. epidermidis* density decreased by 47.9%, and *S. saprophyticus* density decreased by 38.1% with the application of AMG as compared to controls with only LB broth and bacteria (Figure 6). All three bacterial species demonstrated reductions in the mature biofilm coverage in the 96-well plates Again, varying amounts of reduction for each species was found when compared to the planktonic growth and the biofilm attachment assay. In planktonic growth, *S. saprophyticus* was completely inhibited followed by *S. capitis*, and then *S. epidermidis* and in the biofilm attachment phase, *S. saprophyticus* had the most inhibition, followed by *S. capitis*, and *S. epidermidis*. In the mature biofilm eradication, *S. epidermidis* had the largest reduction compared to controls, followed by *S. capitis*, and then *S. saprophyticus*. Since the mature biofilm has different characteristics than planktonic and even the biofilm attachment phases, it could be that *S. epidermidis* is more

sensitive at this time point verses the attachment phase to the inhibitory effects of AMG than the other Staphylococcal species.

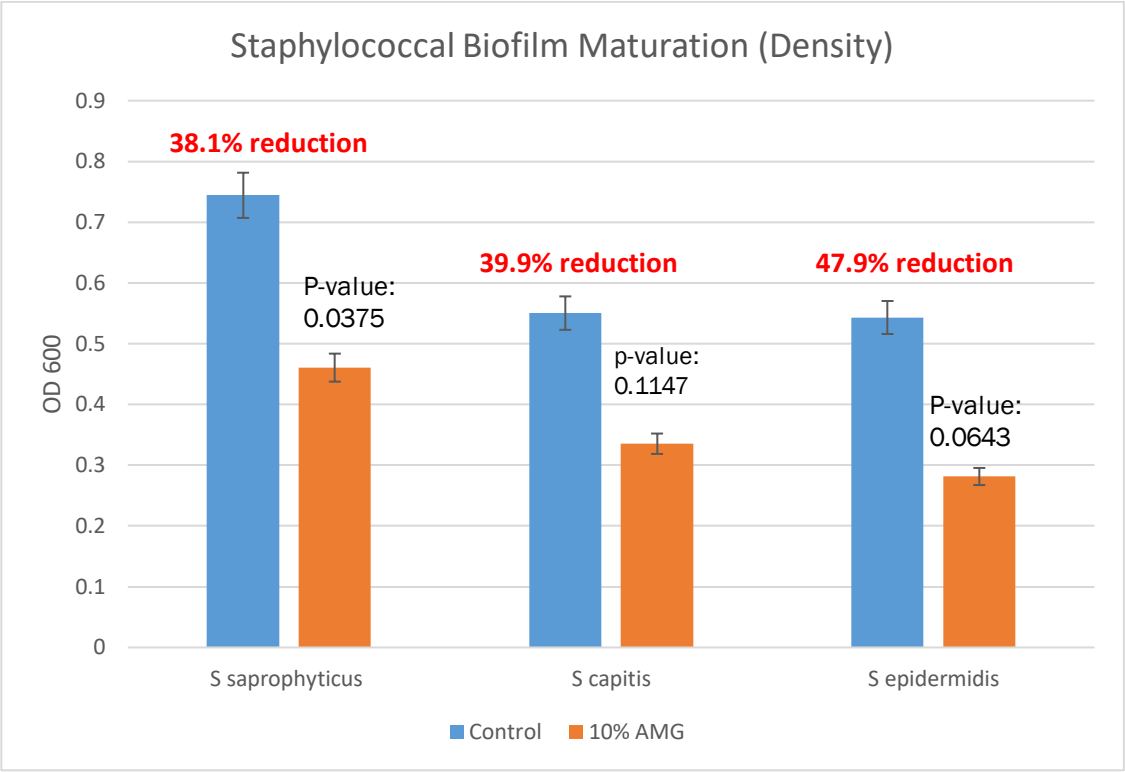

Figure 6: Graphical representation of 24-hour biofilm growth of Staphylococcal species with AMG only added after bacteria have an initial 24 hour growth – Maturation Phase – OD600 test (three separate trials).

The second assessment method again used CV staining and OD595 readings to determine the amount of biofilm density present (Figure 7). At this point we found unexpected results. In the trials using staining for mature biofilm eradication, there was a large increase in experimental verses the control wells for all three Staphylococcal species. These results may be due to errors made during the staining and washing process as this data is in complete odds against all of the other data collected in these experiments, including the OD600 readings of the mature biofilm

data. We are currently exploring and re-examining the staining process and will run additional experiments to determine the cause of these unexpected results.

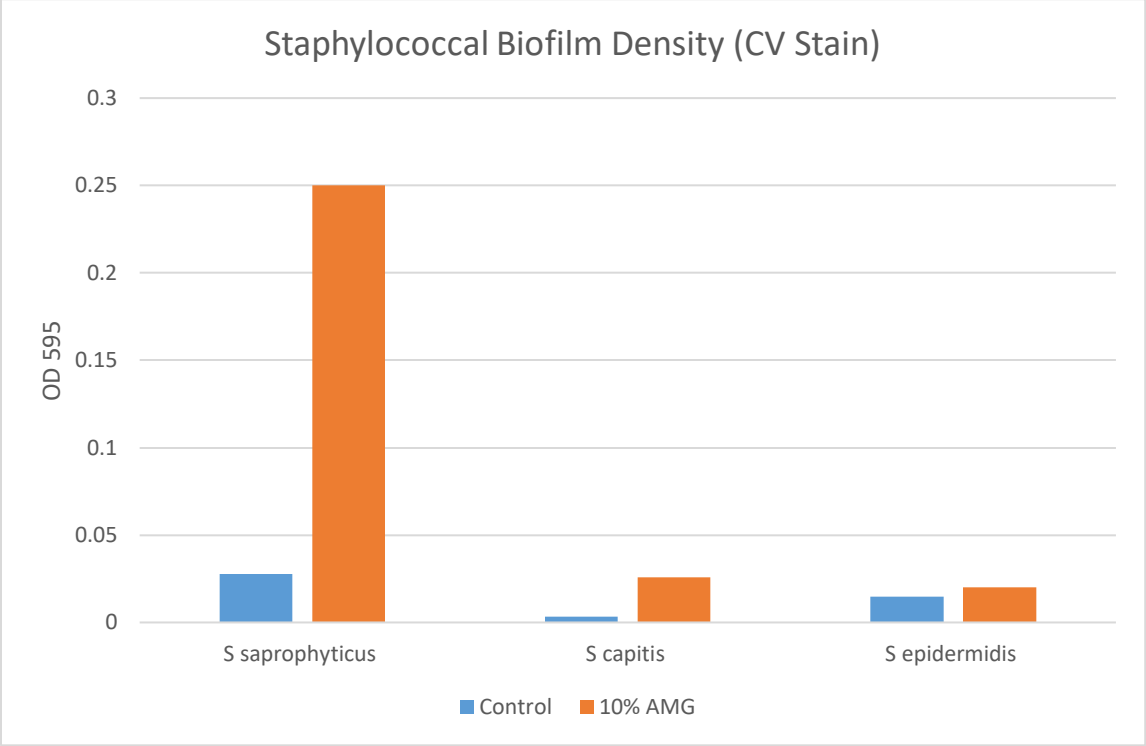

Figure 7: Graphical representation of 24-hour biofilm growth of Staphylococcal species with AMG only added after bacteria have an initial 24 hour growth – Maturation Phase – CV test (three separate trials).

DISCUSSION AND CONCLUSION

Antibiotic resistance is an ever-expanding problem in healthcare and new novel medications are needed in treatment rotation to prevent further resistance. The Staphylococcus genus is the most common cause of early burn would infections and nontoxic treatments are needed to further promote healing of burns without complications. At this time, the majority of treatment for burn wound infections have deficiencies such as toxicity, resistance, targeted specificity, and stability that do not promote positive outcomes for the patient. The AMG presented in this thesis has already shown to be a promising and powerful option for the

treatment of S. aureus infections. The results of this thesis, show that AMG reduced colony forming units and biofilm attachment, suggesting that this could be used as initial treatment after a burn wound has occurred. This data shows that the inhibitory effects of AMG are universally present in all species of Staphylococcus testing. If applied directly after a burn, it could prevent the attachment of Staphylococcus bacteria and inhibit the establishment of biofilms, resulting in the prevention of biofilm maturation that are typically highly drug resistant. AMG was less effective in maturation, suggesting that the biofilm matrix is more complex once completely developed and may prevent the AMG from reaching all cells. AMG significantly reduced the colony forming units, completely depleting *S saprophyticus* colonies. This suggests that it has a greater effect on individual planktonic bacteria than biofilms.

These results support that AMG reduced Staphylococcus biofilm attachment, maturation, and planktonic forms of growth. Future plans include evaluating Gram-positive bacteria and other cocci bacteria to evaluate the broad spectrum coverage of AMG. Additionally, varying concentrations of AMG will be used to determine if an increase in AMG would be better at inhibiting the hardy mature biofilms. Lastly, we plan to delineate the mechanism of action AMG has on bacteria, specifically Staphylococcus. AMG should be studied further to examine its benefits in application to not only burn treatments, but also as a means to be used prophylactically on medical devices and in conjunction with other treatments to prevent antibiotic resistance.

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