Elucidating the Mechanisms of Antibiotic Tolerance During Co-Infection of *Staphylococcus aureus* and *Streptococcus agalactiae* in Chronic Wounds

Lane McCoy

Abstract:

Polymicrobial infections are some of the most financially demanding issues in the healthcare system, requiring over $25 billion in treatment annually in the United States alone. This results from their increased virulence, infectivity, and tolerance of common antimicrobial treatments, part of a process termed synergy. While interactions between bacteria have been appreciated for decades, we do not completely understand the exact mechanisms for how these organisms interact within the infection itself. Furthermore, questions remain about how these interactions depend on local microbes, on the environment of their host, or on spatial arrangement in the wound. Here, we sought to identify and understand novel interactions between *Staphylococcus aureus* and *Streptococcus agalactiae* (Group B Streptococcus, GBS) in a chronic wound environment. To accomplish this, we assessed tolerance to various antibiotics of each organism individually and in co-culture in our *in vitro* wound models. Furthermore, we sought to understand how a *diabetes mellitus* type II environment impacted interactions between these microbes by evaluating how a hyperglycemic environment altered antibiotic tolerance. Our preliminary results suggest that *S. aureus* has increased tolerance to specific antibiotics when co-cultured *in vitro* with *S. agalactiae* and saw an increase in tolerance when grown in our wound models. Our data also shows that *S. agalactiae* has increased tolerance to specific antibiotics when co-cultured in wound models individually and decreased tolerance when grown with *S. aureus*. These results suggest a heightened importance of the environment on the role of antibiotic tolerance development in microorganisms when grown together, and further tests are needed to determine the exact mechanisms by which the environment alters the physiology of these two species. These investigations could be essential in producing more effective treatment strategies and hindering the progression of chronic wounds.

Introduction:

Wounds harbor many types of microorganisms, which contribute to the chronicity and overall progression of these wounds. Despite being some of the most prevalent cases in hospitals, research into microbial physiology in co-infection within the wound infection environment is lacking. Each year, chronic wounds deplete the health of patients, reduce their quality of life, and pose a substantial financial burden to healthcare systems due to their elevated levels of pathogenicity and tolerance towards common antibiotics. It is predicted that the increased levels of antibiotic tolerance within chronic wounds can be attributed to the presence of various organisms since most organisms individually are susceptible to common treatments; however, the mechanisms that establish antibiotic recalcitrance are unknown. Bacteria can interact with each other in a variety of ways, for example: they are capable of secreting sensory molecules in a process known as quorum sensing; they can modify their environment, secreting exopolysaccharides and other materials to construct a biofilm; they can share metabolites or even

lyse competitors. Knowledge of physiological alterations in common wound microbes throughout an infection is critical to develop effective antimicrobial therapies for chronic wounds. Not only this, but it is essential to investigate the microbial ecology within the wound environment to elucidate key mechanisms that allow microorganisms to resist treatment.

For this study, it was critical to use the organisms that were likely to be found together in chronic wounds. Previous studies have reported that *Staphylococcus aureus* is one of the most common organisms to be isolated from chronic ulcers, with up to 93% of the sampled wounds containing strains of *S. aureus*, many of which were methicillin resistant (1). Further metatranscriptomic analyses within human wound infections revealed a positive correlation between *Streptococcus agalactiae* and *S. aureus*, suggestive of an interaction between these two organisms in chronic wound development, because they are commonly identified together in wound samples (2). Experiments were then conducted by our laboratory to observe if these organisms affected each other's growth rates within undefined Todd Hewitt broth and Lubbock wound models, and our findings suggested a significant impact on the growth of *S. agalactiae* in co-culture, providing support of interactions between these microbes within a wound environment. Finally, the impact of the environment on chronic wound development and treatment was suggested by past research on ulcers and other diseases typically associated with *diabetes mellitus* type II. Previous work suggests that the condition provides a more optimal environment for chronic wounds and allows for the communities of microorganisms within them to tolerate antibiotics at elevated levels (3).

In this study, we sought to identify mechanisms used by these microbes to establish antibiotic tolerance in chronic infections. Specifically, we wanted to determine the effects of different environments on the capabilities of *S. aureus* and *S. agalactiae* to tolerate antibiotics when in co-culture in chronic wounds, and we hoped to identify key molecular mechanisms at play in the development of antibiotic recalcitrance. We hypothesized that access to glucose in diabetic conditions would provide favorable conditions for the synthesis of essential components to tolerate different antibiotics more effectively. To assess these characteristics, growth curves were performed in different environmental conditions to determine their effects, and antibiotic tolerance assays were conducted to measure the tolerance rates of *S. aureus* and *S. agalactiae* individually and in co-culture within these environments. Many classes of antibiotics were tested, including aminoglycosides, beta-lactams, and non beta-lactams. Since each antibiotic has a unique mechanism of action, distinct mechanisms of defense could be hypothesized if the two organisms show resistance to a given treatment. Furthermore, we could determine if the presence of excess glucose – comparable to diabetic blood conditions – had any significant impact on the mechanisms by which polymicrobial infections could tolerate antibiotics.

Materials and Methods:

Minimum Growth Inhibitory Concentration Determination

To effectively determine the amount of antibiotic necessary for an antibiotic challenge, the Minimum Inhibitory Concentration (MIC) must be estimated. Every antibiotic used in these experiments had identical MIC determination tests performed. One day before the experiment, cultures were prepared by inoculating a tube of 5mL Brain Heart Infusion (BHI) broth with *S. aureus* and another identical tube with *S. agalactiae*. The *S. aureus* tube was incubated overnight in a shaker, while the *S. agalactiae* tube was incubated statically. The next day, a 12-well plate was filled with 3mL of Brain Heart Infusion broth in 8 wells. The overnight cultures were

retrieved, and the optical density at 600nm was recorded using a spectrophotometer. This information was used to calculate how much sample would be added to bring the total solution OD600 to 0.1 for each bacterium. 4 wells were inoculated with the amount calculated for *S. aureus* and another 4 with the amount calculated for *S. agalactiae*, and all wells were incubated for 3.5 hours. Once this time passed, 30uL from each well was taken and diluted to 10^-8 in 1:10 dilutions. 10uL of the 10^-5 through 10^-8 dilutions was taken and spot-plated using a multi-tip pipette on one BHI Agar plate in 4 separate rows. Various concentrations of antibiotics were added to each well, including: 125ug/mL, 256ug/mL, 512ug/mL, and a 0ug/mL control. The wells were then incubated for 2 hours, and the procedure for collecting and spot-plating samples from above was repeated. All plates were incubated overnight, and the CFU/mL was calculated using the number of colonies observed. The minimum concentration that halted the most growth was determined to be the MIC for each individual organism challenged with an individual antibiotic.

Antibiotic Challenge in BHI and Wound-Like Media

One day before the experiment, cultures were prepared by inoculating a tube of 5mL Brain Heart Infusion (BHI) broth with *S. aureus* and another identical tube with *S. agalactiae*. The *S. aureus* tube was incubated overnight in a shaker, while the *S. agalactiae* tube was incubated statically. The next day, a 12 well plate was filled with 3mL BHI broth in each well. The overnight cultures were retrieved, and the optical density at 600nm was recorded using a spectrophotometer. This information was used to calculate how much sample would be added to bring the total solution OD600 to 0.1 for each bacterium. 4 wells were inoculated with *S. aureus* and another 4 wells were inoculated with *S. agalactiae*. The last 4 wells were inoculated with both organisms; however, the amount added was ½ of the amount added to each organism individually to ensure total bacterial inocula remained constant. The wells were then homogenized, and 30uL from each well was taken and diluted to 10^-8 in 1:10 dilutions. 10uL of each dilution from 10^-5 to 10^-8 were taken and spot-plated in four rows on one BHI agar plate. The co-culture samples were also spot-plated onto another BHI plate infused with 2.5ug/mL Tetracycline to isolate the *S. agalactiae* colonies. Afterwards, the wells and plates were transferred to the incubator. After 3.5 hours, the wells were removed, and the same procedure regarding dilution and spot-plating was performed. The chosen antibiotic was then added to 2 wells of each condition at the Minimum Inhibitory Concentration. The wells were then returned to the incubator for 2 hours. Afterwards, the same procedure used for sampling, diluting, and spot plating was performed. All plates were then transferred to the incubator for overnight incubation, and the CFU/mL was calculated at each time point for each condition using the number of colonies observed the next morning.

For the antibiotic challenge in wound-like media, the procedure is like the challenge in BHI with the following differences. The Lubbock wound model was constructed with 50% bovine plasma suspended in deionized water, 45% filter sterilized Bolton Broth, and 5% laked horse blood. After homogenization, 600uL of media was added to sterile 16mm glass tubes and capped with sterile aluminum foil, with 4 tubes for *S. aureus*, 4 for *S. agalactiae,* and 4 for the co-cultures. The inoculating procedure and initial time point data collection are the same as above. However, the bacteria are allowed to incubate for a much longer period. Time points were collected at 0 hours, 24 hours, 48 hours, 72 hours, and 168 hours. The antibiotic was added after the 48-hour time point at the Minimum Inhibitory Concentration to 2 of the 4 tubes for each condition. At each time point, the data collection procedure is the same. 30uL is taken from each tube and diluted to 10^-8, which is then spot plated from 10^-5 to 10^-8 on BHI agar plates, and co-cultures are also spot-plated on 2.5ug/mL Tetracycline plates. CFU/mL values for each time

point were recorded the day after the data was collected, which would be the same day as the next time point being collected.

Hyperglycemic Growth Curve in BHI and Wound-Like Media

One day before the experiment, cultures were prepared by inoculating a tube of 5mL Brain Heart Infusion (BHI) broth with *S. aureus* and another identical tube with *S. agalactiae*. The *S. aureus* tube was incubated overnight in a shaker, while the *S. agalactiae* tube was incubated statically. The next day, a 12 well plate was filled with 3mL BHI broth in 6 wells. The other 6 wells were supplemented with dextrose to bring the glucose concentration to 4mmol, assuming the original concentration of BHI was 2mmol. The overnight cultures were retrieved, and the optical density at 600nm was recorded using a spectrophotometer. This information was used to calculate how much sample would be added to bring the total solution OD600 to 0.1 for each bacterium. 4 wells were inoculated with *S. aureus* and another 4 wells were inoculated with *S. agalactiae*. The last 4 wells were inoculated with both organisms; however, the amount added was ½ of the amount added to each organism individually. Each condition had 2 wells that were supplemented with dextrose. The wells were then homogenized, and 30uL from each well was taken and diluted to 10^-8 in 1:10 dilutions. 10uL of each dilution from 10^-5 to 10^-8 were taken and spot-plated in four rows on one BHI agar plate. The co-culture samples were also spot-plated onto another BHI plate infused with 2.5ug/mL Tetracycline to isolate the *S. agalactiae* colonies. Afterwards, the wells and plates were transferred to the incubator. Every 1.5 hours, a time point was taken using the same procedure for sampling, diluting, and spot-plating detailed above. The procedure was repeated 5 times, once at 0 hours, 1.5 hours, 3 hours, 4.5 hours, and 6 hours. All plates were then transferred to the incubator for overnight incubation, and the CFU/mL was calculated at each time point for each condition using the number of colonies observed the next morning.

For the hyperglycemic curve in wound-like media, the procedure is like the growth curve in BHI with the following modifications. The wound model was created using the same procedure detailed in the wound-media antibiotic challenge. 6 sterile 16mm tubes were filled with 600uL of media, while the other 6 sterile 16mm tubes were filled with 600uL of media and supplemented with 4mmol of dextrose, assuming the original media is minimal in glucose levels. All tubes were capped with sterile aluminum foil. 4 tubes were for *S. aureus*, 4 for *S. agalactiae*, and 4 for the co-culture, and each condition used 2 tubes of media supplemented with dextrose. The inoculating procedure and initial time point data collection are the same as above. However, the bacteria are allowed to incubate for a much longer period. Time points were collected at 0 hours, 24 hours, 48 hours, 72 hours, and 168 hours. At each time point, the data collection procedure is the same. 30uL is taken from each tube and diluted to 10^-8, which is then spot plated from 10^-5 to 10^-8 on BHI agar plates, and co-cultures are also spot-plated on 2.5ug/mL Tetracycline plates. CFU/mL values for each time point were recorded the day after the data was collected, which would be the same day as the next time point being collected.

Antibiotic Challenge in Hyperglycemic Wound-Like Media

One day before the experiment, cultures were prepared by inoculating a tube of 5mL Brain Heart Infusion (BHI) broth with *S. aureus* and another identical tube with *S. agalactiae*. The *S. aureus* tube was incubated overnight in a shaker, while the *S. agalactiae* tube was incubated statically. The next day, the wound model was constructed with the same procedure as described in the antibiotic challenge in wound media, and it was supplemented with dextrose to bring the concentration to 4mmol. 12 16mm tubes with aluminum foil caps were sterilized and used for

this experiment. Each tube was filled with 600uL of the hyperglycemic wound models. The overnight cultures were retrieved, and the optical density at 600nm was recorded using a spectrophotometer. This information was used to calculate how much sample would be added to bring the total solution OD600 to 0.1 for each bacterium. 4 tubes were inoculated with *S. aureus* and another 4 tubes were inoculated with *S. agalactiae*. The last 4 tubes were inoculated with both organisms; however, the amount added was ½ of the amount added to each organism individually. The wells were then homogenized, and 30uL from each well was taken and diluted to 10^-8 in 1:10 dilutions. 10uL of each dilution from 10^-5 to 10^-8 were taken and spot-plated in four rows on one BHI agar plate. The co-culture samples were also spot-plated onto another BHI plate infused with 2.5ug/mL Tetracycline to isolate the *S. agalactiae* colonies. Afterwards, the wells and plates were transferred to the incubator. In total, time points were taken at 0 hours, 24 hours, 48 hours, 72 hours, and 168 hours. The procedure for sampling, data collecting, and spot-plating was repeated as described for every time point. CFU/mL values for each time point were recorded the day after the data was collected, which would be the same day as the next time point being collected.

Results:

The two antibiotics that were primarily used for these experiments included Tobramycin, an aminoglycoside, and Methicillin, a beta-lactam. For the antibiotic assays, it was crucial to determine the minimum inhibitory concentration (MIC) that would be the most bactericidal throughout growth. To determine these MICs, we examined the effects of various concentrations of antibiotic on bacterial growth after three and a half hours of incubation and observed the minimum bactericidal concentration for each antibiotic.

Bacteria	Antibiotic	MIC (ug/mL)
S. aureus	Tobramycin	256ug/mL
S. aureus	Methicillin	512ug/mL
S. agalactiae	Tobramycin	256ug/mL
S. agalactiae	Methicillin	125ug/mL

Figure 1: Minimum Inhibitory Concentration of *S. aureus* and *S. agalactiae* to Two Different Antibiotics. These results were obtained by recording the minimum concentration that caused a CFU/mL < 10^6 for a given bacteria after 3.5 hours of growth. These values gave the concentrations of antibiotics used in the challenge, where the concentration was equal to the greatest MIC of the two organisms to a given antibiotic.

These results indicate a diverse range of tolerance to the different antibiotics tested for the given experiment. These show that the MIC for the co-cultures with Tobramycin would be 256ug/mL and the MIC for the co-cultures with Methicillin would be 512ug/mL. Each result shows the minimum concentration where extraordinarily little to no growth was detected for each condition. It is also noted that the *S. aureus* strain used is methicillin resistant, so its true minimum inhibitory concentration is likely much higher than the expected result.

Following the confirmation of minimum inhibitory concentrations, we sought to analyze how the tolerance of *S. aureus* and *S. agalactiae* would be impacted when they were grown individually vs in co-culture. It was hypothesized that both organisms would benefit in increased tolerance when grown together due to high correlations previously established in the literature and

metatranscriptomic analysis. The tolerance of each organism to tobramycin, individually and in co-culture, was assayed in BHI media and our *in vitro* wound models. It was observed that *S. aureus* tended to grow significantly better ($p<0.05$) when incubated with *S. agalactiae* in BHI and challenged with tobramycin compared to individually. Figure 7 suggests that the survivability of *S. aureus* in co-culture was higher than individual survival rates. Further, growth of *S. aureus* in wound-like media demonstrates a significant difference in growth; however, it was observed that the organism did significantly better individually ($p<0.05$) rather than in co-culture, according to Figures 4 and 7. It was also observed that *S. agalactiae* had significantly greater tolerance in both BHI and wound conditions ($p<0.05$) where it was incubated individually. Figures 3, 5, and 6 demonstrate that *S. agalactiae* typically survived and grew better without the presence of *S. aureus*. This is indicated by the significantly lower numbers and survival frequencies observed in co-culture.

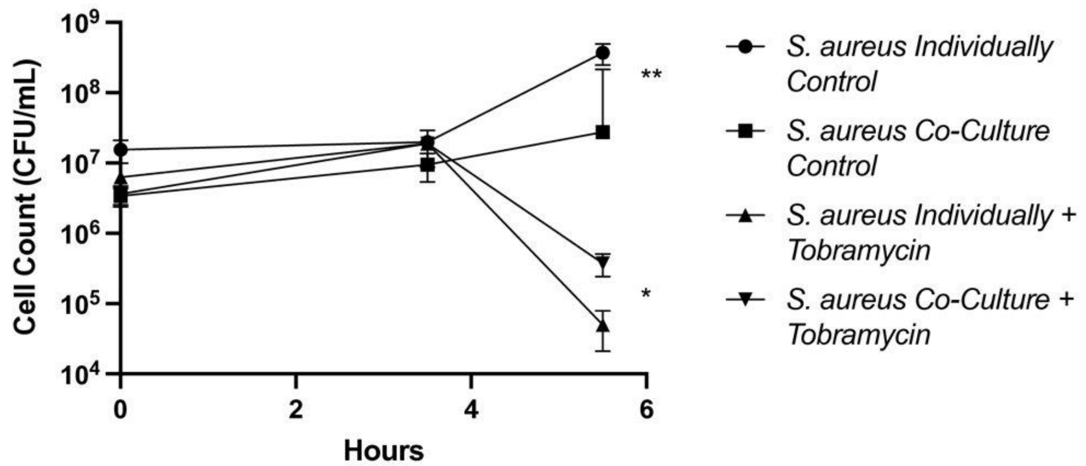


Figure 2: Tobramycin Assay of *S. aureus* Individually and in Co-Culture in Brain Heart Infusion media. The data was collected by counting the number of colonies that appeared on each dilution spot and dividing it by the dilution. These values were then averaged across the four rows, and then the data was averaged across replicates of the experiment. The averages were also used to calculate standard deviations and standard error at each point. The CFU/mL was then plotted with time to gauge the appropriate growth rates in each condition.

Tobramycin Assay of *S. agalactiae* Individually and in Co-Culture in BHI

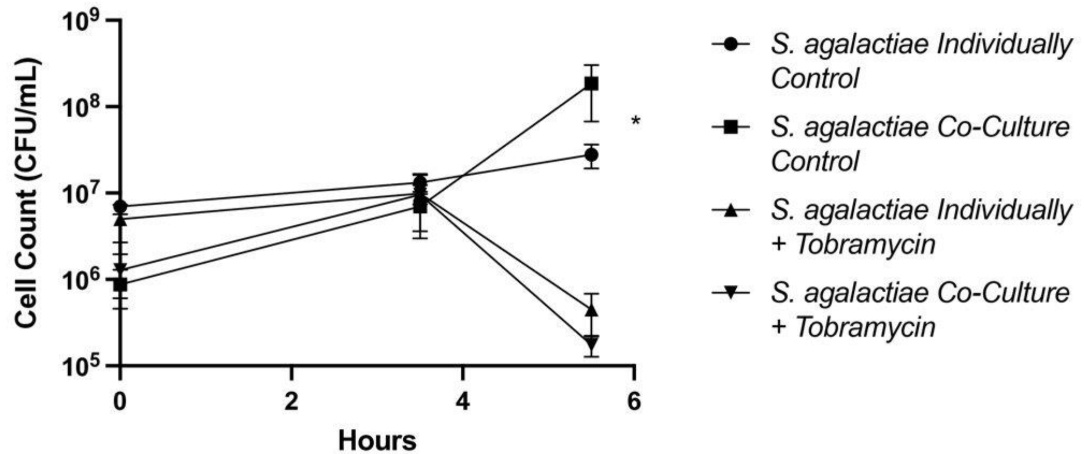


Figure 3: Tobramycin Assay of *S. agalactiae* Individually and in Co-Culture in Brain Heart Infusion media. The data was collected by counting the number of colonies that appeared on each dilution spot and dividing it by the dilution. These values were then averaged across the four rows, and then the data was averaged across replicates of the experiment. The averages were also used to calculate standard deviations and standard error at each point. The CFU/mL was then plotted with time to gauge the appropriate growth rates in each condition.

Tobramycin Assay of *S. aureus* Individually and in Co-Culture in Wound Media

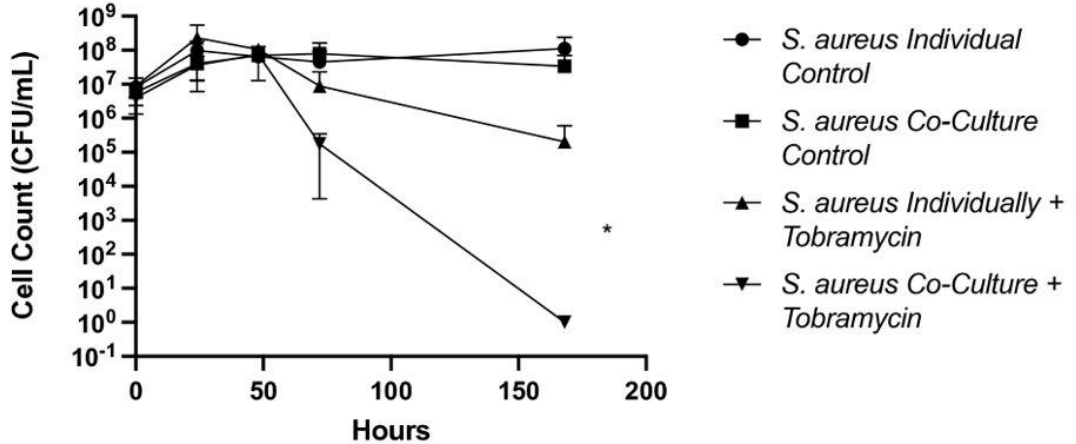


Figure 4: Tobramycin Assay of *S. aureus* Individually and in Co-Culture in Lubbock wound media. The data was collected by counting the number of colonies that appeared on each dilution spot and dividing it by the dilution. This procedure was the same across time points. These values were then averaged across the four rows, and then the data was averaged across replicates of the experiment. The averages were also used to calculate standard deviations and standard error at each point. The CFU/mL was then plotted with time to gauge the appropriate growth rates in each condition.

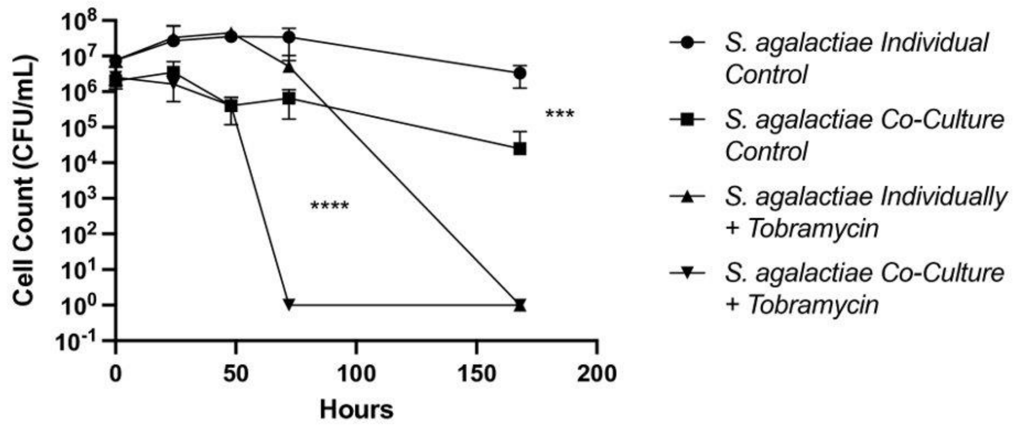
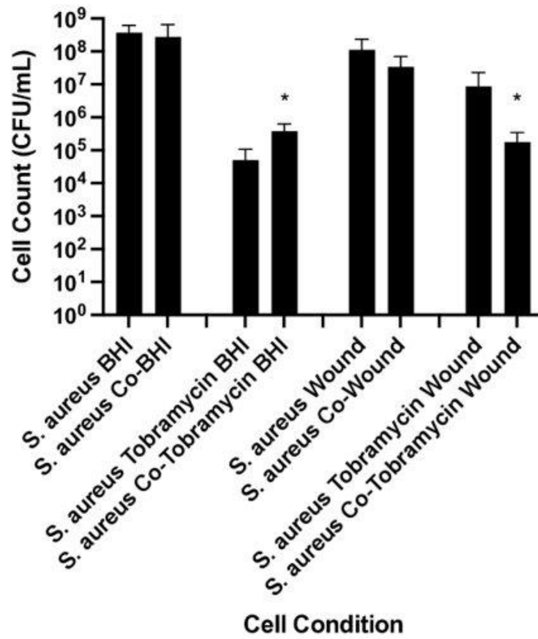


Figure 5: Tobramycin Assay of *S. agalactiae* Individually and in Co-Culture in Lubbock wound media. The data was collected by counting the number of colonies that appeared on each dilution spot and dividing it by the dilution. This procedure was the same across time points. These values were then averaged across the four rows, and then the data was averaged across replicates of the experiment. The averages were also used to calculate standard deviations and standard error at each point. The CFU/mL was then plotted with time to gauge the appropriate growth rates in each condition.

Final Counts of *S. aureus* in Multiple Conditions when Challenged with Tobramycin



Final Counts of *S. agalactiae* in Multiple Conditions when Challenged with Tobramycin

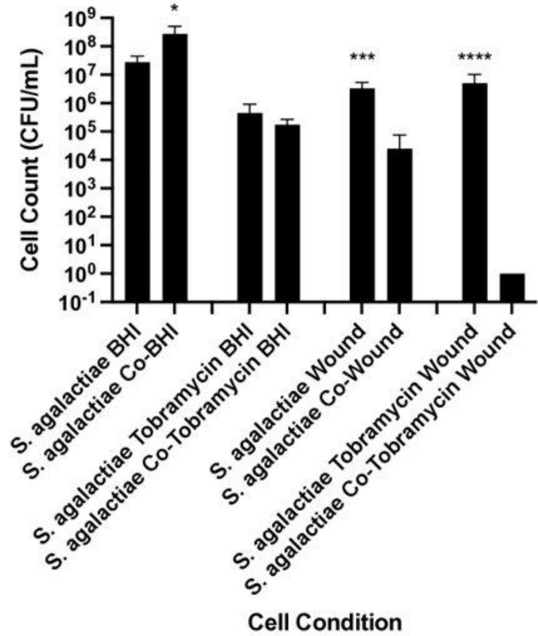
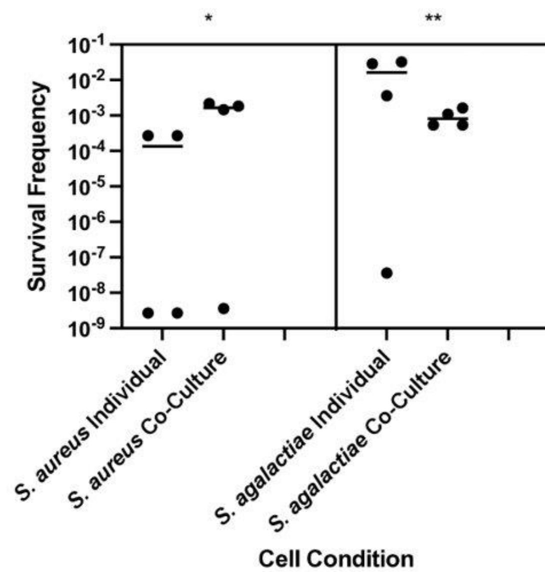


Figure 6: Final Counts of *S. aureus* and *S. agalactiae* in Multiple Conditions when Challenged with Tobramycin. This data was collected by finding the difference in growth for each condition when challenged with Tobramycin. This was achieved for the BHI and wound models by subtracting the final CFU/mL at the last time point from the original CFU/mL at the first time point. These values were then averaged across replicates and the numbers were used to calculate the standard deviation and standard error for each condition. These values were then plotted to see if the growth rate was different between individual bacteria and co-cultures.



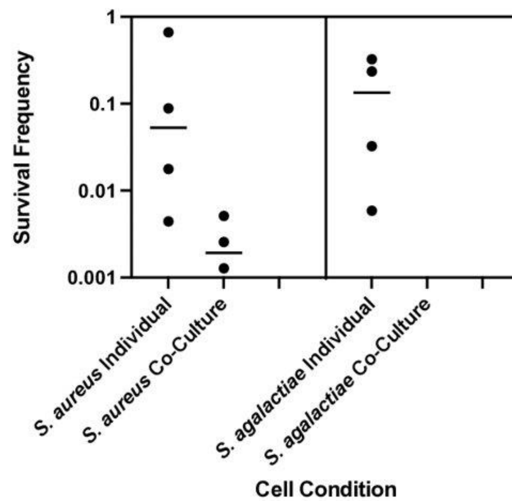


Figure 7: The Survival Frequencies of *S. aureus* and *S. agalactiae* when Challenged with Tobramycin in BHI and Wound media. This data was collected by dividing the final CFU/mL obtained in the condition with Tobramycin by the average final CFU/mL of the corresponding condition without Tobramycin. These values were then averaged, and the data was used to calculate the standard deviation, first and third quartiles, and project these values on a boxplot. This plot shows the variation of the survival percentages for each condition when challenged with Tobramycin.

After addition of the antibiotic to the BHI cases at 3.5 hours and the wound cases at 48 hours, we observed that the survival frequencies of *S. aureus* tended to fluctuate based on the environment and condition, while the survival frequency patterns of *S. agalactiae* remained essentially unchanged. It was also noticed that both organisms survived much better in the wound media compared to the BHI media due to the higher survival frequencies in wound media observed in Figure 7. When analyzing Figures 2 and 6, it is also observed that the growth of *S. agalactiae*

when challenged with tobramycin was not significantly impacted when co-cultured in BHI media; however, survival frequencies indicate that these growths were significant, indicating that this organism is at a detriment when it is challenged with *S. aureus*.

Following these observations, we sought to assess the effects of a hyperglycemic environment on the growth rates of *S. aureus* and *S. agalactiae* to determine if interactions between these organisms in these conditions are possible. Growth curves of *S. aureus* and *S. agalactiae* were performed individually and in co-culture in two different environments: hyperglycemic BHI and hyperglycemic wound-like media, each environment supplemented with dextrose to bring the concentration to 4mmol. The CFU/mL for each species was monitored at each time point for 6 hours.

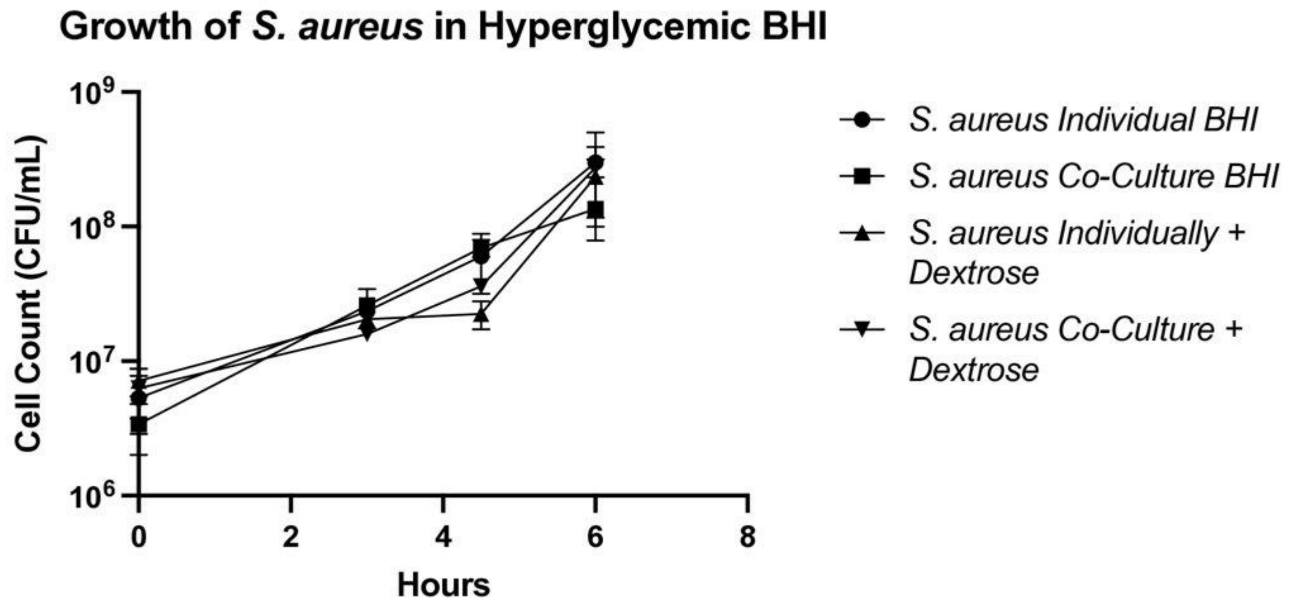


Figure 8: Growth Curve of *S. aureus* in Diabetic BHI Conditions. The data was collected by counting the number of colonies that appeared on each dilution spot and dividing it by the dilution. This procedure was the same across time points. These values were then averaged across the four rows, and then the data was averaged across replicates of the experiment. The averages were also used to calculate standard deviations and standard error at each point. The CFU/mL was then plotted with time to gauge the appropriate growth rates in each condition.

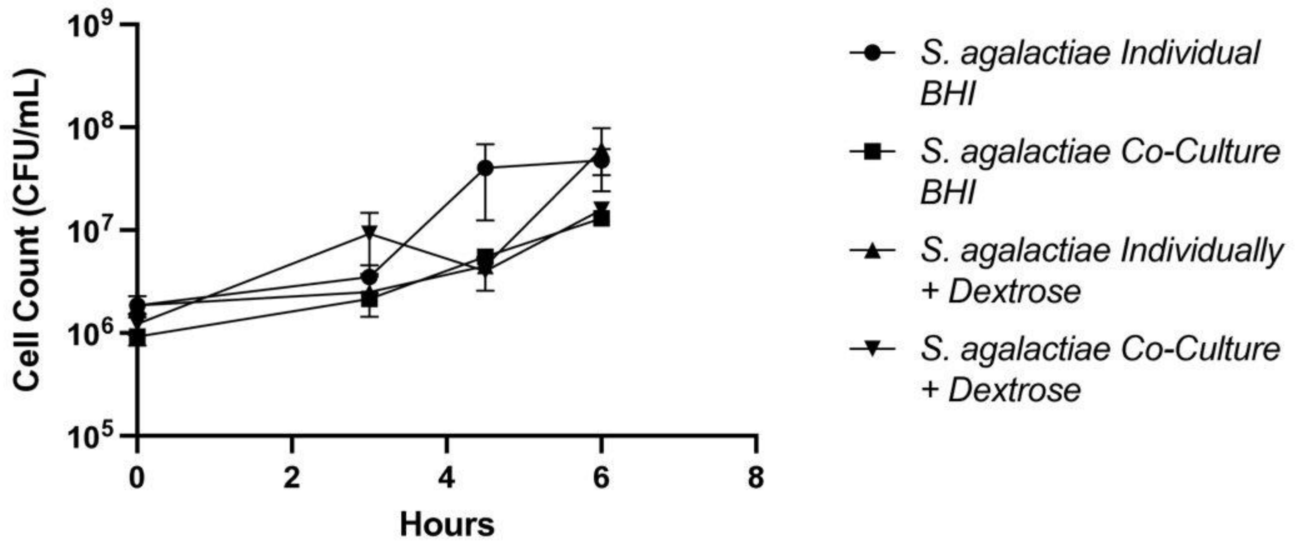


Figure 9: Growth Curve of *S. agalactiae* in Diabetic BHI Conditions. The data was collected by counting the number of colonies that appeared on each dilution spot and dividing it by the dilution. This procedure was the same across time points. These values were then averaged across the four rows, and then the data was averaged across replicates of the experiment. The averages were also used to calculate standard deviations and standard error at each point. The CFU/mL was then plotted with time to gauge the appropriate growth rates in each condition.

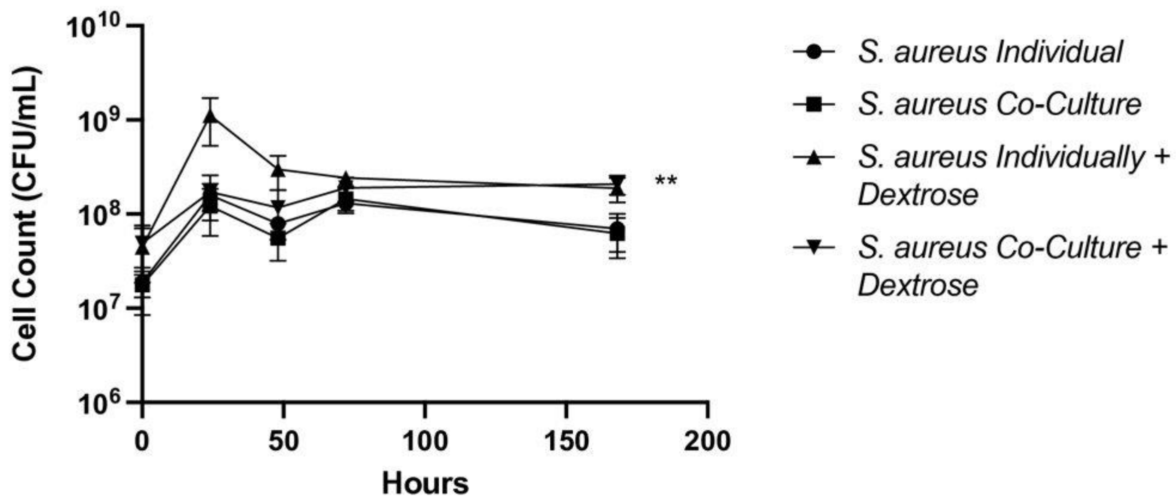


Figure 10: Growth Curve of *S. aureus* in Hyperglycemic Wound Conditions. The data was collected by counting the number of colonies that appeared on each dilution spot and dividing it by the dilution. This procedure was the same across time points. These values were then averaged across the four rows, and then the data was averaged across replicates of the experiment. The averages were also used to calculate standard deviations and standard

error at each point. The CFU/mL was then plotted with time to gauge the appropriate growth rates in each condition.

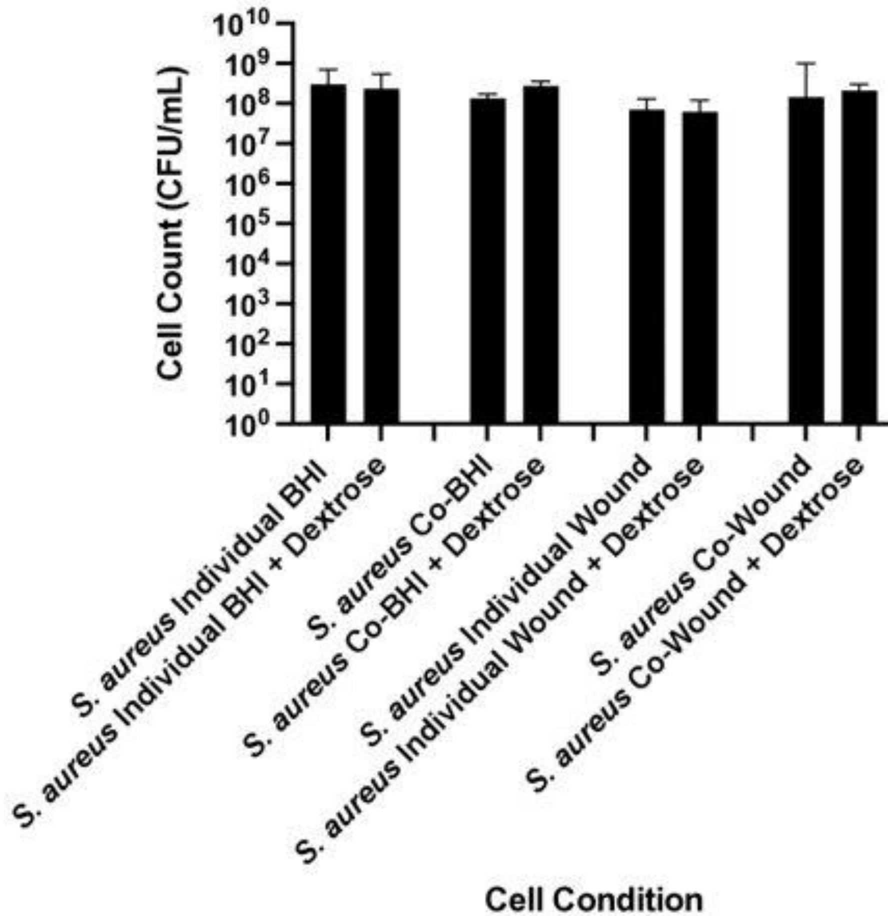


Figure 11: The Final Cell Counts of *S. aureus* in a Diabetic Growth Curve in Brain Heart Infusion Broth. The data was collected by taking the final CFU/mL for every point in a condition and averaging them together to get the final cell counts. These values were also used to calculate a standard deviation and standard error for each condition. These values were then plotted to compare the final cell counts in conditions that were not supplemented with dextrose vs conditions that were.

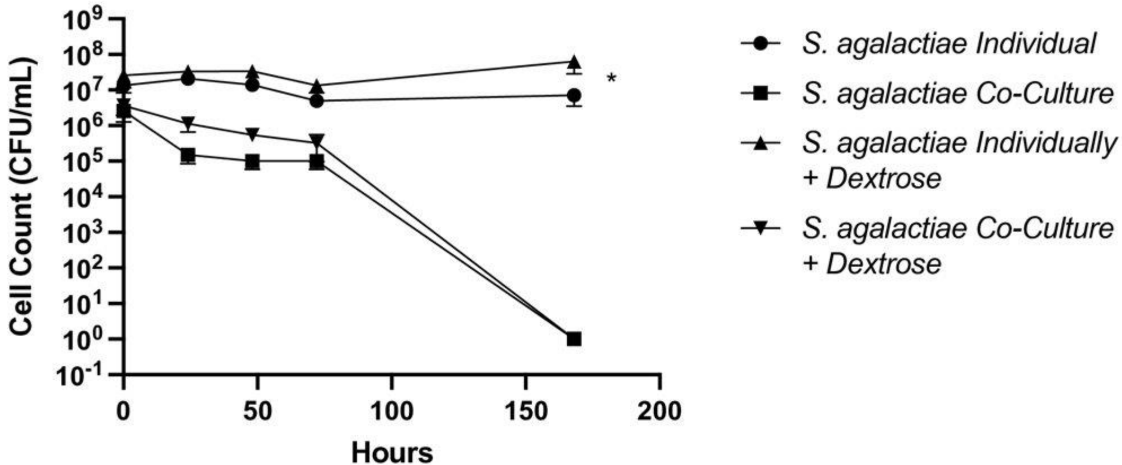


Figure 12: Growth Curve of *S. agalactiae* in Hyperglycemic Wound Conditions. The data was collected by counting the number of colonies that appeared on each dilution spot and dividing it by the dilution. This procedure was the same across time points. These values were then averaged across the four rows, and then the data was averaged across replicates of the experiment. The averages were also used to calculate standard deviations and standard error at each point. The CFU/mL was then plotted with time to gauge the appropriate growth rates in each condition.

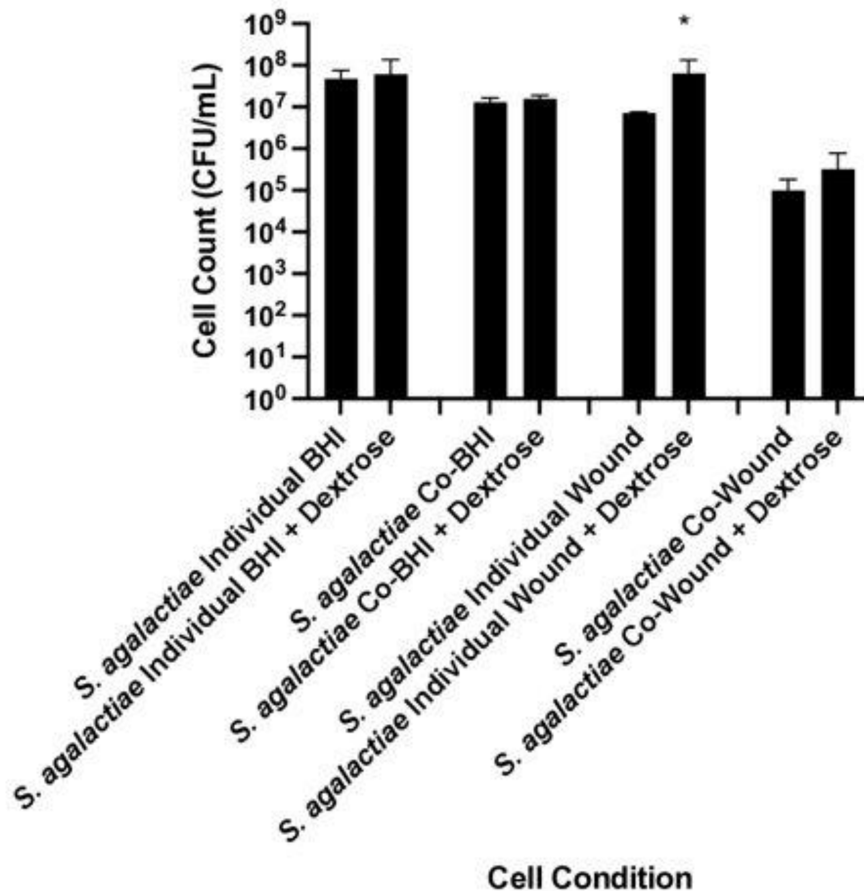


Figure 13: The Final Cell Counts of *S. agalactiae* in a Diabetic Growth Curve in Brain Heart Infusion Broth. The data was collected by taking the final CFU/mL for every point in a condition and averaging them together to get the final cell counts. These values were also used to calculate a standard deviation and standard error for each condition. These values were then plotted to compare the final cell counts in conditions that were not supplemented with dextrose vs conditions that were.

In the hyperglycemic BHI models, the growth rates of *S. aureus* and *S. agalactiae* remained remarkably similar in the individual conditions no matter if dextrose was present. Notable observations were made in the organisms grown in co-culture in Figures 8 and 9. When compared with our previous studies, the co-cultured organisms, primarily *S. agalactiae*, were never able to reach individual levels when grown in non-hyperglycemic BHI media. For the rest of the conditions, there were hardly any differences observed in growth rates or numbers in hyperglycemic BHI. In our hyperglycemic wound models, however, noticeable differences were observed in both trends of growth in *S. aureus* and *S. agalactiae*. For *S. aureus*, the conditions with hyperglycemic media both outperformed their non-hyperglycemic controls by a statistically significant margin ($p<0.05$). For *S. agalactiae,* the individual condition in hyperglycemic media grew significantly better; however, both co-cultures ended up having similar death rates and times. The hyperglycemic co-culture, on the other hand, was able to maintain significantly higher cell counts throughout the curve itself.

Discussion:

Based on the results for the various assays, there are numerous conclusions that can be made. When analyzing the results of the Tobramycin assays in BHI and Lubbock-wound like media, we observe that *S. aureus* performed better against Tobramycin in BHI and worse in wound-like media. We also see that *S. agalactiae* performed better against Tobramycin in wound-like media and worse in BHI. This is reflected in the percent survival distributions, and both disparities were found to be statistically significant ($p < 0.05$). What this shows is that the tolerance of each organism tended to shift whenever the environment changed, which indicates that the environment played a significant role in developing antibiotic tolerance for both species to aminoglycosides like Tobramycin. Furthermore, this indicates that each species could have secreted different virulent factors in each environment, which may have also contributed to the ability to tolerate antibiotics. While these factors cannot be conclusively determined by this study, we can conclude that the environment plays a role in antibiotic tolerance, and further studies can be done to elucidate what exactly would be the main contributor to these differences.

Based on these conclusions, determining growth rates in diabetic conditions became an essential next step to investigating the effects of the environment on antibiotic tolerance. Based on the results, we can conclude that diabetic conditions did not seem to make any difference in the growth of either organism in BHI media. However, it was noted that for *S. aureus*, all growth rates were nearly the same individually and in co-culture. In our previous growth curves, it was noted that *S. aureus* did worse when grown in co-culture, so this finding may indicate that the availability of extra glucose allowed for *S. aureus* to achieve regular growth rates when in the presence of *S. agalactiae*. The same result can be observed for *S. agalactiae*. The extra glucose added to the media did not cause any spike in growth rates in BHI; however, it eliminated the competition between *S. aureus* and *S. agalactiae* that was observed in non-hyperglycemic replicates. This suggests that hyperglycemic environments are favorable for the sustained growth and maintenance of bacteria levels in chronic infections. In our wound models, preliminary data suggests that the addition of glucose caused a huge spike in growth for both *S. aureus* and *S. agalactiae*. When compared to the control, growth rate differences were much more noticeable. Since the wound-media contains less glucose than regular BHI – equivalent to normal blood sugars –, this difference indicates that diabetic blood is a huge contributing factor to the growth of chronic wounds. Future tests will then observe if this environmental factor has any effect on the development of antibiotic tolerance in co-cultures.

While this study is useful for suggesting the potential for interactions that lead to antibiotic tolerance, it is not guaranteed to identify a precise mechanism for which this occurs. Future studies should investigate the molecular aspects of these interactions to reveal novel RNA or protein mechanisms that might contribute to antibiotic tolerance in chronic wounds. Furthermore, it is also suggested that this study does not account for increased *S. agalactiae* virulence in chronic wounds. Past research suggests that *S. agalactiae* virulence is triggered in a wound hyperinflammatory response, characterized by hordes of neutrophils that release granular molecules designed to attack the bacteria community in a chronic wound (4). Studies that repeat these experiments while accounting for *S. agalactiae* activation would be useful to accurately determine the role of *S. agalactiae* in antibiotic tolerance development within a chronic wound. These studies may help advance the knowledge of microbial interactions between these two organisms in chronic wounds. While this study does not account for the entire microbiome within the wound environment, it is critical to isolate the key community mechanisms for

establishing and maintaining infection so that future treatments can be designed to attack these crucial factors, saving millions in chronic healthcare treatment.

References:

1. **Fazli M, Bjarnsholt T, Kirketerp-Møller K, Jørgensen B, Andersen AS, Krogfelt KA, Givskov M, Tolker-Nielsen T**. 2009. Nonrandom distribution of Pseudomonas aeruginosa and Staphylococcus aureus in Chronic Wounds. Journal of Clinical Microbiology **47**:4084–4089.
2. **Ibberson, Carolyn**. Unpublished.
3. **DG; G**. Wound healing and diabetes mellitus. Clinics in plastic surgery. U.S. National Library of Medicine.
4. **Keogh RA, Haeberle AL, Langouët-Astrié CJ, Kavanaugh JS, Schmidt EP, Moore GD, Horswill AR, Doran KS**. 2022. Group B Streptococcus adaptation promotes survival in a hyperinflammatory diabetic wound environment. Science Advances **8**.