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The Effect of the Combined Use of Garlic Extract and Sodium Salicylate on the Inhibition and Eradication of *Staphylococcus epidermidis* Biofilms Grown on Titanium

Angel Huang

Implant associated infections (IAIs) place stress on patients and burden the healthcare system, and *Staphylococcus epidermidis* is a common pathogen involved. This study analyzed the effects of aqueous garlic extract (AGE) and sodium salicylate (SOD SAL) on the inhibition and eradication of *S. epidermidis* biofilms grown on titanium to find a potential remedy for IAIs. In the inhibition assay, bacterial suspensions were incubated with titanium discs and either AGE, SOD SAL, or both. In the eradication assay, bacteria cultured on titanium were treated with the treatments. The absorbance in optical density (OD) of each sample after treatment was analyzed using a spectrophotometer. AGE inhibited >40% of biofilm growth, whereas AGE and the combination treatment eradicated 17% of bacteria. It was concluded that AGE is better at inhibiting biofilm growth, but all treatments showed the same efficacy in eradicating pre-grown biofilm. Therefore, AGE could be used to treat and prevent IAIs.

Keywords: Staphylococcus epidermidis, titanium, garlic extract, sodium salicylate

Introduction

Orthopedic implants are primarily used to restore function in a patient's affected joint by replacing joints or by fixing bones (Zimmerli, 2014). As the population ages, the number of orthopedic surgeries increases. In fact, it is projected that the demand for primary hip and knee orthopedic surgery will increase by 174% and 673%, respectively, from 2005 to 2030 (Montanaro et al., 2011). Unfortunately, some of these implants become infected during or after surgery, causing tissue damage, impaired function, and sometimes death (Zimmerli, 2014; Antoci et al., 2008). The rate of infection after elective orthopedic surgery ranges from 0.7% to 4.2%, but this percentage can be much higher in other operations (Moriarty et al., 2016). Implant-associated infections (IAIs) require long courses of antibiotic therapy and multiple revision surgeries; not only does this place a strain on the patient physically, emotionally, and financially, but it burdens the overall healthcare system due to the extra expenditure of resources (Zhai et al., 2014).

The most common pathogens causing IAIs are *Staphylococci*, which make up about 75.3% of all strains, where *S. aureus* is the most common pathogen, followed by *S. epidermidis* (Montanaro et al., 2011). The major obstacle in treating infections caused by these bacteria is that some strains have become resistant to antibiotics, meaning certain antibiotics are no longer effective in killing the bacteria (Moriarty et al., 2016). Furthermore, *Staphylococci,* especially *S. epidermidis,* form biofilms, which are communities of bacteria residing in a polysaccharide slime they produce (Otto, 2018). These bacteria can attach to implant surfaces like titanium and such biofilms provide a layer of protection to the bacteria residing within, making them less susceptible to antibiotics (Zhai et al., 2014). Titanium implants are often colonized by

bacteria, and these microbes can infect the surrounding tissue, leading to further inflammation (Riool et al., 2014). These factors contribute to the difficulty in treating IAIs, which has led to more research on unconventional treatment methods.

Natural substances, such as garlic, have been tested for antimicrobial properties. When crushed, garlic releases a compound called allicin (Wu, Santos, & Fink-Gremmels, 2015). Allicin possesses antibacterial properties that occur when it reacts with enzymes containing sulfhydryl groups (Pérez-Giraldo et al., 2003). Fresh garlic extract containing allicin therefore has antibacterial properties as well, and it has been shown that garlic extract is able to inhibit and eradicate *S. epidermidis* biofilms (Wu, Santos, & Fink-Gremmels, 2015). This exhibits the potential for garlic extract to be used as a treatment for IAIs.

Like garlic extract, sodium salicylate is another compound that has antimicrobial characteristics. In fact, it is used in medicine as a non-steroidal anti-inflammatory drug (National Center for Biotechnology Information, 2020). According to Polonio et al., sodium salicylate is able to enhance the antibacterial activity of some antibiotics; for example, it enhances the activity of amikacin against another pathogen, *Klebsiella pneumoniae* (2001). Salicylate functions against *S. epidermidis* by reducing the production of biofilm constituents, which inhibits the overall growth of biofilm (Muller et al., 1998). It is clear that salicylate could hold a potentially major role in treating orthopedic infections.

Because of the challenges surrounding IAIs, the proposed research question is: To what degree can the combined use of garlic extract and sodium salicylate inhibit the formation of and eradicate *Staphylococcus epidermidis* biofilms?

Literature Review

Antibiotic Treatment

Antibiotic therapy is a current form of treatment for IAIs. Such therapy can be systemic or local (Moriarty et al., 2016). Due to the potential development of antibiotic resistance in bacteria, most infections are treated with a combination of antibiotic drugs (Moriarty et al., 2016). Rifampin, generally regarded as the most effective antibiotic against *S. epidermidis,* must be used in combination with other antibiotics to prevent bacterial resistance (Zimmerli, 2014). A study by Gomes et al. explores different combinations of common antibiotics and their effects on *S. epidermidis* biofilms (2012). The authors found that antibiotics alone were generally ineffective against the biofilms, but combinations of antibiotics that included rifampin were able to achieve reasonable bactericidal levels (Gomes et al., 2012). This concurs with a study done in 1996 by Isiklar et al., where rabbits infected with *S. epidermidis* were treated with various antibiotics; it was found that vancomycin alone, a strong antibiotic, was not as effective in curing infection as a combination of vancomycin and rifampin. Hamad et al. (2015) agree that the combination of antibiotics is more effective in eradicating biofilms; however, they found that the rate of resistance to rifampin in bacteria was rather high. While they acknowledge the importance of including rifampin in antibiotic therapy, they found that using another powerful antibiotic called doxycycline with rifampin may be more effective in eliminating biofilms. This is because most of the bacteria in their study were susceptible to doxycycline even if they were resistant to rifampin.

Instead of using two strong antibiotics, Polonio et al. (2001) used vancomycin in combination with sodium salicylate in their study of *S. epidermidis* biofilm inhibition and eradication. In this particular experiment, treating biofilms with a combination of sodium salicylate and vancomycin had a significantly higher bactericidal effect than treating them with the substances alone. Moreover, the combination treatment inhibited biofilm growth by over 99.9%. This research provides insight into the potential use of sodium salicylate in antibiotic therapy. However, it was found that some *S. epidermidis* strains have become resistant to vancomycin, thus further limiting the options for treating infections (Pinheiro et al., 2014). In this case, other treatment methods could be effective in eliminating infection.

Garlic Extract and Allicin

As a result of increasing antibiotic resistance in *S. epidermidis,* the exploration of using natural substances to treat infections has expanded, specifically on garlic and its properties. Garlic, when crushed, re-

leases allicin which has antibacterial properties (Wu, Santos, & Fink-Gremmels, 2015). One such study on the effects of fresh garlic extract and allicin on *S. epidermidis* biofilms discovered that the minimum biofilm inhibitory concentration (MBIC) of allicin was 12.5 μg/mL, and the MBIC for garlic aqueous extract and garlic ethanol extract was 1.56 and 0.78 μg/mL (Wu, Santos, & Fink-Gremmels, 2015). This indicates that garlic extract and allicin are able to inhibit the growth of *S. epidermidis* biofilms. Another study by Pérez-Giraldo et al. (2003) agrees that allicin has antibacterial properties against *S. epidermidis*. In this study, strains of bacteria that were and were not methicillin-resistant both showed reduction in biofilm growth after being treated with allicin (Pérez-Giraldo et al., 2003). Based on these studies, it can be concluded that allicin and garlic extract may be used to prevent IAIs.

Surgical Intervention

In all cases of device-related infections, surgical intervention is required to remove the infected tissue through debridement, and/or to remove the implant itself (Moriarty et al., 2016). Antibiotic treatment is also required to treat implant infections. Depending on the situation, the implant may be left in the patient, or it could be removed and replaced (Moriarty et al. 2016). A two-stage exchange of an implant involves removal of all dead tissue and the implant followed by a course of antibiotic treatment before a new implant is attached (Zimmerli, 2014). While this method yields higher success rates of eliminating infection than a one-stage exchange, it is highly invasive and can cause functional impairment (Zimmerli, 2014).

Modified Biomaterials

The best way to reduce implant associated infections is to prevent them from the start. In an effort to prevent the attachment and growth of bacteria on implant surfaces, researchers have modified biomaterials in ways such as loading them with antibiotics or by altering the surface structure (Montanaro et al., 2011). Doping biomaterials can have advantages over taking antibiotics systemically because the drug is delivered to the site of infection and can reach higher concentrations without the patient having to take more antibiotics (Moriarty et al., 2016). However, the concentrations of antimicrobial drugs in these doped biomaterials can cause local or systemic toxicity (Montanaro et al., 2011). Additionally, constant exposure to these drugs can contribute to the development of new antibiotic-resistant strains of bacteria (Montanaro et al., 2011). Similarly, loading biomaterials with antibiotics could be effective in preventing infection, but the risk of developing antibiotic-resistant strains limits this field. Nevertheless, a study by Antoci et al. (2008) shows the remarkable effectiveness of vancomycin-modified titanium alloy against *S. epidermidis*. The modified biomaterial effectively prevented the adhesion and production of biofilm, and its antimicrobial activity continued even after being challenged by bacteria (Antoci et al., 2008). Moreover, when tested for resistant *S. epidermidis,* the zone of inhibition of the bacteria remained the same after four weeks of being exposed to the modified biomaterial (Antoci et al., 2008).

Coating implant surfaces with certain substances that can minimize bacterial adhesion is another way to reduce infection (Montanaro et al., 2011). These substances function by modifying the hydrophilic properties of a biomaterial surface or by hindering protein adsorption onto the surfaces (Montanaro et al., 2011). Certain metals have shown antibacterial activity, and one particular metal that has exceptional potential is silver (Moriarty et al., 2016). Silver has antimicrobial properties against a broad spectrum of bacteria, and it is effective at low concentrations with low toxicity (Moriarty et al., 2016). However, bacteria may develop resistance to silver, and silver poses a health risk, though minimal, to humans (Moriarty et al., 2016).

Summary

While these studies analyzed a multitude of treatments for IAIs, there are still some gaps that need to be filled. Previous studies on combinations of antibiotics have not used sodium salicylate with natural antibiotic substances, so it is unknown whether it can enhance antibacterial properties in such substances. Similarly, garlic extract has not been used in combination with other antibiotics, so its full antibiotic potential has not been realized. Previous studies involving

antibiotics also did not use titanium as a model implant surface, and as titanium is a common implant material, it is important to use it in experiments as an implant surface for bacteria to attach to so as to model real life infections. Therefore, this study sought to bridge this gap by analyzing the effect of combining garlic extract and sodium salicylate on *S. epidermidis* biofilms grown on titanium.

Materials

- Tryptic Soy Broth (Carolina Biological)
- Sodium salicylate, $C_7H_5NaO_3$ (HiMedia)
- SpectroVis™ Plus Spectrophotometer (Vernier)
- 24-well flat bottom plates
- 10mL test tubes
- 600mL beakers
- Parafilm
- Laptop computer
- Micropipette
- Bunsen burner
- Surgical blade
- Blender
- Forceps
- Coffee filter
- Garlic bulbs
- Distilled water
- 1 strain of *Staphylococcus epidermidis* (ATCC® 35984™)
- 1 packet of Dulbecco's Phosphate Buffered Saline, Without Calcium and Magnesium (HiMedia)
- Titanium discs, commercially pure grade 1, low iron, 9mm dia, 2mm thick (Kemira Chemicals)

Methodology

The purpose of this experiment was to analyze the effects of aqueous garlic extract (AGE), sodium salicylate (SOD SAL), and a combination of both substances on the inhibition and eradication of *S. epidermidis* biofilms grown on titanium. Both eradication and inhibition tests were performed to compare the efficacy of each treatment before and after biofilms were grown.

Preparation of Treatment Solutions

There were three treatment solutions used in this experiment. Treatment A was 16 mg/mL AGE, treatment B was 5 mM SOD SAL, and treatment C was a combination of 16 mg/mL AGE and 5 mM SOD SAL. The concentration of AGE was determined based on a study by Belguith et al. (2010), where the allicin content in different concentrations of AGE was approximated; the authors estimated that there was 8.1 µg/mL in 10 mL of AGE. After establishing an approximate allicin content per 1 mL of AGE, another study that found the minimum inhibitory concentration (MIC) of AGE against *S. epidermidis* was referred to. Its authors found that the MIC occurred at a concentration of 12.5 µg/mL allicin (Wu, Santos, & Fink-Gremmels, 2015). Hypothetically, the AGE that was prepared in this experiment would have had an allicin concentration of 12.96 µg/mL, based off of the study by Belguith et al. However, because this research was not conducted in a professional lab, it was anticipated that the prepared AGE would not contain as high an allicin content. Therefore, a concentration of 16 mg/mL for the AGE was used so that while it would exhibit a bactericidal effect, the AGE concentration would not be too high and eradicate all the bacteria, as that would impede the ability to accurately compare the efficacy of each treatment. Therefore, this concentration ensured that there would be data from each sample to analyze and compare. As for treatment B, a study by Polonio et al. (2001) used 5 mM SOD SAL, so the same concentration was used in this experiment.

Bacteria Strain

The bacteria strain used in this study was *S. epidermidis* RP62A (ATCC 35984) due to its common occurrence in IAIs and its ability to form biofilms. In order to perform the inhibition and eradication tests, the original strain of bacteria was incubated with 6 mL of tryptic soy broth (TSB) in a tube according to the instructions provided on the *S. epidermidis* product sheet (ATCC, 2020). The bacteria was incubated at 37°C for 24 h. Afterwards, this bacterial solution was diluted using a spectrophotometer to reach an optical density (OD) value of 0.2 when analyzed at a wavelength of 600 nm. The wavelength at which OD values were taken was determined to be 600 nm because it

ensured that the bacteria's absorbances were being measured, as opposed to the absorbance of other substances in the samples. To dilute the solution, the tube was first shaken vigorously to create a homogenous mixture, and then the solution was distributed into cuvettes to find the OD values. Phosphate buffered saline (PBS) was added to each cuvette until the OD values reached 0.2. Afterwards, aliquots of 20 µL of the diluted bacterial suspension were dispensed into 210 wells of 24-well flat bottom plates that each contained 1 mL of TSB and a titanium disc (shown in Figure 1A). A micropipette was used to ensure accurate measurements.

Biofilm Inhibition Assay

To find the inhibitory effects of each treatment, 100 µL of each treatment was allocated in 90 wells with 30 wells for each treatment. Another 30 wells contained only the bacterial suspension and broth to serve as a control group. These wells were incubated at 37°C for 6 days to ensure growth of mature biofilms, which Cao et al. had previously done in their study (2018). However, these wells were taken out of the incubator after 22 h to monitor bacterial growth; then 1 mL of TSB was added to each well, and parafilm was placed over the plates to prevent evaporation. The plates continued to incubate until they reached 6 days. After the 6th day, the biofilms on the titanium discs were scraped into tubes containing 5 mL of broth. To do this, the discs were rinsed with PBS to remove non-adherent bacteria. Then they were scraped with a flame-sterilized scalpel. The scraping of the discs was performed as such: a disc was held by the hand, both sides were scraped, and the scalpel was swirled vigorously in a tube to remove the scraped biofilm (as shown in Figures 2A and 3A). This method was repeated for all discs, and the tubes were incubated at 37°C for 18 h. After incubation, the cultures were vigorously swirled so that they became homogeneous, and they were analyzed for absorbance values using the spectrophotometer. After each culture was transferred into and out of a cuvette, the cuvette was rinsed with PBS to prevent cross-contamination. All OD values were recorded using Vernier Spectral Analysis.

Biofilm Eradication Assay

To find the effect of each treatment on pre-grown biofilms, 90 of the 210 wells were incubated for 24 h at 37°C without any treatments. The discs from these wells were scraped in the same manner as mentioned above; then the tubes were incubated at 37°C for 18 h. After incubating, 100 µL of each treatment were dispensed into the tubes, allocating 30 tubes for each. The tubes were incubated once more for 24 h at the same temperature as before. After the final incubation, the cultures were swirled and analyzed for OD values in the same manner as the inhibition assay.

Statistical Analysis

A one-way analysis of variance (ANOVA) test was conducted to analyze the statistical differences between each treatment in the inhibition and eradication tests. The alpha value was 0.05, with $p<0.05$ showing statistical differences. A post-hoc Tukey test was conducted for both assays to determine between which groups statistical differences existed. Outliers were not considered in the data analysis.

Results

In the inhibition assay, it was found that all treatment groups produced an average OD value lower than that of the control group, indicating fewer bacteria in treatment groups. This can be seen in Table 1, where the mean OD for the control group was 0.407, whereas the means of treatments A, B, and C were 0.243, 0.377, and 0.367, respectively. Treatment A produced the lowest mean, inhibiting 40.3% of biofilm growth. Table 1 also indicates that the medians of each treatment group were lower than that of the control group. Moreover, it can be seen that the minimum OD value out of all samples occurred with treatment A, while the minimums for treatments B and C were higher than the control group minimum. However, the control group produced the maximum OD value out of all samples, and treatment A yielded the lowest maximum. Treatment C showed the lowest range, and the control group showed the highest. These data are visually represented through a boxplot in Figure 1.

Table 1: Descriptive Statistics for Inhibition Assay OD Values

Treatment	n	Mean	Std. Dev.	Min	Q1	Median	Q3	Max	Range	Mean OD as a percentage of the control
A	30	0.243	0.052	0.114	0.218	0.247	0.287	0.317	0.204	59.7%
B	30	0.377	0.035	0.292	0.357	0.378	0.403	0.426	0.134	92.6%
C	30	0.367	0.035	0.303	0.347	0.368	0.389	0.431	0.127	90.2%
D (Control)	30	0.407	0.067	0.274	0.370	0.416	0.440	0.572	0.298	100%

Table 1 shows the descriptive statistics for the inhibition assay data. Outliers were not considered in calculations. It can be seen that Treatment A had the lowest mean and the control group had the highest.

Figure 1: Boxplot of Inhibition Assay OD Values

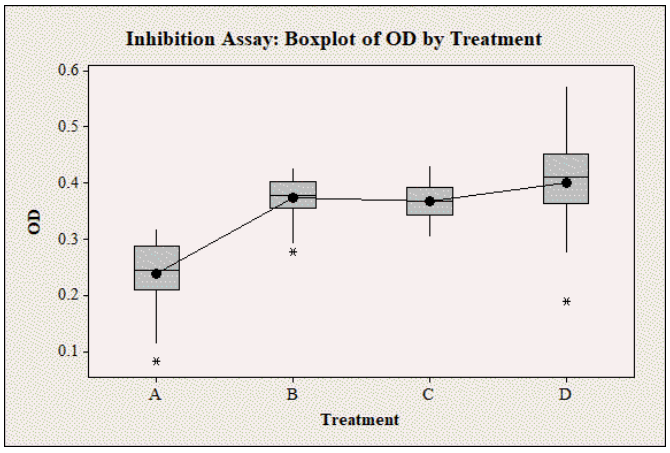


Figure 1 shows a boxplot of the OD values in the inhibition test. Outliers are represented with the star symbol. It c an be seen that all treatments had a lower average OD than the control (treatment D).

The results of the eradication assay showed that on average, all treatment groups had fewer bacteria after treatment. The mean OD value of the control group was 0.657, shown in Table 2, whereas the means for treatments A, B, and C were 0.545, 0.571, and 0.545, respectively. Treatments A and C eliminated the most bacteria at 17%. Table 2 also shows that the medians for all treatment groups were lower than the median of the control group. The lowest minimum was produced with treatment B at 0.443, and the highest minimum occurred with the control group. Similarly, the highest maximum value was also produced in the control group, and the lowest maximum was produced with treatment A. The highest range was 0.220, occurring in treatment B. These values are visually represented in Figure 2.

Table 2 shows the descriptive statistics for the eradication assay data. Outliers were not considered in calculations. All treatments produced a mean OD

Table 2: Descriptive Statistics for Eradication Assay OD Values

Treatment	n	Mean	Std. Dev.	Min	Q1	Median	Q3	Max	Range	Mean OD as a percentage of the control
A	30	0.545	0.039	0.461	0.516	0.548	0.583	0.603	0.142	83%
B	30	0.571	0.053	0.443	0.529	0.583	0.603	0.663	0.220	86.9%
C	30	0.545	0.030	0.467	0.529	0.549	0.564	0.610	0.144	83%
D (Control)	30	0.657	0.044	0.549	0.630	0.654	0.688	0.752	0.203	100%

lower than the control group. Treatment B showed the greatest variation in OD.

Figure 2: Boxplot of Eradication Assay OD Values

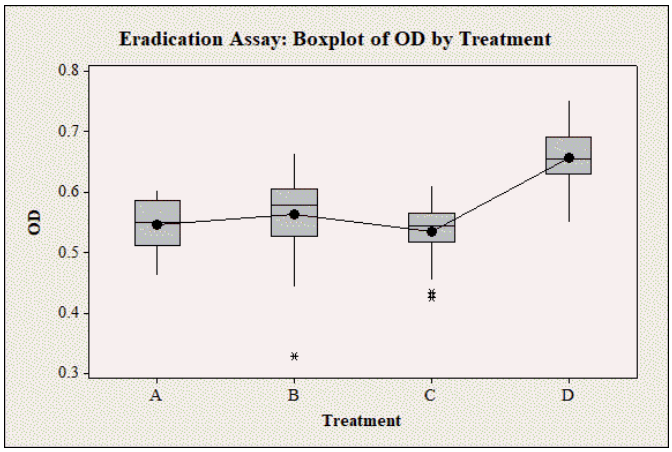


Figure 2 shows a boxplot of the OD values in the eradication test. Outliers are represented as the star symbol. The control group yielded the highest average OD, and treatment C had the most outliers.

To determine the statistical significance of the data, a one-way ANOVA test was conducted with a confidence level of 95%. In the inhibition assay, the null hypothesis was that there would be no difference between the average amount of biofilm grown between samples exposed to each treatment. The p-value was <0.001, which was lower than the set alpha (α) value of 0.05, so the null hypothesis was rejected. This means that there was a significant difference in OD values between groups, as seen in Table 3. A Tukey test, shown in Figure 3, was conducted to determine the areas of statistical difference. It was found that treatment A had a significantly lower average OD value than all other groups; however, there was no statistically significant difference between treatments B, C, and the control group.

Table 3 shows the table values of the ANOVA test where $\alpha = 0.05$, and $p < 0.001$; S = 0.05511, R-Sq = 57.52%, R-Sq(adj) = 56.42%. Because $p < \alpha$, the results were significant.

Figure 3 shows the Tukey post-hoc test for the inhibition assay. Statistical difference between means are shown by the (---*--) symbols not overlapping, meaning only treatment A had a statistically significant different mean OD.

A one-way ANOVA was also conducted on the eradication assay data, and the null hypothesis was that there would be no difference in the average amount of surviving biofilm between each treatment. The p-value was <0.001, thus rejecting the null hy-

Figure 3: Tukey Test on Mean OD Values for the Inhibition Assay

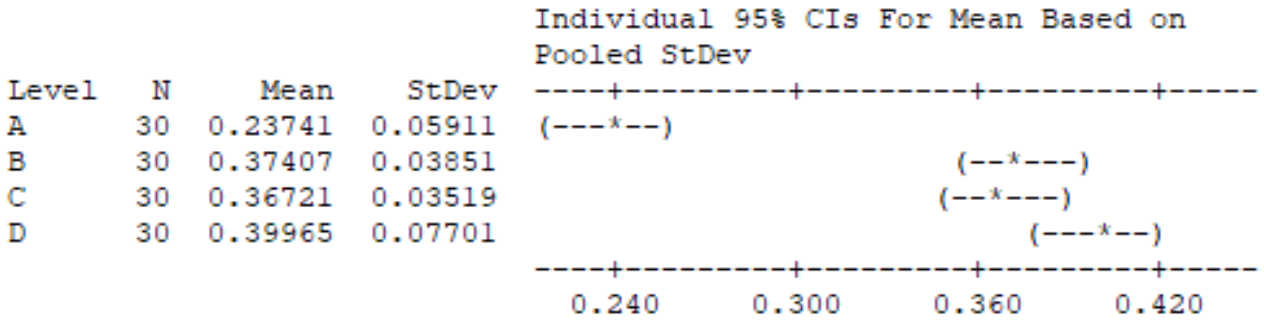


Table 3: One-way ANOVA Test on Mean OD Values for the Inhibition Assay

Source	DF	SS	MS	F	P
Treatment	3	0.47698	0.15899	52.36	<0.001
Error	116	0.35227	0.00304		
Total	119	0.82925			

Table 4: One-way ANOVA Test on Mean OD Values for the Eradication Assay

Source	DF	SS	MS	F	P
Treatment	3	0.27937	0.09312	36.93	<0.001
Error	116	0.29249	0.00252		
Total	119	0.57186			

pothesis and demonstrating statistical significance between groups. Table 4 shows the values of the ANOVA test. As shown in Figure 4, a post-hoc Tukey test was conducted and showed a statistically significant difference between each treatment group and the control group. However, there was no significant difference between the individual treatment groups.

Table 4 shows the table values of the ANOVA test where $\alpha = 0.05$, and $p < 0.001$; S = 0.05021, R-Sq = 48.85%, R-Sq(adj) = 47.53%. Because $p < \alpha$, the results were statistically significant.

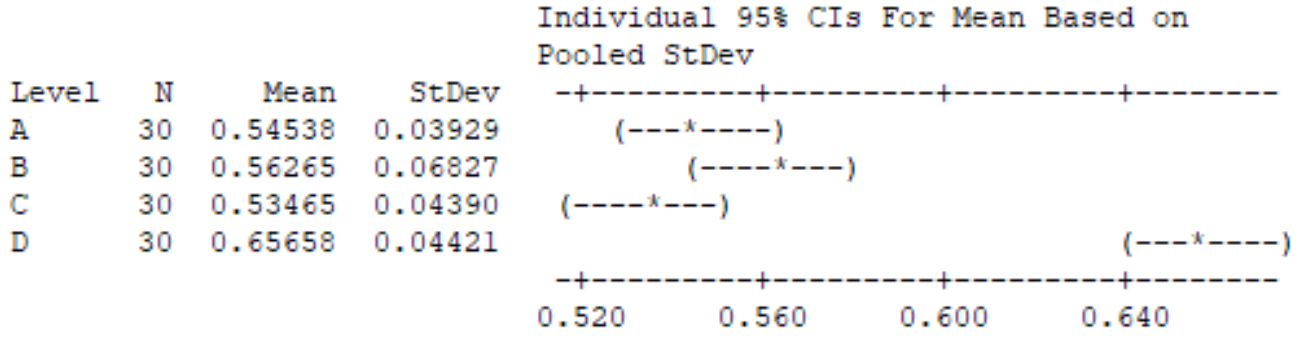
Figure 4 shows the Tukey post-hoc test for the eradication assay. Statistical difference between means are shown by the (---*--) symbols not overlapping, meaning there was no statistically significant difference between the treatments, but all treatments produced a mean OD significantly lower than the control group.

Discussion

The purpose of this study was to find a novel potential treatment for orthopedic implant infections against *Staphylococcus epidermidis* biofilms. *S. epidermidis* is able to attach to implant surfaces like titanium and cause infection in a patient during or after surgery. Infections must be treated with combinations of antibiotics, and since garlic extract and sodium salicylate are substances with antimicrobial properties, the goal of this research was to determine if sodium salicylate could enhance those activities of AGE.

The hypothesis that treatment C, a combination of AGE and SOD SAL, would be most effective in inhibiting and eradicating *S. epidermidis* biofilms grown on titanium was not supported. In the inhibition assay, it was found that treatment A, AGE alone, produced the lowest mean OD value, and the Tukey test confirmed that it was statistically significantly lower than all other treatments as well as the control. This means that AGE was the most effective inhibitory agent against *S. epidermidis* grown on titanium, whereas SOD SAL and the combination treatment did not inhibit a significant amount of bacteria grown. In other words, AGE is superior to the other treatments in preventing the initial growth of bacteria. This might be because the integrity of SOD SAL diminishes over time when in an aqueous solution, whereas allicin retains antimicrobial effects for a long time. Similarly, while treatment C had an average OD value lower than the other groups for eradication, the Tukey test demonstrated that the treatments did not eradicate significantly different amounts of bacteria from each other, but rather they all eradicated a significant amount of bacteria when compared to the control group. This essentially

Figure 4: Tukey Test on Mean OD Values for the Eradication Assay



demonstrates that the efficacy of each treatment in eradicating pre-existing bacteria was about the same. It can be concluded that AGE could be used to prevent and kill bacterial biofilms in IAIs as it inhibited 40.3% of biofilm growth and eradicated 17% of biofilms in this study.

The results of this study are generally concordant with previous research, specifically the results of AGE. Wu, Santos, & Fink-Gremmels (2015) found that AGE was able to inhibit at least 90% of *S. epidermidis* biofilm formation at a concentration of 1.56 μg/mL. Conversely, in regards to the results of sodium salicylate, a previous study showed that 5 mM sodium salicylate did not have a significant effect on the eradication of biofilms (Polonio et al., 2001), whereas there was significant eradication in this experiment. Similarly, there was no significant inhibitory effect of sodium salicylate observed in this study, but Muller et al. (1998) found that 5 mM salicylic acid, of which sodium salicylate is a sodium salt, had a biofilm inhibitory effect of up to 55%.

Though AGE showed the most potential for use in the medical field to combat IAIs, it is unknown based on this research what is the most effective and safe method by which this could be done. This study also did not explore the use of AGE and SOD SAL for use as a topical cream, so it is unknown whether that is possible. Since implants are located underneath tissue, topical creams may not be effective. Because garlic can cause irritation in humans, it may prove beneficial to extract pure allicin from garlic in contrast to using fresh garlic extract. This allicin could be used in a lavage (flushing saline solutions through a body cavity), which is what Zhai et al. (2014) performed in their study with rabbits. While the combination of AGE and SOD SAL did not inhibit biofilm growth, SOD SAL could still be used in a solid form rather than as an aqueous solution to prevent inflammation. For example, in an infected patient, a combination of ingesting SOD SAL and using an allicin lavage may be more effective in eliminating bacteria on implant devices as well as relieving symptoms like inflammation. Nevertheless, this research project did not analyze the *in vivo* effects of the two substances, so effects on humans cannot be determined.

Conclusion

Based on the results, it is concluded that AGE is a more effective substance in inhibiting the growth of *S. epidermidis* biofilms than SOD SAL or a combination of both. Furthermore, all treatments have the same level of effectiveness in eradicating pre-grown biofilms. A few sources of error are apparent in this study. One possible error is contamination of bacteria samples, which could have led to more or less bacteria being cultured, as well as the introduction of different microbes. Another error could have been imprecise scraping of the titanium discs; if the biofilm on a disc was not completely scraped off, it would lead to less bacteria grown or an inaccurate final OD measurement.

In order to mitigate these issues, samples should be incubated in a separate incubator from other research samples, and well plates and tubes should be covered with cases and lids/stoppers instead of parafilm to prevent contamination. Furthermore, instead of scraping the discs with a blade, the discs could be incubated in a tube from the beginning, and the blade could be vortexed afterwards to achieve a higher bacterial retrieval rate.

Future research could be conducted to analyze the efficacy of each treatment when in different concentrations—especially the concentration ratios of treatment C. Different ratios of sodium salicylate and garlic extract together may be more or less effective. Moreover, different types of garlic extract could be tested, such as garlic ethanol extract. Instead of using sodium salicylate, salicylic acid could be used with garlic extract to analyze its efficacy in inhibiting and eradicating *S. epidermidis*. These different treatments could also be tested on different strains of bacteria commonly involved in infections, or they could be tested on alternative implant materials to observe any differences in their effects between materials.

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Appendix A

Figure 1A: Setup of Well Plates

All wells contained a titanium disc and TSB. Samples in the inhibition assay also contained treatments.

Figure 2A: Scraping of Biofilm

Titanium discs were held by the hand and scraped on both sides. The retrieved biofilms were placed into tubes shown in Figure 3.

Figure 3A: Setup of Test Tubes

Scraped biofilms were placed into tubes containing TSB.

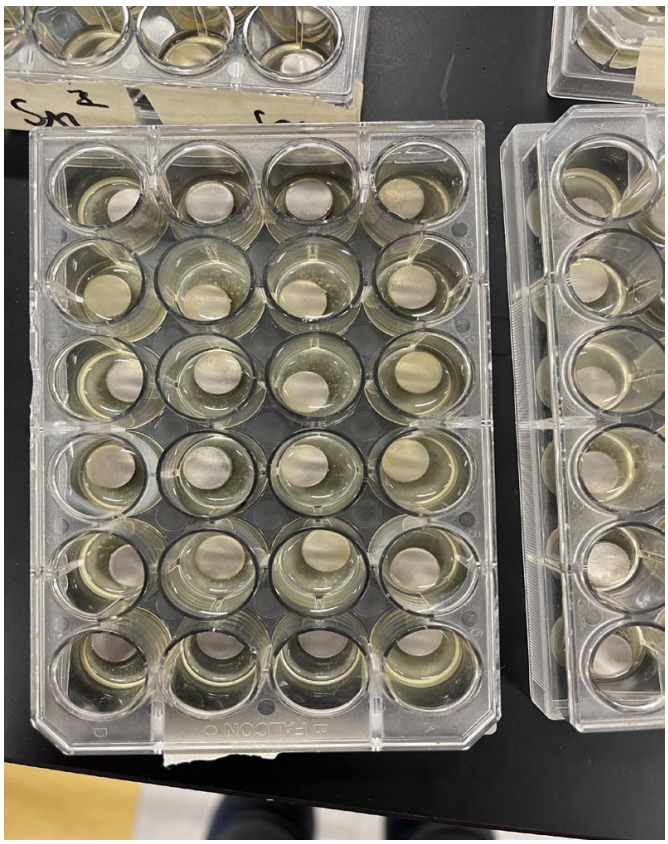

Figure 4A: Experimental Design Diagram

Title of the Experiment

The Effect of the Combined Use of Garlic Extract and Sodium Salicylate on the Inhibition and Eradication of *Staphylococcus epidermidis* Biofilms Grown on Titanium

Hypothesis

If *S. epidermidis* biofilms grown on titanium were treated with garlic extract, sodium salicylate, and a combination of both, the combination would eradicate the most bacteria.

If *S. epidermidis* were treated with the same treatments before incubation, the combination treatment would inhibit the most bacterial growth.

This is because it has been found that garlic extract and sodium salicylate alone are able to inhibit *S. epidermidis* biofilm growth as well as kill pre-grown bacteria. Moreover, combinations of antibiotics are stronger than antibiotics alone, so if these two substances were combined, a synergistic antimicrobial effect on biofilms could occur.

Independent Variable

Type of treatment solution, concentration in mM for salicylate and mg/mL for garlic extract

Levels of Independent Variable	No treatment	100 µL Garlic extract [16 mg/mL]	100 µL 5 mM Sodium salicylate	100µL [16 mg/mL] Garlic extract & 5 mM sodium salicylate
Number of Repeated Trials	30	30	30	30

Dependent Variable

The amount of S. epidermidis biofilm grown after treatment, measured by absorbance as optical density (OD).

Control Group

The S. epidermidis cultures that did not receive any treatments.

Constants

Incubation time, incubation temperature, size of titanium, amount of each treatment solution, and amount of bacterial suspension in each well.

Appendix B

Table 1B: OD Values of all Samples in the Inhibition Assay

	OD Values of Each Treatment			
Trial #	A	B	C	D (Control)
1	0.2851874475	0.2780993314*	0.3679498607	0.3571902314
2	0.317455084	0.3653009454	0.3973563441	0.1889349879*
3	0.2183491188	0.3275391492	0.4280891533	0.2744655196
4	0.2868359322	0.4028392951	0.3467153612	0.4202966985
5	0.1532724261	0.4213877327	0.3077033426	0.2988368019
6	0.1738755035	0.4190405195	0.3676739741	0.5720113041*
7	0.2303741967	0.4028410775	0.3213650118	0.3046155284
8	0.1885259178	0.2923834051	0.3686520986	0.3514231196
9	0.08270700268	0.3997633401	0.3469546148	0.4347474441
10	0.2242883382	0.3787593748	0.3062669656	0.463856493
11	0.1135580666	0.3896502077	0.3925079884	0.366233598
12	0.2927436265	0.3574365066	0.4114510355	0.4061312031
13	0.2262340895	0.36369118	0.3676604172	0.381776472
14	0.2220043235	0.3352127724	0.3684093396	0.3780267482
15	0.2490341234	0.3713568071	0.4201377702	0.2872811513
16	0.2867781143	0.3035676927	0.4307128638	0.460815044
17	0.3142047876	0.4263244002	0.3670192373	0.392046906
18	0.2883847039	0.3587978558	0.3727388047	0.4164609565
19	0.2110208651	0.4216752685	0.3769069798	0.3942075214
20	0.2817411641	0.3536996975	0.3792590655	0.4738556058
21	0.3067921244	0.419805875	0.3734818766	0.4806301821
22	0.2236561755	0.4061964286	0.3033035261	0.4730547453
23	0.2687024658	0.3547592097	0.3305159769	0.4222641553
24	0.2408869848	0.3529607783	0.3303753594	0.4497005586
25	0.1576889091	0.3776734686	0.3578821298	0.4402125265
26	0.2474524941	0.3889287911	0.3661230898	0.3696597282
27	0.3099243974	0.3782264974	0.3909390096	0.3782724155
28	0.2053520714	0.374275919	0.383784318	0.4201847145
29	0.249703317	0.3994027324	0.4099836515	0.4372440608
30	0.2657005147	0.4003981333	0.3244872706	0.4951938012
Average	0.2374144762	0.3740664798	0.3672135479	0.3996543408

Table 1B shows the raw data of the inhibition assay. OD values of each sample in each treatment are listed, with outliers marked with an asterisk (*) and highlighted red.

Table 2B: OD Values of all Samples in the Eradication Assay

	Treatment			
Trial #	A	B	C	D (Control)
1	0.5275587127	0.6504282032	0.4334718749*	0.6551254771
2	0.5267575373	0.5917214972	0.5040065365	0.6473124179
3	0.4938386897	0.492122447	0.5553153643	0.612443668
4	0.5036701248	0.5838696926	0.4250348902*	0.5491903691
5	0.5073578466	0.6240485271	0.5423893977	0.64299824
6	0.4610022143	0.557186929	0.5778259661	0.6698555443
7	0.544033014	0.6343816309	0.4536768836*	0.629575358
8	0.508701091	0.5917653667	0.5191298103	0.6203853632
9	0.5264017294	0.6627897494	0.6104175432	0.6938001773
10	0.5120972084	0.6014129049	0.4665836495	0.6986188644
11	0.4734846189	0.6351421648	0.5312648929	0.6280712285
12	0.4961067332	0.328119895*	0.5549292261	0.6380900841
13	0.5363889189	0.4873234177	0.5493949925	0.606029388
14	0.5504904216	0.5636789124	0.5248361812	0.6526609985
15	0.5533794519	0.5268581446	0.4953810022	0.6087457702
16	0.5508952309	0.4979209229	0.5445475637	0.5740213953
17	0.5792801464	0.6026758793	0.5576183082	0.6842155739
18	0.5920577904	0.5529664703	0.5496882933	0.6513808127
19	0.590223611	0.6142248316	0.5417689056	0.6928423226
20	0.5879899729	0.5202059285	0.5267610785	0.685375371
21	0.5973508956	0.529066082	0.5085420918	0.6799894618
22	0.6011285913	0.524008598	0.5665900461	0.6323236136
23	0.589705701	0.4427459611	0.5691342891	0.7003428563
24	0.6029407318	0.609886592	0.5422114608	0.6649698628
25	0.5379962112	0.583225149	0.5628091786	0.6693946832
26	0.5843032645	0.6033665532	0.577547242	0.7303928342
27	0.5580918406	0.5829126387	0.5614703985	0.7517253385
28	0.5665890398	0.570219111	0.5757693929	0.6331514619
29	0.5457093153	0.57290202	0.578591386	0.7059940283
30	0.5559399599	0.5422569915	0.5329275236	0.688315795
Average	0.5453823538	0.5626477737	0.5346545123	0.6565779453

Table 2B shows the raw data of the eradication assay. OD values of each sample in each treatment are listed, with outliers marked with an asterisk (*) and highlighted red.

Figure 1B: OD Analysis Using the Spectrophotometer

Figure 1B shows the method used to analyze the bacterial samples for absorbance values in OD.

Figure 2B: Lab Setup of Biofilm ScrapingFigure

3B: Image of Bacterial Sample