Elucidating *Streptococcus agalactiae* and *Staphylococcus aureus* Polymicrobial Interactions in Diabetic Wound Infections

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I. Abstract

Diabetic foot ulcers (DFUs) are a leading complication of diabetes mellitus due to their propensity for infection and poor healing, which results in significant morbidity and financial burden for patients. Various pathogenic species, representing several bacterial and fungal genera, perpetuate polymicrobial infections within the wound environment. Particularly prevalent species include *Streptococcus agalactiae,* or Group B Streptococcus (GBS), and *Staphylococcus aureus,* which serve as the focus of this study. Preliminary data suggest that *S. aureus* may contribute to an increased burden of GBS in diabetic wounds through synergistic interactions, enhancing the overall severity of infection. This study examines mono- and dual-species biofilm assays, bacterial growth curves, and investigation of nutrient sharing between GBS and *S. aureus* in clinical DFU isolate samples collected at the University of Colorado Anschutz. These methods serve to elucidate the mechanisms driving polymicrobial synergy between GBS and *S. aureus* within the DFU environment. Moving forward, the dynamics of these interactions could inform the development of novel DFU infection management approaches that inhibit polymicrobial pathologies, ultimately improving patient outcomes.

Keywords: diabetic foot ulcers, polymicrobial interactions, biofilms, streptococci, staphylococci

II. Background

Diabetes mellitus (DM) affects nearly 537 million adults aged 20-79 internationally, with a predicted increase to 783 million by 2045 (Sun et al., 2022). As such, DM represents a significant public health concern that will continue to grow in the future. While approximately 50% of DM cases remain undiagnosed due to either a lack of symptoms or detection, in diagnosed cases, up to 94% of people with diabetes report experiencing complications (Hossain et al., 2024; International Diabetes Federation, 2023). There are a variety of diabetes-related complications that can manifest over the course of an individual's lifetime in both Type 1 and Type 2 DM, many of which are severe. Among these complications are diabetic kidney disease, retinopathy, stroke, heart disease, peripheral vascular disease (PVD), and peripheral neuropathy (Tomic et al., 2022). PVD and peripheral neuropathy are the leading cause of diabetic foot ulcers. Approximately 19 to 34% of people with diabetes develop a diabetic foot ulcer over the course of their lifetime, and the recurrence rate of ulcers is 65% within five years of the initial wound (Armstrong et al., 2017). DFUs are characterized by a breach of both the epidermal and dermal skin layers and are classified based on wound depth, size, tissue necrosis, ischemia, and presence of infection (Wang et al., 2022). Generally, these ulcers develop in the context of disturbed blood supply, neuropathy, and defective immune responses, which provide favorable conditions for microbial colonization and infection (L. R. Kalan & Brennan, 2019). Moreover, diabetic foot ulcers, especially those that are infected and non-healing, are a major predictor of lower-limb amputation in diabetic patients, as described in Figure 1 (Frykberg et al., 1998; Kumar et al., 1994; Pecoraro et al., 1990; Reiber et al., 1983; Uccioli et al., 2015). In recent years, lower-limb revascularization efforts have risen in accordance with an increase in DFU prevalence. As limb-preserving interventions have been developed and improved, overall amputation rates have

declined among diabetic individuals. However, the risk of lower limb amputation in those with DFUs remains at 19% (McDermott et al., 2023; Mills et al., 2014; Rodrigues et al., 2022).

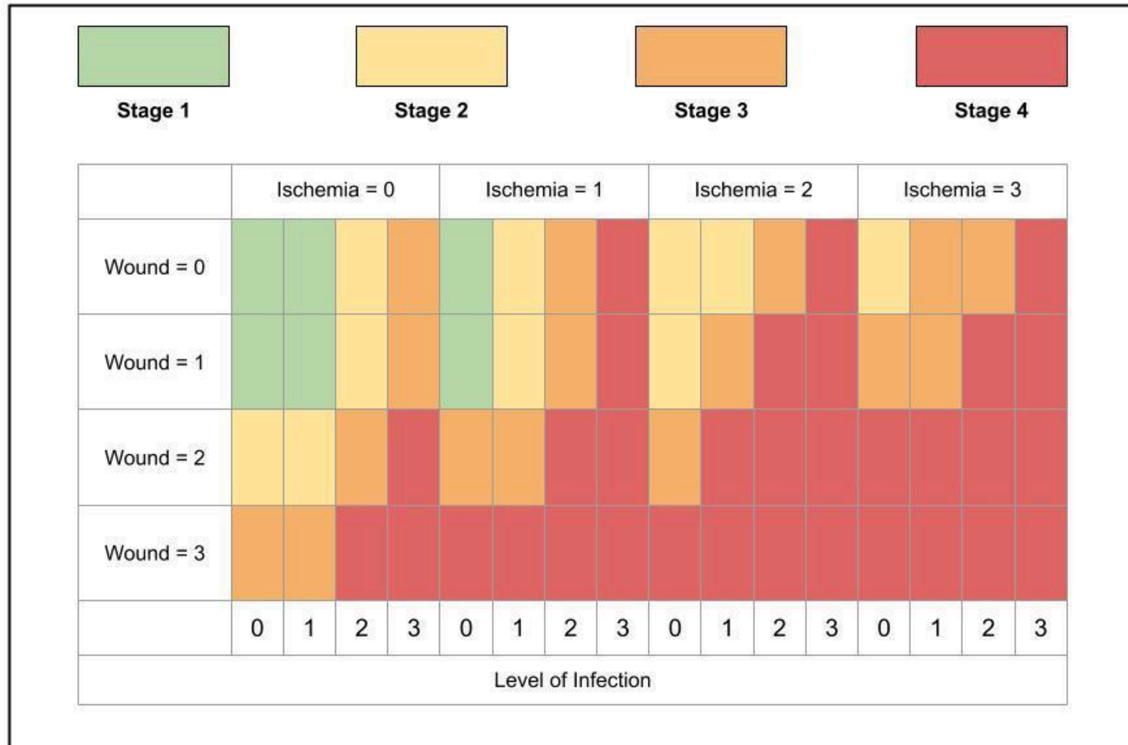

Figure 1. *Wound, ischemia, and foot infection classification in relation to risk of minor or major lower-extremity amputation according to the SVS WIfI class system, developed by the Society for Vascular Surgery. Green (Stage 1) indicates very low risk of amputation, yellow (Stage 2) indicates low risk of amputation, orange (Stage 3) indicates moderate risk of amputation, and red (Stage 4) indicates high risk of amputation. As wound class, level of infection, and ischemia increase, the risk for amputation increases. Adapted from McDermott et al., 2023 and Mills et al., 2014.*

Among infected wounds, there are diverse microbial populations that inhabit the epithelium. Prior studies have shown that *Staphylococcus aureus, Pseudomonas aeruginosa,*

Escherichia coli, Streptococcus agalactiae, and Klebsiella pneumoniae are some of the most common bacterial species isolated from DFUs (Banu et al., 2015; Kalan et al., 2019, Macdonald et al., 2021). During infection progression, the DFU microbiome can change and evolve. As the chronicity of infection and wound severity increases, the presence of anaerobic pathogens, aerobic gram-positive cocci, and aerobic gram-negative bacilli grows (Sadeghpour Heravi et al., 2019). Furthermore, the presence of fungal species in the wound environment can delay healing and worsen overall infection intensity. Commonly isolated fungal species include *Cladosporium herbarum* and *Candida albicans,* which can form interkingdom biofilms with yeasts and bacteria (L. Kalan et al., 2016). Biofilms are dense communities of microbes, which grow on and within surfaces. They are more challenging to treat with antimicrobials than bacterial, fungal, or yeast cells living in non-biofilm structures. Some bacterial species, particularly *S. aureus*, are also adept biofilm formers that thrive in both monomicrobial and polymicrobial environments (Boles et al., 2010; Peng et al., 2022). In turn, this robust characteristic of biofilm formation often confers increased resistance to treatment and evasion of the host immune response in DFUs. Even in the absence of interkingdom fungal or yeast infections, *S. aureus* retains the capability to form biofilms, especially when cohabitating with other bacteria.

Streptococcus agalactiae, otherwise referred to as Group B Streptococcus (GBS), is another bacterial species commonly isolated from diabetic wounds (R. A. Keogh et al., 2022; Waldman et al., 2023). Despite this, some of the mechanisms behind GBS infectivity and pathogenesis in the DFU environment have only been recently described. According to Keogh et al., GBS mediates inflammation in murine diabetic wound models (2022). Furthermore, in a recent wound excision model, mice were infected with GBS alone, *S. aureus* alone, and GBS and *S. aureus* together to understand the microbial consequences of dual-species burden in DFUs. It

was recently discovered in the Doran lab that the burden of GBS was significantly increased in the presence of *S. aureus*, suggesting that *S. aureus* could enhance the survival of GBS *in vivo* (R. A. Keogh et al., 2024). A similar effect was observed in *E. faecalis*: the presence of *S. aureus* enhances *E. faecalis* burden in the wound, though less significantly compared to *S. aureus* and GBS.

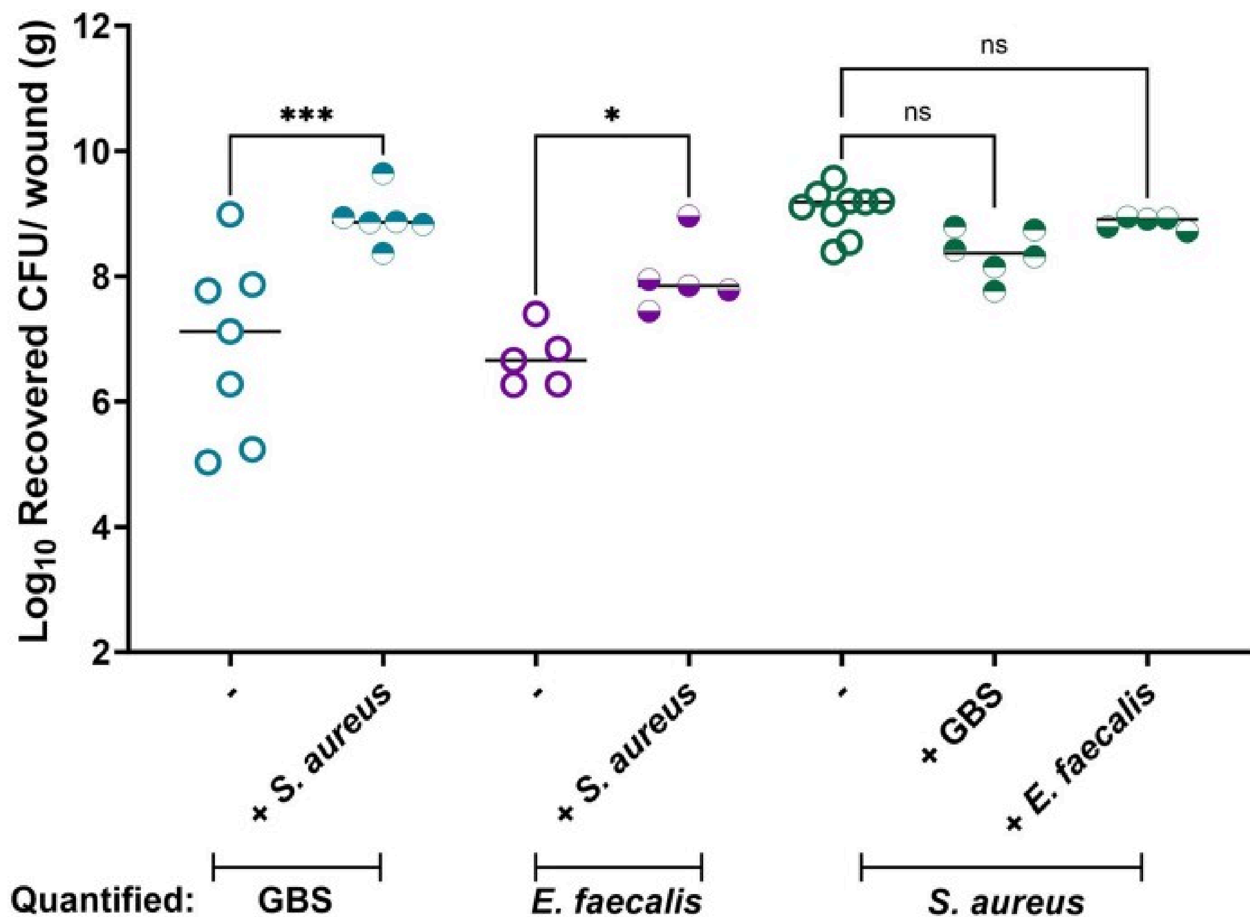

Figure 2.[†] *Log_{10} recovered CFU from diabetic mouse wound tissue was quantified. Mice were inoculated with S. aureus, E. faecalis, and GBS, as well as these species combined. Infection occurred over the course of 4 days: 3 days with adhesive attached, and 24 hours with adhesive*

*removed. GBS and E. faecalis burden is increased in the presence of S. aureus. Statistical significance was determined via one-way ANOVA with Sidak's multiple comparisons. *** Indicates $p < 0.001$, * Indicates $p < 0.05$. (R. A. Keogh et al., 2024).*

† = Data published in Keogh et al., 2024, of which this defendant was the second author.

However, most studies focused on GBS are limited to vaginal and lung epithelial models. Since GBS colonizes approximately 20 to 30% of women's vaginal tracts in the United States, it poses a risk for those who are pregnant; the bacteria can be passed on to neonates and cause severe cases of pneumonia, meningitis, and resulting sepsis (M. A. Khan et al., 2015; Morgan et al., 2025; Regan et al., 1991). As such, much of our knowledge regarding GBS pathogenesis is focused on these populations. For example, GBS production of β-hemolysin mediates lysis of red blood cells and other eukaryotic cell types, fibrinogen-binding proteins facilitate bacterial adhesion, and plasminogen-binding proteins are largely responsible for inflammation and immune evasion in the vaginal and lung epithelial environments (Brokaw et al., 2021; Buscetta et al., 2016; Doran et al., 2002; Nizet, 2002; Rosa-Fraile et al., 2014). The ability of GBS to adhere, form biofilms, and grow while cohabitating with *S. aureus* and other bacteria is still largely unknown in the context of DFUs.

III. Introduction

Three primary features of bacterial survival were explored in this study: biofilms, aggregation, and growth. Biofilms are clumps of bacteria that attach to both a surface and each other. Proteins, polysaccharides, and environmental DNA are embedded within the matrix. This multidimensional structure of biofilms helps confer protection to the bacteria within it (Vestby et al., 2020). The biofilm-forming properties of *S. aureus* have been extensively studied in the

diabetic wound environment (Afonso et al., 2021; Liu et al., 2024; MacLeod, 2019; Pouget et al., 2020). However, other bacterial species thrive in the wound environment, and their biofilm-forming ability, particularly in the case of GBS, has not been elucidated. Moreover, co-species biofilm formation between *S. aureus*, as well as other prominent biofilm formers, and GBS in DFUs is scarcely understood. GBS and *S. aureus* were the second- and fourth-most common key pathogens, respectively, isolated from diabetic foot ulcers in our previous work (R. A. Keogh et al., 2024). *S. aureus* biofilms enhance its resistance to antimicrobial therapies and evasion of the host immune response in diabetic wounds. Data in-progress indicates that *S. aureus* also increases the GBS burden both *in vivo* and *in vitro*, although the molecular mechanisms driving this interaction are not well defined. Furthermore, it is generally understood that polymicrobial environments promote co-aggregation in chronic wound environments, such as DFUs (Anju et al., 2022). Aggregation is one of the first steps in biofilm formation; essentially, this process involves bacteria, adhesins, and other molecules sticking together and attaching to surfaces, which forms the basis of a biofilm (Kragh et al., 2016). This phenomenon has not been studied between *S.* aureus and GBS in the wound environment, though it may contribute to robust biofilm development. This lack of further knowledge necessitates the following study. The findings from this study will build upon the knowledge of polymicrobial interactions in diabetic wound infection, one of the major complications leading to morbidity and mortality among diabetic patients. During this study, biofilm formation, aggregation, growth enhancement, and nutrient sharing were selected as the primary forms of measurement for polymicrobial collaboration. Such investigations will help us further understand how *S. aureus* enhances the survival and proliferation of GBS, therefore highlighting new therapeutic targets that may be disrupted to improve clinical outcomes for patients with DFUs. This could

ultimately translate into a decrease in both lower limb amputations and complex polymicrobial infections among diabetics and other patients with chronic wounds.

IV. Results

Diverse Bacterial Species are Isolated from DFU Debridement Tissue[†]

To illustrate the diverse microbiome within the DFU environment, we analyzed debridement tissue that was received from patient samples (n = 108) at the University of Colorado Anschutz. Out of all patients sampled, 77 (71.3%) had Class I wounds, 24 (22.2%) had Class II wounds, and 7 (6.48%) had Class III wounds (Fig. 3A). Classification was based upon criteria developed by Dr. Garrett Moore, which resembles the University of Texas (UT) system of classification: Class I represents no visible infection, Class II represents concern for colonization, and Class III represents the appearance of infection. Wound ischemia and depth were not considered in this study. Following the preparation described in the methods below, MALDI analysis was completed at Children's Hospital Colorado to isolate and identify bacterial species. The two most predominant species isolated were *Enterococcus faecalis* and *Staphylococcus aureus*, with 39.3% and 36.4% of patients, respectively, exhibiting colonization. *Streptococcus agalactiae* (GBS) was also recovered at 19.6% prevalence (Fig. 3B). Unlike previous meta-analyses, we found that *Escherichia coli* and *Pseudomonas aeruginosa* were not among the most common bacteria in this local patient population; however, they were still present (Idrees et al., 2024; Macdonald et al., 2021). In total, over 28 species were identified in DFU samples, indicating a flourishing microbial community within this environment.

[†] = Data published in and modified from Keogh et al., 2024, of which this defendant was the second author. All figures are original and were created by this defendant using updated data.

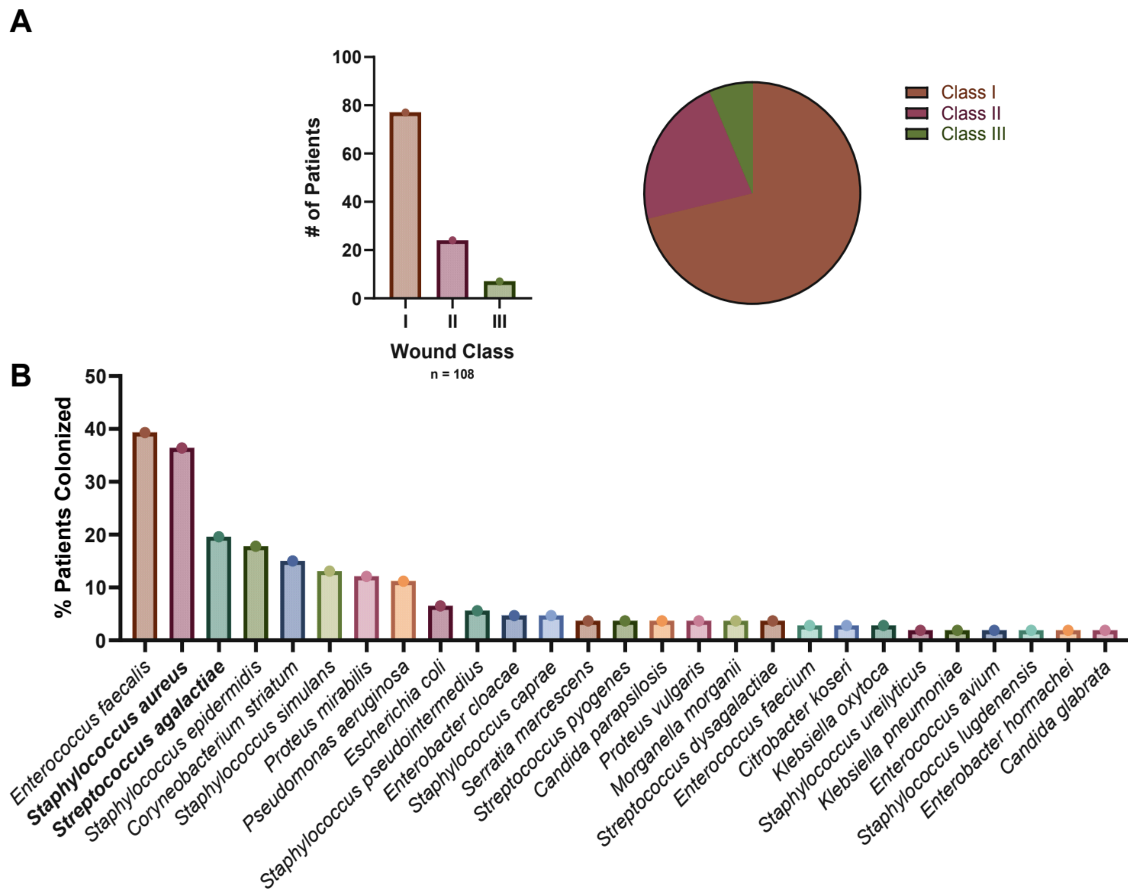

***Figure 3. (A)** Numerical and percentage breakdown of patient wound sample (n = 108) classification. The bar graph indicates numerical delineation, while the pie chart visually indicates percentages. **(B)** Percentage breakdown of patient wound sample bacterial species. A total of 28 species are represented. Single occurrences (i.e., species with n = 1) were not included in these graphical representations.*

S. aureus and GBS are Often Found Co-Isolated in the Wound†

Moving forward, we then analyzed the percentage of patient samples that contained both *S. aureus* and GBS, as well as *E. faecalis*, henceforth referred to as co-colonization. In samples that contained *S. aureus*, 33% of these samples also contained GBS (Fig. 4A). In samples that

contained GBS, 50% also contained *S. aureus* (Fig. 4B). In samples that contained *E. faecalis*, 42.9% contained *S. aureus* while only 16.7% contained GBS (Fig. 4C). Given the relationship between *S. aureus* and GBS co-colonization, combined with the knowledge of the increased burden in our murine model of infection *in vivo,* further investigation focused on the interaction between these species. Our initial determination of species co-colonization allowed us to establish that not only was GBS common in the diabetic wound environment, but its growth also appears to be positively associated with and impacted by the presence of *S. aureus.*

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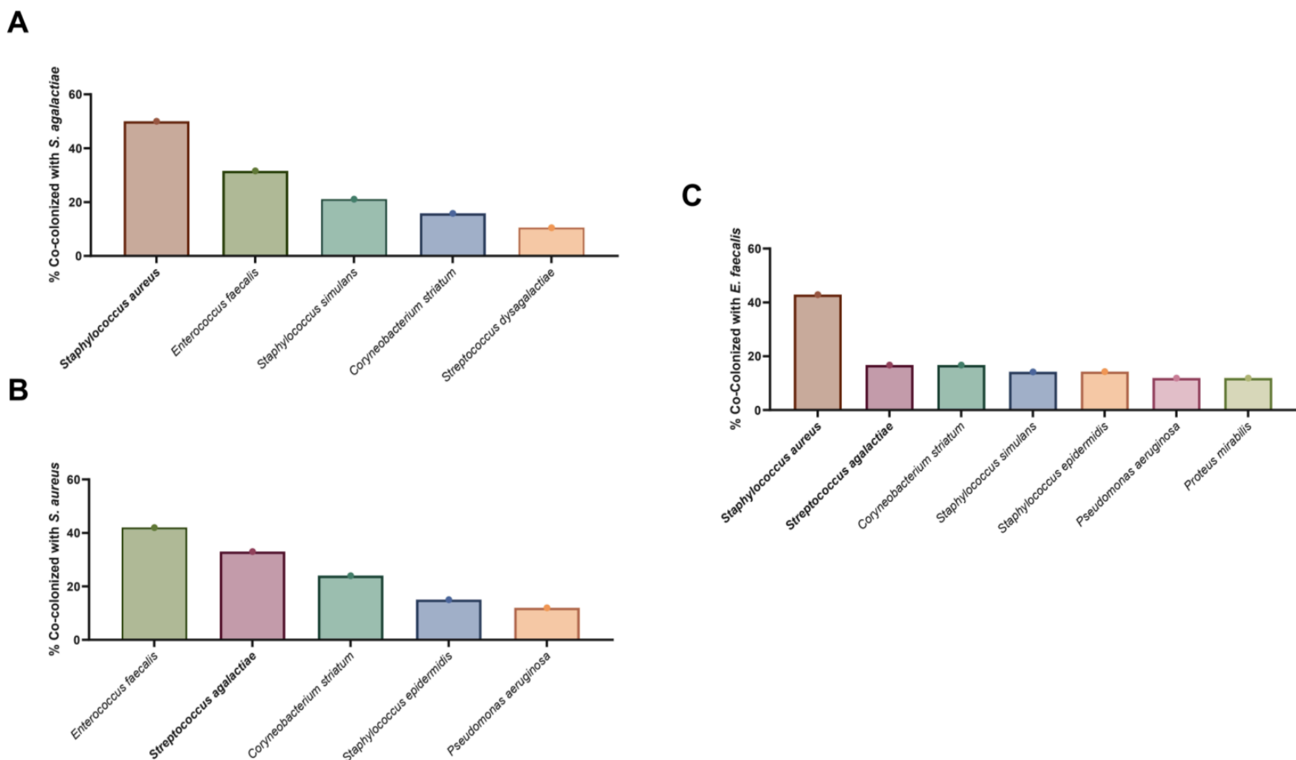

Figure 3. ***(A)*** *Wound samples containing S. aureus were isolated and analyzed for percent co-colonization. 42% of S. aureus samples also contained E. faecalis, and 33% of samples also contained GBS.* ***(B)*** *Wound samples containing GBS were isolated and analyzed for percent*

co-colonization. 50% of GBS samples also contained S. aureus, while 31.6% of samples also contained E. faecalis. ***(C)*** *Wound samples containing E. faecalis were isolated and analyzed for percent co-colonization. 42.9% of E. faecalis samples also contained S. aureus, whereas only 16.7% contained GBS. For all sample analyses, only species that exhibited at least 10% co-colonization with S. aureus, GBS, and E. faecalis were included.*

Biofilm Formation is Species-Dependent†

To determine whether biofilm formation was conserved between clinical isolates and wild-type strains of GBS*, S. aureus,* and *E. faecalis*, static mono-species biofilm assays were analyzed following 24 hours of incubation in a 96-well plate. The laboratory strains included were A909 (GBS*)*, LAC (*S. aureus),* OG1RF, and V583 (*E. faecalis)*. Numbered strains indicate clinical isolates obtained from DFU samples. Biofilm was measured as biomass following crystal violet staining using a Tecan absorbance reader at OD_{595}. GBS generally acted as a poor biofilm former, with an average OD_{595} of 0.115 (Fig. 4A). Similarly, *E. faecalis* had an average OD_{595} of 0.218 (Fig. 4C). While *S. aureus* biofilm varied based on strain, the average OD_{595} was 0.547 across the laboratory strain LAC and clinical isolates (Fig. 4B). When analyzed using a one-way ANOVA, overall biofilm formation in *S. aureus* was significantly higher than biofilm formation in both GBS and *E. faecalis* (Fig. 4D).

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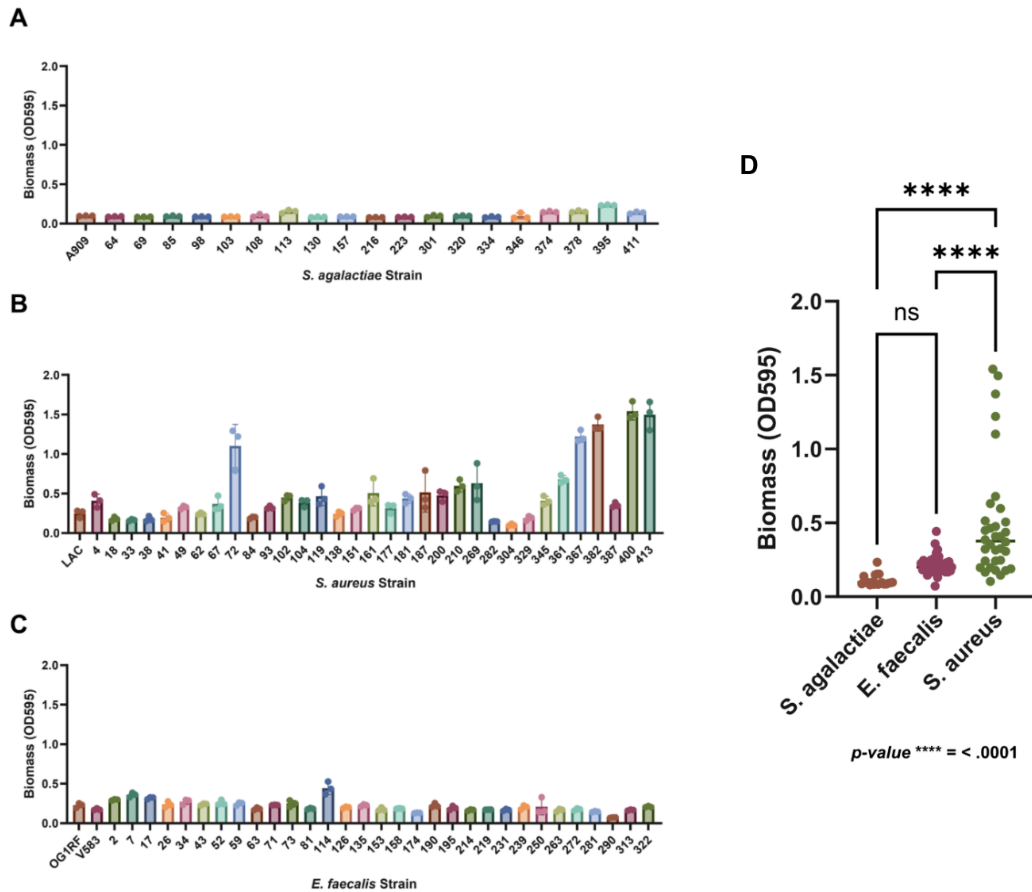


Figure 4. ***(A)*** *Biofilm formation of various GBS strains measured as biomass at OD_{595} following 24 hours of incubation at 37°C. An average OD_{595} of 0.115 was calculated, indicating poor biofilm formation capability.* ***(B)*** *Biofilm formation of various S. aureus strains measured as previously described. An average OD_{595} of 0.547 was calculated, indicating significant biofilm formation capability.* ***(C)*** *Biofilm formation of various E. faecalis strains measured as previously described. An average OD_{595} of 0.218 was calculated, indicating poor biofilm formation capability, like GBS.* ***(D)*** *Biofilm-forming capability of all strains was compared using a one-way ANOVA. Each dot represents a clinical isolate or laboratory strain. S. aureus exhibited significantly higher biofilm formation as compared to GBS and E. faecalis at 24h. **** Indicates significance at $p < 0.0001$.*

S. aureus Enhancement of GBS Biofilm Formation Varies

Since *S. aureus* was found to be a strong biofilm former alone, we wanted to determine whether a wild-type strain could also enhance GBS biofilm formation *in vitro*. The same biofilm assay was completed by halving the amount of bacteria added (from 8uL to 4uL per strain) and combining both LAC and A909 in the same well. Biomass was then measured in a similar fashion at OD_{595}, 24 hours post-inoculation. Furthermore, a $\log_{10}$ colony-forming unit (CFU) count was performed to compare the number of viable bacterial cells from each species in both the planktonic, non-biofilm-associated environment and the biofilm. This determined whether the biofilm burden was predominantly *S. aureus* (LAC), GBS (A909), or a mix of both. Ultimately, we found that there was a similar number of both species located in the planktonic and biofilm-associated environments (Fig. 5A). These results indicate that *S. aureus* does not form robust biofilms alone in the polymicrobial environment: GBS biofilm formation may also be enhanced. This was further supported by the absorbance reading associated with the CFU count. In these technical replicates alone, there was a significant difference in biofilm formation between LAC alone, A909 alone, and LAC + A909 combined (Fig. 5B). When this data was combined with three other biological replicates, significance between monomicrobial and polymicrobial biofilms was even more pronounced (Fig. 5C).

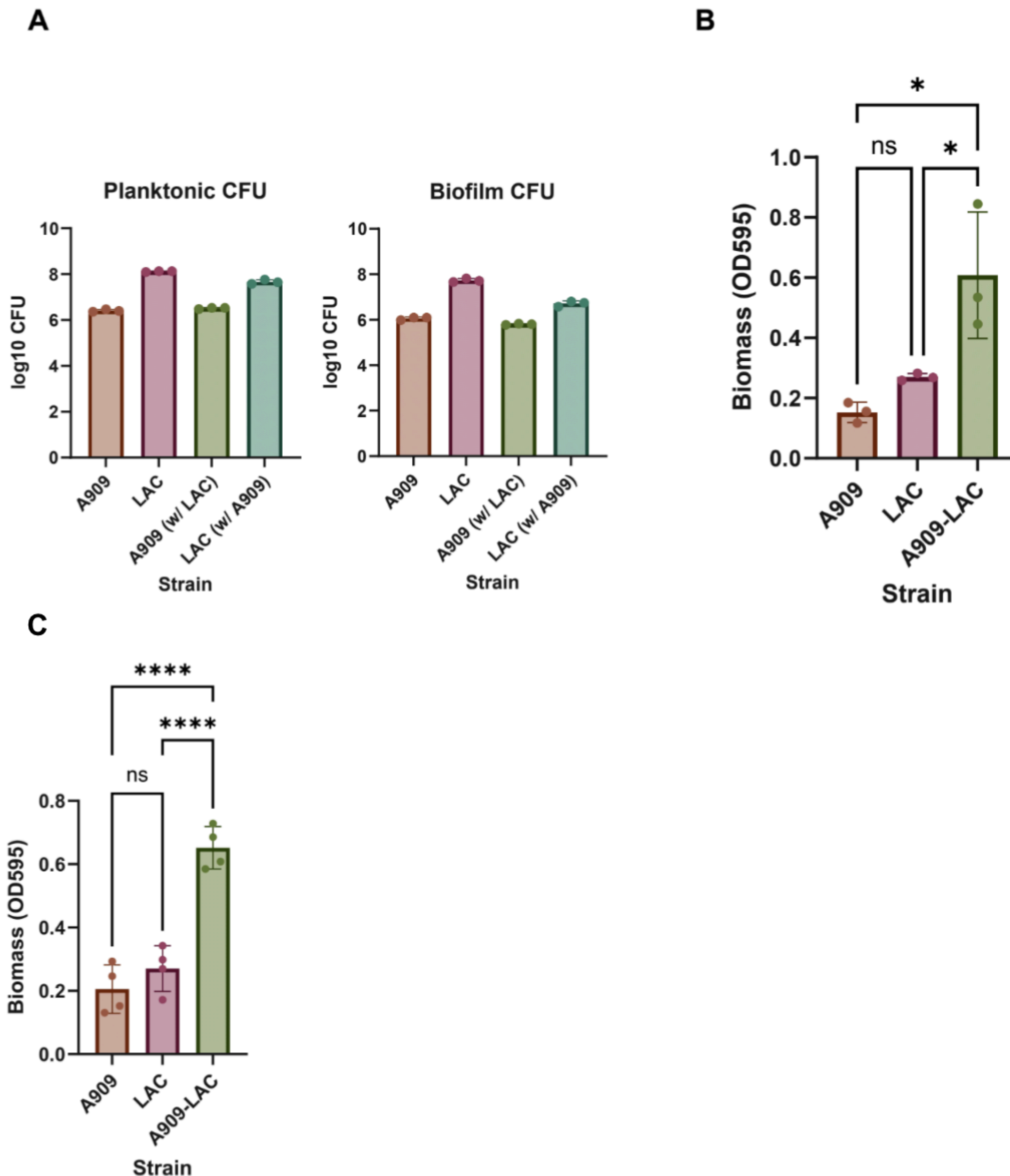


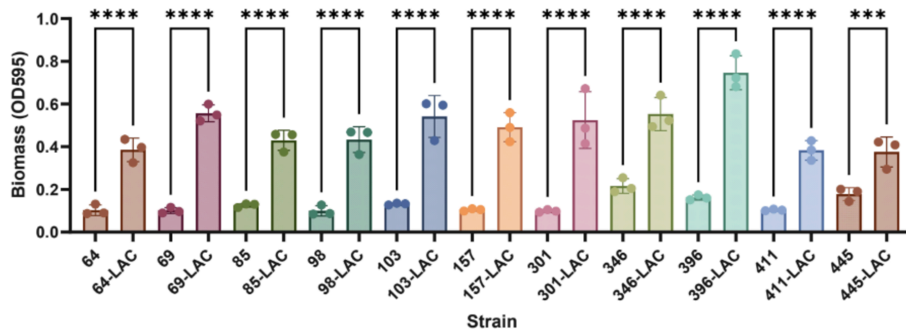
Figure 5. (A) *Colony forming units (CFUs) were quantified at 24 hours post-plate preparation. CFU was compared between planktonic and biofilm-associated environments; no significant difference was found between CFU counts, regardless of monomicrobial or polymicrobial inoculation.* ***(B)*** *Biomass measured as absorbance at OD_{595} was compared to CFU count originating from the same technical replicates. There was a significant difference in biofilm formation between A909 alone and LAC alone, versus A909 and LAC together. A one-way*

ANOVA was completed, indicating significance at $p < 0.05$. ***(C)*** *Data from four biological replicates was averaged and combined to determine significance across multiple samples receiving the same treatment. A one-way ANOVA was completed, indicating significance at $p < 0.0001$.*

Our next aim was to measure polymicrobial biofilm formation in clinical isolates. Three conditions were measured: GBS clinical isolates in the presence of wild-type *S. aureus* (LAC), *S. aureus* clinical isolates in the presence of wild-type GBS (A909), and clinical GBS and *S. aureus* strains co-isolated together. Data varied largely between these conditions. When GBS clinical isolates were combined with LAC, every strain was statistically significant for increased biofilm formation in polymicrobial conditions compared to GBS alone (Fig. 6A). However, when compared to LAC alone, the effect of polymicrobial biofilm formation is less prominent. Some combinations, such as 69-LAC and 346-LAC, are still significant at $p < 0.0001$. Other combinations are either less significant or exhibit no significance (Fig. 6B). Similarly, when *S. aureus* clinical isolates were combined with A909, none of the resulting biofilms were significant compared to *S. aureus* alone. This indicates that LAC, rather than A909, may predominantly drive biofilm formation in these polymicrobial environments. Ultimately, we can deduce that some clinical isolates are phenotypically similar to wild-type species, while others behave differently. This was further corroborated when clinical GBS and *S. aureus* isolates were combined. None of the clinical isolate species showed significant biofilm formation in monomicrobial versus polymicrobial environments (Fig. 6C). Species were specifically combined based on co-isolation status. For example, strain 62 is a *S. aureus* isolate found with GBS strain 64 in the same patient. Even though these species were co-isolated in the wound, biofilm formation was not enhanced when combined *in vitro*. This suggests that biofilm

formation largely depends on each isolate's biological makeup. In short, while significance is found in A909 and LAC, it is not conserved across all GBS and *S. aureus* variants.

A



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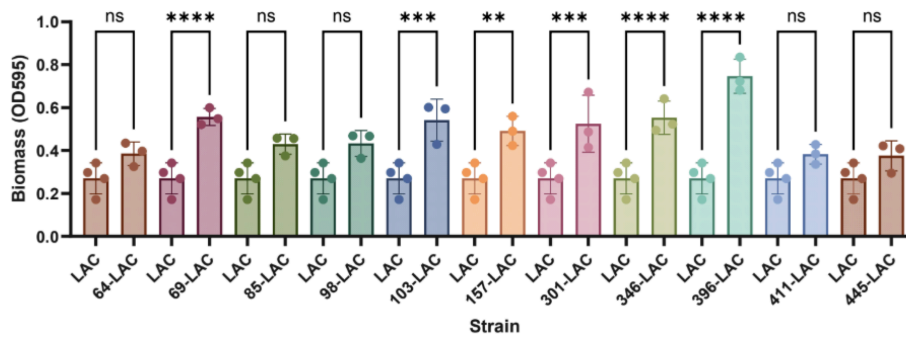

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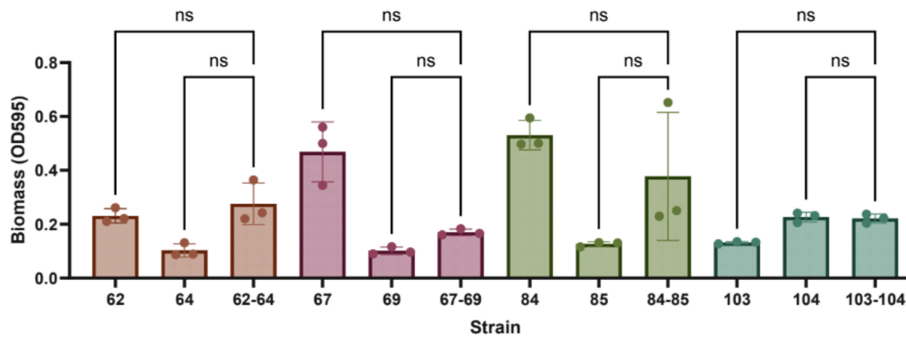

Figure 6. (A) *Polymicrobial biofilm formation between GBS clinical isolates and LAC, compared to clinical isolates alone. A one-way ANOVA with Sidak's multiple comparisons was completed to determine significance. All species combinations were found to be significant.* ***(B)*** *Polymicrobial biofilm formation between GBS clinical isolates and LAC, compared to LAC alone. A one-way ANOVA with Sidak's multiple comparisons was completed to determine significance. Some combinations were found to be significantly different, while others were not.* ***(C)*** *Polymicrobial biofilm formation between GBS and S. aureus clinical isolates, compared to the isolates alone. None of the combinations were found to be significant when a one-way ANOVA was conducted. **** Indicates significance at $p < 0.0001$, *** Indicates significance at $p < 0.001$, and ** Indicates significance at $p < 0.01$.*

S. aureus and GBS Co-Colonization Increases Percent Aggregation

Aggregation, or clumping, was the next focus of our study. We wanted to determine whether aggregation is increased in polymicrobial GBS and *S. aureus* environments compared to these species alone. Aggregation was chosen because it is a phase of biofilm formation and a method of protection against antibiotics and other stressors. Over five hours, 100µL of a resuspended A909-LAC mixture was pipetted into a 96-well plate, and absorbance was measured using OD_{595}. Percent aggregation was then calculated by dividing the absorbance value by the initial absorbance at hour 0. This assay aimed to illustrate the percentage of bacteria that clumped together and precipitated out of solution over time. It was found that when A909 and LAC were combined, percent aggregation at hour five had increased significantly (Fig. 7). This indicates that aggregation is potentially enhanced when GBS and *S. aureus* are together. Aggregation in clinical isolates, however, was not tested due to the large variability found in

biofilm formation. Since aggregation is involved in the development and maintenance of biofilms, it is likely that clinical isolates behave differently based on their genetic variability, as indicated by the previous biofilm results.

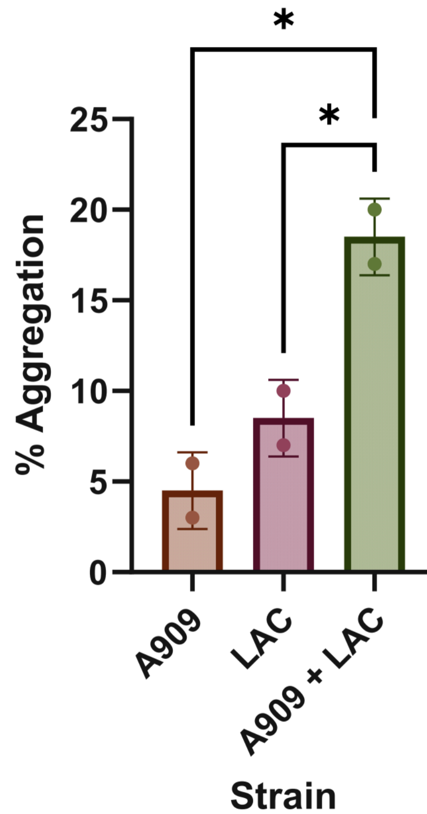

Figure 7. *Aggregation was measured via Tecan plate reader (OD_{595}) every hour over the course of a five-hour time curve. Values were then divided by initial absorbance to yield percent aggregation. A909 and LAC alone exhibited significantly less aggregation as compared to A909 and LAC together at hour five. The graph above represents two biological replicates, with three averaged technical replicates in each experiment. * Indicates significance at $p < 0.05$.*

S. aureus Supernatant Increases GBS Growth Across all Isolates

After investigating biofilm-related properties of *S. aureus* and GBS, our next goal was to elucidate the effect of *S. aureus*-produced products on the growth of GBS, as well as the effect of

GBS-produced products on *S. aureus*. As such, we completed a series of growth curves including various strains and their respective supernatant. Supernatant was utilized to represent products derived from bacteria, but not the bacteria themselves. Since supernatant does not contain any bacterial cells, a growth increase in the presence of supernatant would be due to bacterial products alone. First, a growth curve was completed using A909 with LAC supernatant at three concentrations: 10%, 20%, and 40%. This measured both the dosage and overall effect of *S. aureus* metabolites on GBS growth. We found that 40% and 20% LAC supernatant significantly increased A909 growth, measured in absorbance at OD_{595}, at hours five and six in the growth curve: this constitutes the exponential phase of bacterial growth before plateauing near hour seven (Fig. 8A and B). 10% LAC supernatant, however, did not impose a significant effect on growth. A CFU count was then completed to determine whether *S. aureus* bacteria was contaminating the growth of GBS, and to see whether CFU matched absorbance at each corresponding time point. While there were slight differences between absorbance and CFU, a similar trend was illustrated. At hours six and seven, CFU in the 40% LAC growth conditions were significantly higher than A909 without added supernatant (Fig. C and D). At hour seven alone, all three supernatant concentrations significantly increased A909 growth. Doubling time was compared across all four conditions: ultimately, the addition of 40% LAC supernatant significantly decreases A909 doubling time from 0.92 hours (55.2 min) to 0.77 hours (46.2 min) (Fig. 8E). Conversely, there was no significant impact on LAC growth by A909 supernatant, suggesting that there is a one-way relationship between the species. *S. aureus*-derived products positively impact GBS growth, while GBS-derived products do not necessarily affect *S. aureus* growth (Fig. S1).

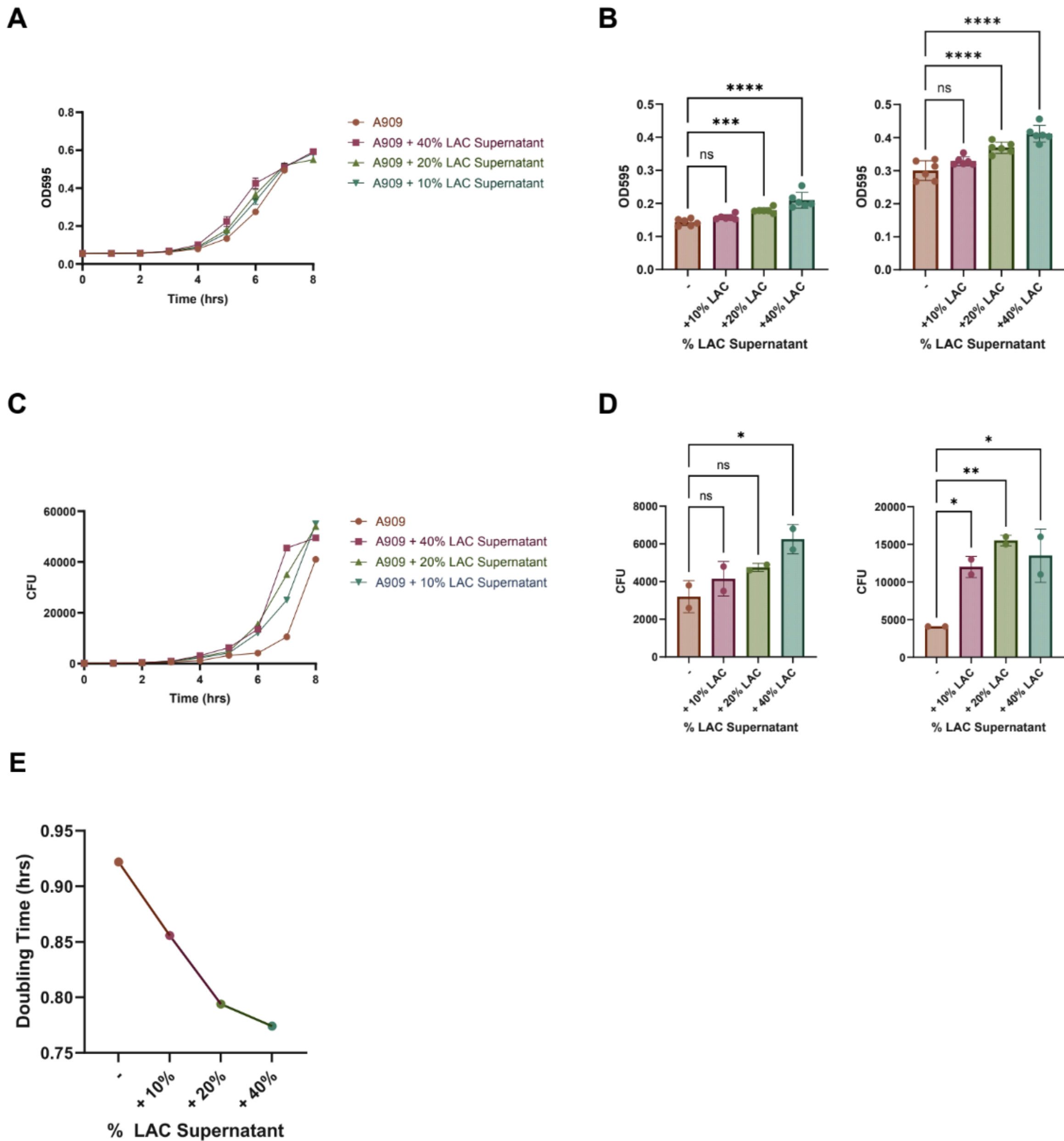

Figure 8. ***(A)*** *Growth of GBS (A909) was quantified via absorbance (OD_{595}) hourly over an eight-hour time curve. The exponential phase of bacterial growth is visualized at approximately hour four through seven, with growth subsequently plateauing at hour eight. Growth was measured under four conditions, as shown above.* ***(B)*** *Growth of A909 during hours five and six across all four conditions. A one-way ANOVA with Sidak's comparisons was utilized to*

*determine significance. At hours five and six, 20% and 40% LAC supernatant significantly increases GBS burden. *** Indicates significance at $p < 0.001$, **** Indicates significance at $p < 0.0001$.* ***(C)*** *Growth of GBS (A909) was quantified via CFU hourly over an eight-hour time curve. Similarly to Fig. 8A, the exponential phase of growth is visualized at approximately hour five through seven, with growth plateauing at hour eight.* ***(D)*** *Growth of A909 during hours six and seven across all four conditions, measured as CFU. A one-way ANOVA using Sidak's comparisons was again utilized to determine significance. At hour six, only 40% LAC supernatant significantly increased GBS growth. At hour seven, 10%, 20%, and 40% LAC supernatant significantly affected growth. ** Indicates significance at $p < 0.01$, * Indicates significance at $p < 0.05$.* ***(E)*** *Doubling time was compared across 40%, 20%, and 10% LAC supernatant concentrations compared to A909 alone. A decrease in doubling time of 0.15 hours, or 9 minutes, was observed between growth in 40% LAC supernatant versus no supernatant. Doubling time = ln(2) / growth rate.*

After determining that LAC supernatant enhances A909 growth, the next goal was to elucidate the effect of *S. aureus* clinical isolate supernatant on A909, as well as LAC supernatant on GBS clinical isolates. Using the same growth curve methodology, it was discovered that *S. aureus* supernatant improves A909 growth consistently across all our isolates and laboratory strains (LAC and JE2) (Fig. 9A). Regardless of potential genetic variability and differences between clinical isolates, the ability to improve A909 growth remained constant. When the opposite effect (LAC supernatant on GBS clinical isolate growth) was tested, each GBS isolate experienced an increase in growth. However, this growth enhancement was not consistent across all isolates (Fig. 9B).

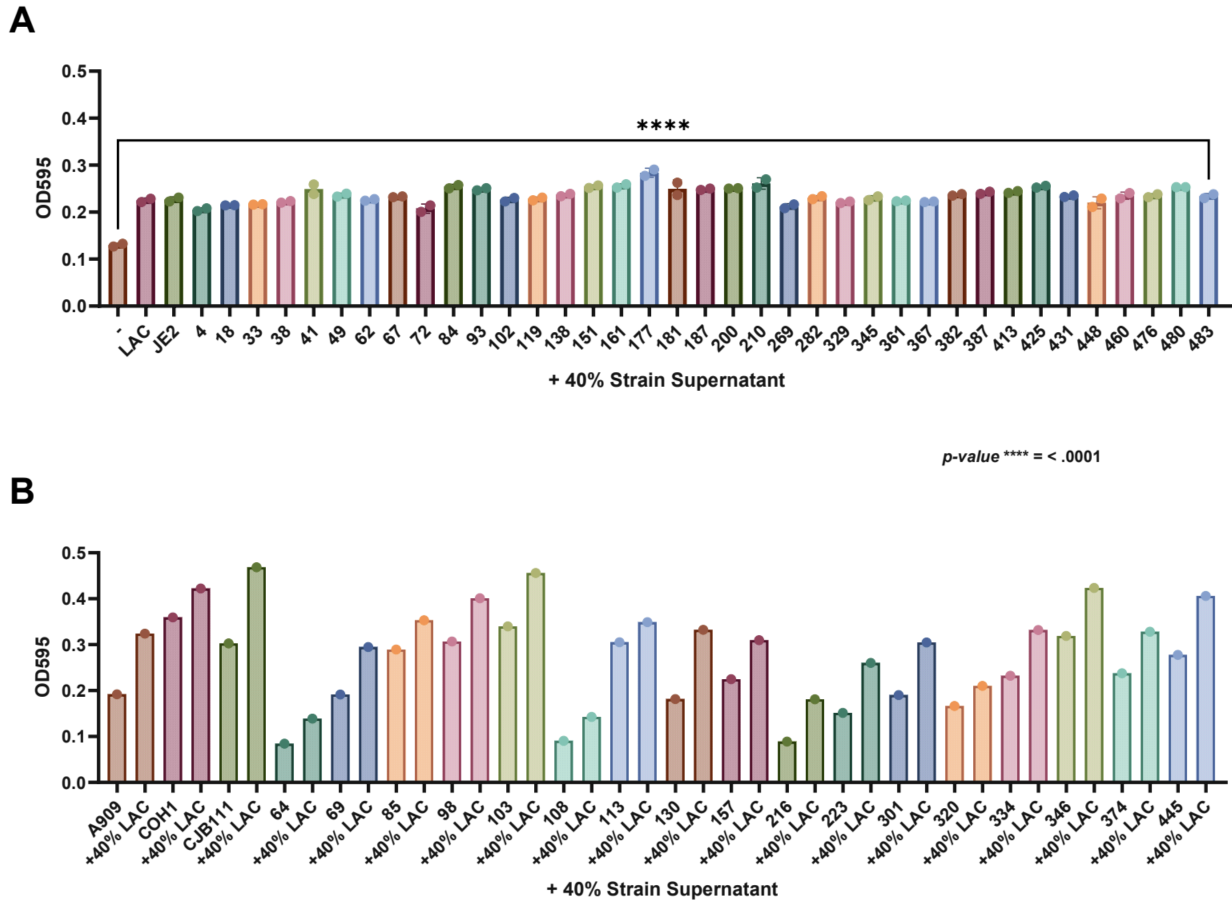

Figure 9. (A) *A growth curve including A909 alone (denoted as -) compared to A909 with 40% S. aureus strain supernatant. Growth of A909 was enhanced in all strains and isolates, with absorbance reading consistently between 0.2 - 0.3* OD_{595}*. A one-way ANOVA with Sidak's multiple comparisons was performed to determine significance. All strains showed significance at p <0.0001 (****).* ***(B)*** *A growth curve including wild-type GBS (A909) and clinical isolates with 40% LAC supernatant. GBS growth was enhanced in all strains and isolates, however the degree of growth enhancement varied. Values represent average growth across three technical replicates.*

GBS Growth in Arginine-Deficient Environments is Rescued by Arg-Producing *S. aureus*

The final goal of this project was to determine how *S. aureus* may be aiding GBS growth. Based on previous data, it is now understood that *S. aureus* supernatant alone can increase the growth of GBS, particularly laboratory strain A909, but the mechanism behind this interaction is unknown. There are several amino acids that GBS is a known auxotroph for, including arginine, glycine, histidine, tryptophan, threonine, valine, tyrosine, phenylalanine, leucine, isoleucine, methionine and serine (Rajagopal et al., 2005). We hypothesized that *S. aureus* could produce some of these amino acids and enhance GBS growth by providing essential nutrients. Arginine is an example of an amino acid that *S. aureus* produces, but GBS does not.

Furthermore, it has been previously documented that loss-of-function mutations in the *S. aureus* ArgH gene dismantle the bacterium's ability to produce arginine (Nuxoll et al., 2012). However, much of this work has been completed in the context of strain JE2, a derivative of laboratory strain LAC. Thus, moving forward, JE2 was used as a baseline to compare to *S. aureus* missing functional *argH* (Δ*argH*). After completing a growth curve in CDM lacking arginine, it was determined that adding 40% Δ*argH* supernatant enhanced growth significantly less than 40% JE2 supernatant in the context of A909 (Fig. 10A and B). While growth with Δ*argH* supernatant was still significantly higher than A909 alone, this data illustrates that *argH* is an important mediator of growth in *S. aureus* enhancement of GBS. A 40% spent BHI media control was also used to determine whether ambient arginine (i.e., arginine in media) contributed to increased GBS growth. We found that it significantly increases A909 growth in arginine-deficient conditions. This ultimately suggests that arginine is necessary for GBS growth, and that both the environment and other bacteria provide it since GBS cannot produce it alone. Finally, DNA extraction, polymerase chain reaction (PCR), and gel electrophoresis were

completed to confirm the presence of an insertion in the *argH* gene, producing Δ*argH*. An ordered mutant library, the Nebraska Transposon Mutant Library (NTML), was utilized to select arginine-deficient mutants in *S. aureus*. A confirmatory gel electrophoresis illustrated that an insertion in the selected Δ*argH* strain was present in the *argH* gene, as shown at approximately 5000bp (Fig. 10C).

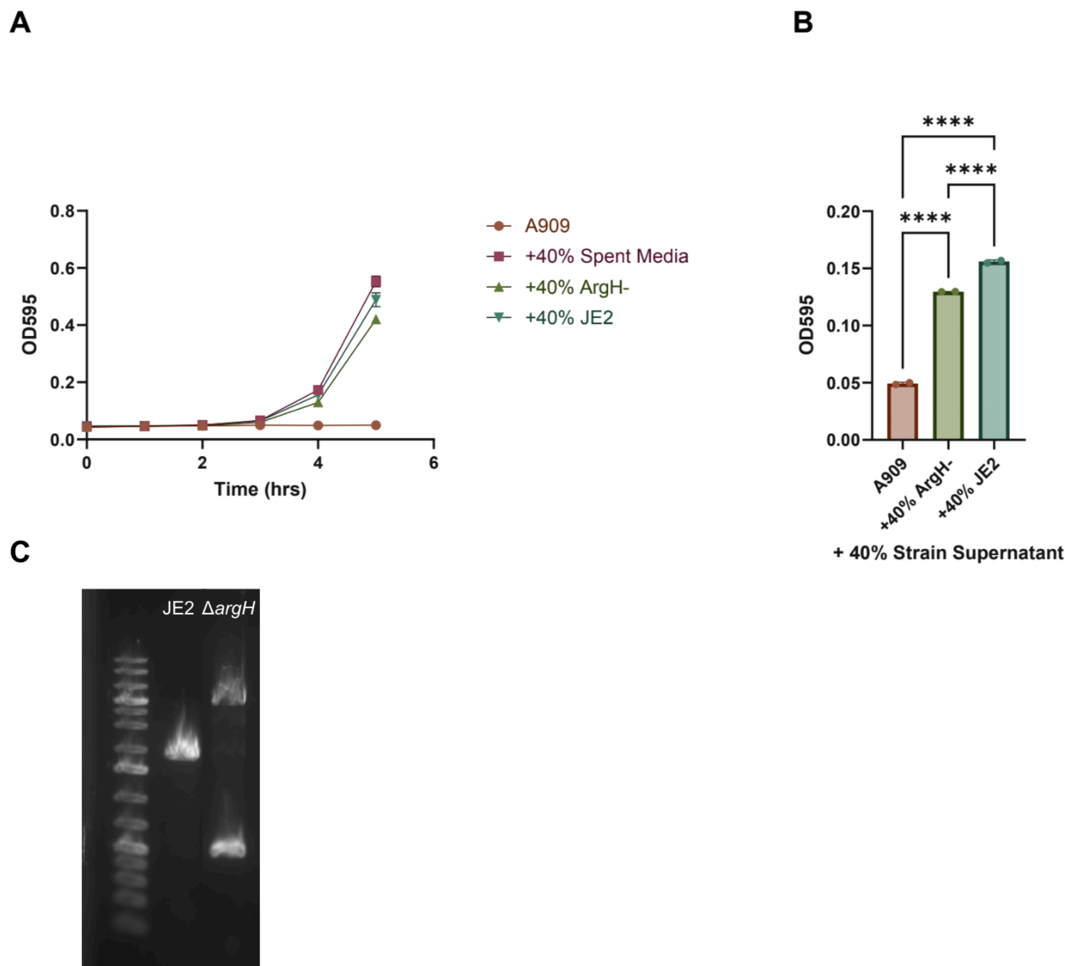

Figure 10. (A) *A growth curve was completed over five hours in CDM lacking arginine. A909 alone did not grow. When 40% strain supernatant and 40% BHI spent media supernatant was included, growth increased significantly, with the exponential phase occurring from hours four to five.* ***(B)*** *Differences in growth between A909 alone, 40% ΔargH supernatant, and 40% JE2*

supernatant are shown. While ΔargH and JE2 both increased growth significantly, ΔargH had a significantly lower effect on growth than JE2. A one-way ANOVA with Sidak's multiple comparisons was performed, indicating significance at p < 0.0001 (****). ***(C)*** *A confirmatory gel electrophoresis was run using DNA from JE2 and the ΔargH strain to determine whether an insertion in the argH gene was present. JE2 lacked the insertion, indicating that arginine was still being produced due to the presence of the argH gene. The ΔargH strain exhibited an insertion in the argH gene, indicating that it did not produce arginine due to the argH gene.*

V. Discussion

This project aimed to determine the identity of bacteria in diabetic foot ulcers, compare their behavior to corresponding laboratory strains through biofilm formation, aggregation, and growth, and elucidate potential mechanisms for polymicrobial collaboration in the wound environment. Our population included diverse samples of wound debridement tissue from patients with varying DFU severity, containing dozens of unique bacterial species. Several other studies have identified *E. faecalis, S. aureus,* and *E. coli* as dominant colonizers of the DFU environment (Anita et al., 2023; Idrees et al., 2024; L. R. Kalan et al., 2019; Lavigne et al., 2021; Liu et al., 2024; Thurlow et al., 2020; Wada et al., 2023; Wolcott et al., 2016). Our study corroborated the prevalence of both *E. faecalis* (39.3%) and *S. aureus* (36.4%), although we found that *E. coli* (6.5%) was not particularly prevalent in our DFU samples (Fig. 1A). However, we also wanted to highlight the presence of GBS in conjunction with these species. GBS was the third most frequently isolated species in our patient population, with 19.6% of samples containing the bacterium. While GBS is generally classified as an opportunistic pathogen residing in the intestinal and vaginal tracts, harboring the potential to cause disease in immunocompromised populations such as neonates and the elderly, it is a rising concern for

those with diabetes (Hanna & Noor, 2022). Not only is it currently a significant cause of morbidity and mortality in diabetic adults, but with the predicted rise in diabetes over the coming decades, GBS prevalence in diabetic infections will only worsen moving forward.

A significant percentage of diabetic wound infections do not occur in a monomicrobial environment (Du et al., 2022; M. S. Khan et al., 2023; Macdonald et al., 2021). In fact, many infections harbor a variety of microbial species. Including both bacteria and fungi, 76.6% of our DFU samples contained at least two distinct organisms, indicating polymicrobial infection (Table S1). The ability of these species to interact, collaborate, and compete in the DFU environment varies. Focusing on the three most isolated species in our samples, *E. faecalis, S. aureus,* and GBS, polymicrobial infection was overwhelmingly prevalent. Out of all *E. faecalis* infections, 2.4% were monomicrobial. Of all *S. aureus* and GBS infections, only 7.7% and 10% were monomicrobial, respectively (Table S1). Furthermore, many of those infected had two or more of these species together. As shown in Fig. 3A and B, we found that in samples containing GBS, 50% also contained *S. aureus*, and in samples containing *S. aureus*, 33% also contained GBS. Despite GBS representing 19.6% of infected samples overall, this species tends to be bidirectionally associated with *S. aureus* in the wound. This relationship, combined with our knowledge of increased GBS burden *in vivo* when associated with *S. aureus* (Fig. 2), motivated our investigation of GBS and *S. aureus* interactions in the DFU environment, which has yet to be studied outside our laboratory.

By completing monomicrobial and polymicrobial biofilm formation assays, aggregation assays, and growth curve analyses, we reached three primary conclusions. First, biofilm formation is not only species-dependent but strain-dependent as well. When monomicrobial biofilm assays were performed with both laboratory strains and clinical isolates, it was found that

S. aureus generally forms strong biofilms. This supports the idea that *S. aureus* is an adept biofilm former in a variety of environments (Peng et al., 2022; Periasamy et al., 2012; Wu et al., 2024). More specifically, we found that laboratory strain LAC is not particularly proficient in biofilm-forming capability, while some clinical isolate strains are suitable biofilm formers (Fig. 4B). This suggests that the ability to form biofilms is strain-dependent, even in a generally strong biofilm-forming species such as *S. aureus*. Biofilm formation was also quantified in *E. faecalis* and GBS, wherein we found that both species cannot construct robust biofilms compared to *S. aureus* (Fig. 4A, C, and D). Subsequently, polymicrobial biofilm assays were completed using *S. aureus* and GBS to determine if biofilm formation was enhanced in the context of both species. We ultimately found a significant increase in polymicrobial biofilm formation between laboratory strains A909 and LAC, compared to these strains alone (Fig. 5). However, similar to the monomicrobial biofilm assays, we found that this effect on biofilm formation is highly strain dependent. When polymicrobial biofilm assays were completed among various clinical isolates, the results were inconsistent. Although every combination of GBS clinical isolate with LAC exhibited an increase in biofilm formation compared to GBS alone, when compared to LAC alone, significance varied between species (Fig. 6A and B). When clinical isolate species of GBS and *S. aureus* were combined to measure co-species biofilm formation, none of the polymicrobial conditions were significant compared to monomicrobial biofilms (Fig. 6C). In conclusion, although significance in biofilm formation is found in A909 and LAC polymicrobial conditions, it is not conserved across all variants of GBS and *S. aureus*.

Our next area of interest was aggregation. Aggregation is an essential facet in generating and maintaining biofilms (Kragh et al., 2016). Therefore, we hypothesized that if biofilm formation was enhanced, aggregation would also be improved in the polymicrobial environment.

Over a five-hour growth period, aggregation was measured in solution containing A909 alone, LAC alone, and A909 and LAC combined. Percent aggregation was then calculated by dividing hourly aggregation by initial aggregation. At hour five, percent aggregation in the A909/LAC solution had increased significantly compared to these species alone (Fig. 7). This suggests that aggregation is enhanced similarly to biofilm formation.

Nevertheless, there were limitations in our measurement of aggregation. First, the graphical representation shown only includes two biological replicates. This assay would need to be repeated at least once more to show a relationship between species cohabitation and aggregation definitively. Second, we did not measure the identity of bacteria involved in the aggregate versus planktonic solution. Therefore, it is unknown whether the aggregate is primarily composed of *S. aureus*, GBS, or a mixture of both. Since *S. aureus* is known to clump and cause coagulation of blood in-vivo, it may be predominantly *S. aureus* that is "falling out" of solution and aggregating (Crosby et al., 2016; Haaber et al., 2012). In the future, it would be advisable to repeat this assay and use flow cytometry or CFU count to reliably measure the type and number of bacteria present in planktonic solution versus aggregate.

Our final investigation in this study was to illuminate the effect of metabolites and other products produced by *S. aureus* on the growth of GBS. Several growth curves were completed to measure this effect. First, growth curves containing A909 with LAC supernatant were utilized to determine laboratory strain growth. Over the course of eight hours, absorbance readings (OD_{595}) and CFU counts were taken hourly to quantify growth. Between approximately hours four and seven, the exponential phase of bacterial growth was observed. Thus, we focused on hours five and six to determine whether the presence and dosage of LAC supernatant significantly impacted GBS growth. At hours five and six, 40% and 20% LAC supernatant enhanced GBS growth, as

shown through absorbance (Fig. 7A and B). To ascertain whether CFU matched absorbance and to ensure that *S. aureus* bacteria was not contaminating growth, a CFU count was performed at corresponding hourly timepoints. We found that CFU generally matched absorbance, with 40% LAC supernatant conditions exhibiting a significant increase in GBS growth at hour six (Fig. 7C and D). However, there were slight differences. At hour seven of the growth curve, absorbance had leveled off into a plateau, whereas CFU still showed significant differences in 40%, 20%, and 10% LAC supernatant conditions compared to A909 alone. Ultimately, though, we concluded that the presence of LAC supernatant enhanced overall A909 growth. In subsequent trials, A909 was exposed to *S. aureus* clinical isolate supernatant to determine whether this effect was conserved across strains. When the growth of A909 alone was compared to A909 + 40% strain supernatant, growth was enhanced in every single combination (Fig. 9A). This insinuates that the ability of *S. aureus* to produce products that aid GBS growth is consistent across many strains. We then quantified the opposite effect: whether 40% LAC supernatant increased growth in various GBS strains. While every combination experienced an increase in growth, the degree of growth varied (Fig. 9B). This is likely due to differences in individual GBS strains. In contrast, many *S. aureus* strain supernatants generally produce a similar effect on laboratory strain A909, and this growth increase is weakened in the presence of clinically isolated GBS strains.

As this project transitioned into discovering the specific mechanisms behind the impact of *S. aureus* on GBS biofilm formation, aggregation, and growth, we decided to focus first on amino acids. GBS is an auxotroph for many amino acids, as previously mentioned. *S. aureus* can produce some of these amino acids, which may be shared with GBS in the DFU environment. We began our inquiry with arginine, which GBS cannot produce. By utilizing JE2, a

LAC-derived *S. aureus* strain that produces arginine, and Δ*argH*, an *S. aureus* strain lacking a functional *argH* gene and thus has decreased arginine-producing ability, we wanted to see whether the addition of 40% strain supernatant impacted the growth of A909 in arginine-deficient conditions. We first confirmed that an insertion in the *argH* gene was present, rendering *argH* unusable by *S. aureus* (Fig. 10C). Although *S. aureus* contains other genes that confer the ability to produce arginine, such as *argG* and *ccpA*, the lack of *argH* alone can decrease arginine production by the bacterium (Nuxoll et al., 2012). After completing growth curves in CDM lacking arginine, we found that A909 did not grow whatsoever, while A909 grew significantly in the presence of 40% JE2 and 40% Δ*argH* supernatant (Fig. A and B). However, it is important to note that growth in 40% Δ*argH* was also significantly lower than in 40% JE2. These data suggest that arginine is necessary for GBS proliferation, and it could be provided by *S. aureus* to rescue growth when ambient arginine is not present. This is an exciting and novel possibility that could be explored further to highlight the impact of *S. aureus*-derived products on GBS survival.

VI. Future Directions

There are several possibilities for future inquiry into the interactions between *S. aureus* and GBS in the context of DFUs. There are two that we found most pertinent in our exploration. First, we would like to extrapolate our work to a cell line model. All the experiments included in this study were completed in 96-well plates without an appropriate animal or cell model. By utilizing a skin cell epithelial model that mimics human keratinocytes, such as NTERTs, we could evaluate polymicrobial interactions in epithelial cell adherence, infection, and wound healing (Smits et al., 2017). Second, we would like to continue our investigation of amino acids that GBS lacks the production capability for and determine whether *S. aureus* could provide

these nutrients to GBS in the wound environment. While we have begun studies on arginine, there are 11 other amino acids that GBS cannot make alone. This provides several possibilities for experimentation with *S. aureus* and other bacterial species, which can provide these nutrients for GBS. Ultimately, the goal of these investigations is to better understand the microbial environment of DFUs. If we can develop a robust and expansive knowledge base regarding the behavior of bacteria and other microbes in diabetic wounds, then it is possible to develop targeted treatment methods that combat and eliminate infection as the prevalence of amputations, complications, and mortality rise due to diabetic wounds.

VII. Materials & Methods

Sample Collection

Specimens were obtained from routine debridement procedures in patients conducted at the University of Colorado Anschutz Medical Campus outpatient facility. Tissues discarded during processing were stored in sterile 2 ml Eppendorf tubes containing 500 µl of PBS and maintained at room temperature to preserve the samples. Using a vortexer, each sample was mixed three times for 10 seconds to ensure homogeneity. Aliquots of 10 µl from that mixture were plated onto a series of culture media: Tryptic Soy Agar (TSA), Sheep's Blood Agar, Luria-Bertani Agar (LBA), Group B Streptococcal CHROMagar, and Candida CHROMagar. Plates were incubated aerobically at 37°C for 48 hours, except for the Candida CHROMagar, which was incubated at 30°C. Following incubation, single colonies were isolated and further cultured on Brain-Heart Infusion (BHI) agar at 37°C for 24 hours for bacterial isolates. Fungal isolates were incubated on yeast extract peptone dextrose agar at 30°C for 24h. All the isolates were later stored in glycerol, from which representative samples were then shipped out to be identified via MALDI.

Bacterial Strains & Growth Conditions

Clinical isolates were prepared as described above. Reference strains for biofilm formation assays included *S. aureus* strains USA300 LAC (LAC), *E. faecalis* strain OG1RF, and GBS strain A909 (Boles et al., 2010; D. Keogh et al., 2016; Madoff et al., 1991). All strains were cultured overnight in BHI broth at 37°C. *S. aureus* was grown under shaking conditions while E. faecalis and GBS were grown statically.

Table 1. *Bacterial strains utilized. Serotype refers to the differences in specific antigens and capsular polysaccharides between GBS strains (Yao et al., 2020).*

Strain	**Description**	**Reference**
A909	Wt GBS strain	(Kavanaugh et al., 2019)
AH1263	Wt CA-MRSA USA300 LAC strain, Erm sensitive	(Kuehl et al., 2020)
OG1RF	Wt *E. faecalis* strain	(Kumar et al., 1994)
V583	Vancomycin resistant *E. faecalis*	(Paulsen et al., 2003)
COH1	Serotype III GBS strain, assoc. with neonatal meningitis	(Vollmuth et al., 2024)
CJB111	Serotype V hypervirulent GBS clinical isolate	(Spencer et al., 2021)
JE2	Community-assoc. USA300 LAC derivative strain, received from Nebraska Transposon Mutant Library (NTML)	(Fey et al., 2013)
Δ*argH*	JE2 strain deficient in *argH*, from NTML	(Fey et al., 2013)

Biofilm Formation Assay

Assays for static biofilm formation were performed using a previously described method by Marroquin et al., 2019. Overnight cultures of the bacterial strains in BHI were diluted 1:50 in a 96-well plate containing 10% human plasma to promote biofilm development. Plates were incubated at 37°C for 24 hours. After incubation, the biofilms were washed twice with sterile PBS to remove non-adhering bacteria. Then, the plate was stained for 5 min at 60°C with 0.05% crystal violet to highlight the adhering biomass. Biofilms were subsequently washed twice more with PBS, and the remaining adherent cells were treated with 100% ethanol to solubilize the crystal violet. Biomass was quantified by measuring the optical density at 595nm (OD_{595}) on a plate reader Tecan Infinite 200pro.

Aggregation Assay

Cultures of *S. aureus* in Tryptic Soy Broth (TSB) and GBS in Todd Hewitt Broth (THB) were grown overnight for 16 to 17 hours at 37ºC. The following day, the cultures were centrifuged at 3,900 rpm for 10 minutes, the supernatant was removed, and the bacterial pellets were washed with PBS. After washing, 1 mL aliquots were transferred to Eppendorf tubes and resuspended in PBS to achieve an OD_{595} of approximately 1.0. Optical density measurements were taken each hour over a five-hour time period using a Tecan plate reader. Percent aggregation was calculated by comparing the OD_{595} values at each time point to the initial reading.

Growth Curve & Colony Forming Unit (CFU) Analysis

Cultures of *S. aureus* and GBS in BHI were grown overnight for 16 to 17 hours at 37ºC. *S. aureus* was grown in shaking conditions, while GBS was grown in static conditions. The following day, GBS strains were diluted 1:500 in 25mL BHI for normal growth, and 25mL conditionally defined media (CDM) lacking arginine for arginine-deficient growth. Then, 1mL aliquots of overnight cultures of *S. aureus* were centrifuged for 3 minutes at 13.3 g (1089 rpm). The resulting supernatant was sterilized using a 0.22µm filter, and 80µL of the sterilized supernatant was added to the top row of a 96-well plate to create a 40% solution in BHI (or CDM). Supernatant was diluted 1:2 down the plate to create 40%, 20%, and 10% concentrations. Finally, 100µL of the 1:500 diluted GBS strain was added to each well, along with the appropriate controls: BHI or CDM alone, *S. aureus* supernatant alone, GBS alone, and spent media alone. Absorbance readings were measured at OD_{595} each hour for eight consecutive hours in BHI and five consecutive hours in CDM.

VIII. Supplemental Material

Supplemental Figure 1

Supplemental Table 1

IX. Acknowledgements

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am now armed with. I cannot wait to give back to the research community as a physician assistant and treat the very patients that I am currently studying!

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