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Virulence characteristics of Gram-positive bacteria isolated from diabetic foot ulcers

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Abstract

Diabetic wound infections including diabetic foot ulcers (DFUs) are a major global health concern and a leading cause of non-traumatic amputations. Numerous bacterial species establish infection in DFUs, and treatment with antibiotics often fails due to widespread antibiotic resistance and biofilm formation. Determination of bacterial species that reside in DFU and their virulence potential is critical to inform treatment options. Here, we isolate bacteria from debridement tissues from patients with diabetes at the University of Colorado Anschutz Medical Center. The most frequent species were Gram-positive including *Enterococcus faecalis, Staphylococcus aureus,* and *Streptococcus agalactiae*, also known as Group B Streptococcus (GBS). Most tissues had more than one species isolated with *E. faecalis* and GBS frequently occurring in polymicrobial infection with *S. aureus*. *S. aureus* was the best biofilm producing species with *E. faecalis* and GBS isolates exhibiting little to no biofilm formation. Antibiotic susceptibility varied amongst strains with high levels of penicillin resistance amongst *S. aureus*, clindamycin resistance amongst GBS and intermediate vancomycin resistance amongst *E. faecalis*. Finally, we utilized a murine model of diabetic wound infection and found that the presence of *S. aureus* led to significantly higher recovery of GBS and *E. faecalis* compared to mice challenged in mono-infection.

Keywords: bacteria; diabetic; gram-positives; isolated; virulence; wounds

Introduction

Diabetic wounds of the foot and lower limbs are a leading cause of global amputation (Pecoraro et al. 1990, Kurnar et al. 1994, Reiber et al. 1995, Frykberg et al. 1998, Uccioli et al. 2015). These infections often become chronic and will not heal due to the presence of multiple bacterial pathogens and an impaired immune response (Perez-favila et al.2019, Brem and Tomic-canic 2007). Gram-positive bacterial species are often the most common in diabetic wound infections with Staphylococci being the dominant genus according to culture based methods, 16 s rRNA data and metagenomic sequencing (Citron et al. 2007, Lipsky et al. 2012, Gardner et al. 2013, Perim et al. 2015, Kalan et al. 2019). However, follow-up studies examining the phenotypic characteristics of the strains recovered from DFUs are important to understand the virulence characteristics of strains persisting in different demographics and help inform treatment options.

Antibiotics are often administered either systemically or topically to the wound, and it is recommended to obtain a bacterial culture from wound tissues prior to antibiotic selection for treatment (Lipsky et al. 2012, Senneville et al. 2023). However, even with proper identification of bacterial species in wound tissue, antibiotics often fail to resolve the infection (Singh and Gupta 2017). Treatment failure has been linked to multiple bacterial mechanisms such as the acquisition of antibiotic resistance, emergence of persister cells, and formation of biofilm communities (Citron et al. 2007, Serra et al. 2015, ALbeloushi et al. 2019, Kalan and Brennan 2019). Thus, identification of a bacterial species alone may be insufficient to inform treatment options without knowledge of potential resistance profiles and biofilm formation capacity.

In this study, we characterize a panel of Gram-positive clinical isolates collected from debridement tissue of diabetic individuals treated at the University of Colorado Anschutz. We found using culture based methods that the most common genera in our cohort were *Enterococcus*, *Staphylococcus*, and *Streptococcus*, with *E. faecalis*, *S. aureus* and GBS as three of the most frequently isolated species. We therefore analyzed all clinical isolates of *E. faecalis*, *S. aureus* and GBS for antibiotic resistance and the ability to form biofilm. We found that *S. aureus* strains have the greatest biofilm formation where *E. faecalis* and GBS isolates had high levels of antibiotic resistance, suggesting varied methods of survival to antibiotic treatment. Further investigation of samples revealed that DFUs with *E. faecalis* or GBS were often polymicrobial with *S. aureus* being the most commonly co-isolated organism. Due to high co-incidence, we utilized a murine model of diabetic wound infection, and found that co- infection with *S. aureus* significantly increased GBS and *E. faecalis* bacterial burden in wound tissues in comparison to mice infected with GBS or *E. faecalis* alone. Collectively, these results provide a wealth of data on strains representing three of the most relevant species in diabetic wound infection, and how they may cooperate during diabetic wound infection.

Materials and methods

Sample collection

Patients from the University of Colorado outpatient facility at Anschutz Medical Campus had routine debridement of tissues resulting in discarded tissues we utilized for our analysis. Tissues were placed into sterile 2 ml Eppendorf tubes with 500 µl

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of PBS. Samples were then vortexed three times for 10 seconds before plating 10 µl onto Tryptic soy agar, Sheep's blood agar, Luria-Bertani agar, Group B Streptococcal CHROMagar, and Candida CHROMagar. All plates were incubated aerobically at 37°C for 2 days except the Candida CHROMagar which was placed at 30°C. Resulting single colonies were further isolated on brain-heart infusion (BHI) agar for 24 h at 37°C for bacteria yeast extract peptone dextrose agar for 24 h at 30°C for fungi. Isolates were then stocked in glycerol and plates sent for identification via matrix associated laser desorption/ionization (MALDI).

Bacterial strains and growth conditions

Clinical isolates were collected as described above. Reference isolates were used for biofilm formation including *S. aureus* strains USA 300 LAC, *E. faecalis* OG1RF and GBS A909 (Madoff et al. 1991, Boles et al. 2010, Keogh et al. 2016). All strains were grown in BHI overnight for 24 h at 37°C with *S. aureus* shaking and *E. faecalis* and GBS grown statically.

Biofilm formation assay

Static biofilm formation was tested as previously described (Marroquin et al. 2019). Overnight bacterial cultures were grown in BHI then diluted 1:50 into a 96-well plate containing 10% human plasma. Plates were incubated at 37°C for 24 h. Following incubation, plates were washed two times with sterile PBS and stained with .05% crystal violet for 5 minutes at 60°C. Resulting biofilms were washed two more times with PBS and the remaining adherent cells treated with 30% acetic acid. Biomass was quantified by OD_{595} in a Tecan Infinite 200pro plate reader.

Antibiotic susceptibility

Bacterial strains were grown overnight in BHI as described and antibiotic sensitivity determined (Burcham et al. 2019). Cultures were normalized to an OD_{600} of 0.1 in PBS and 100 µl of each strain was duplicate plated onto BHI. Antibiotic discs were added to the plates and incubated overnight at the following concentrations (Penicillin 10 U, Clindamycin 2 mg, Erythromycin 15 mg, Vancomycin 30 µg, and Tetracycline 30 µg) (Hudzicki 2012). The following day, zones of inhibition around discs were measured and strains were characterized as susceptible, intermediate or resistant based on the 2020 performance standards for antimicrobial susceptibility testing (Weinstein and Lewis 2020a) as follows: *Staphylococci*: Penicillin (Resistant <28 mm, Susceptible >29 mm), Clindamycin (Resistant <14 mm, Intermediate 15–20 mm, Susceptible >21 mm), Erythromycin (Resistant <13 mm, Intermediate 14–22 mm, Susceptible >23 mm), Vancomycin (Resistant <16 mm Susceptible >17 mm), and Tetracycline (Resistant <14 mm, Intermediate 15–18, and Susceptible >19 mm). *Enterococci*: Penicillin (Resistant <14 mm, Susceptible >15 mm), Clindamycin (intrinsically resistant), Erythromycin (Resistant <13 mm, Intermediate 14–22 mm, Susceptible >23 mm), Vancomycin (Resistant <14 mm, Intermediate 15–16 Susceptible >17 mm), and Tetracycline (Resistant <14 mm, Intermediate 15–18, and Susceptible >19 mm). *β*-hemolytic *Streptococci* (GBS): Penicillin (Susceptible >24 mm, any non-susceptible GBS to be reported to emergence of penicillin resistance), Clindamycin (Resistant <15 mm, Intermediate 16–18 mm, Susceptible >19 mm), Erythromycin (Resistant <15 mm, Intermediate 16-20 mm, Susceptible >21 mm), Vancomycin (Susceptible >17 mm), and Tetracycline (Resistant <18 mm, Intermediate 19–22, and Susceptible >23 mm) (Weinstein and Lewis 2020a).

Table 1. Bacterial strains

Strain	Description	Reference
A909	wt GBS strain	(Kavanaugh et al. 2019)
AH1263	wt CA-MRSA strain LAC, USA300-0114 PFGE type, Erm sensitive	(Kuehl et al. 2020)
OG1RF	wt *E. faecalis*	(Kurnar et al. 1994)

Murine model of diabetic wound infection

Male 7–10 week old mice were given low-dose intraperitoneal injections of streptozotocin at 50 mg/kg followed by 250 µl of 25% glucose for five consecutive days as previously described (Akbari et al. 2023). Mice with glucose levels exceeding 200 mg/dL via glucometer were considered diabetic. Following, we utilized a murine model of chronic wound infection developed by Chong et al. (Chong et al. 2017) that we adapted for GBS infection (Keogh et al. 2022, Akbari et al. 2023). Briefly, mice were anesthetized with 3% isofluorane and the dorsal area was shaved before treatment with Nair to completely remove hair. The following day, the mouse skin was cleaned with iodine and treated with the local anesthetic lidocaine on the back. Mice were then wounded with a 6 mm full thickness excision and bacterial inoculum of 5×10^6 CFU in 5 µl was applied. For polymicrobial infections, each strain, A909, OG1RF and LAC were grown separately and normalized to 5×10^6 CFU in 5 µl before adding 5 µl of each strain (where indicated) directly onto the back of the mouse. Wounds were then covered in the surgical adhesive Tegaderm for three days. Tegaderm was then removed and mice were left for an additional 24 h before being euthanized and wound tissue collected for homogenization and CFU determination. CFU enumeration was completed using media selective and differential for GBS, *E. faecalis* and *S. aureus*. GBS CHROMagar was used for identification of GBS and *E. faecalis* which turn purple and blue on this media, respectfully. *S. aureus* LAC was plated on mannitol salt agar with cefoxitin at a concentration 5.2 µg/µl to inhibit growth of contaminating Staphylococci. These experiments were approved by the committee on the use and care of animals at the University of Colorado–Anschutz Medical Campus in our protocol no. 00987 (Table 1).

Results

Microbial abundance in diabetic debridement tissue

We received debridement tissue from DFUs of 96 patients at the University of Colorado Medical Center at Anschutz. Tissue was obtained from 96 individuals with two recurrent patients (98 samples total) (Table S1). Samples were vortexed and isolated for single species via culture-based methods before being sent for matrix-associated laser dissociation/identification for species identification. A total of 243 microbes were recovered from the 96 patients with 55 unique species represented (Table S2). In cases where the same species was identified multiple times from the same sample, the first isolate corresponding to that species was utilized to avoid double counting. Only two samples (patient 8 and 53) had no recoverable microbes from tissue (Table S1). The most frequently isolated species were *E. faecalis* (33.7% of patients), *S. aureus* (33.7%), *S. epidermidis* (20.4%) and *S. agalactiae* (GBS) (19.4%) (Fig. 1A). These findings are consistent with metagenomic and 16S studies which find *S. aureus* and GBS frequently in DFUs (Gardner et al. 2013, Wolcott et al. 2016, Kalan et al. 2019). Interestingly, *E.*

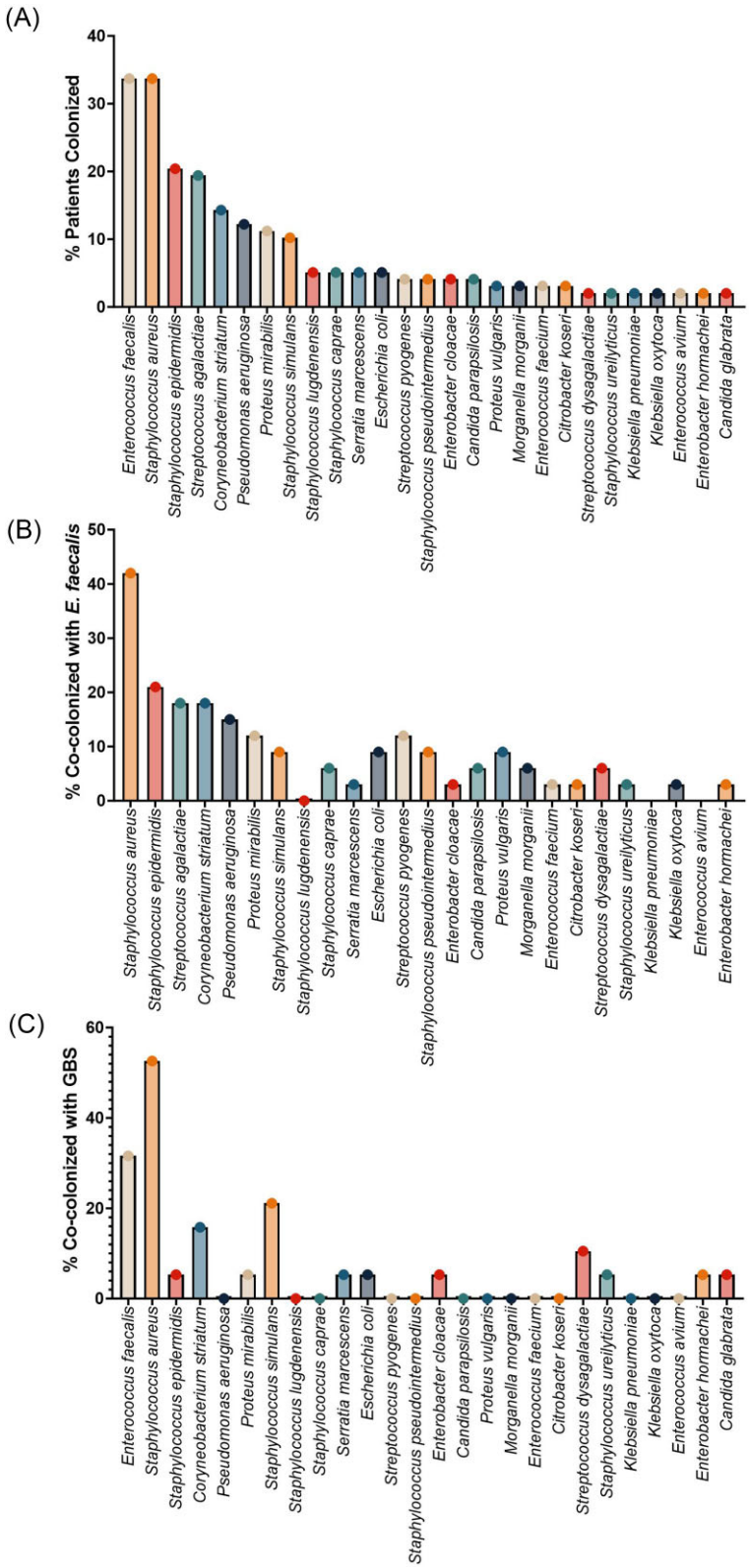

Figure 1. Microbial species in diabetic wound debridement tissues. (A) Percentage of patients in which each species was isolated. Species included were isolated a minimum of two times. (B) Co-incidence of species with *E. faecalis* and (C) GBS.

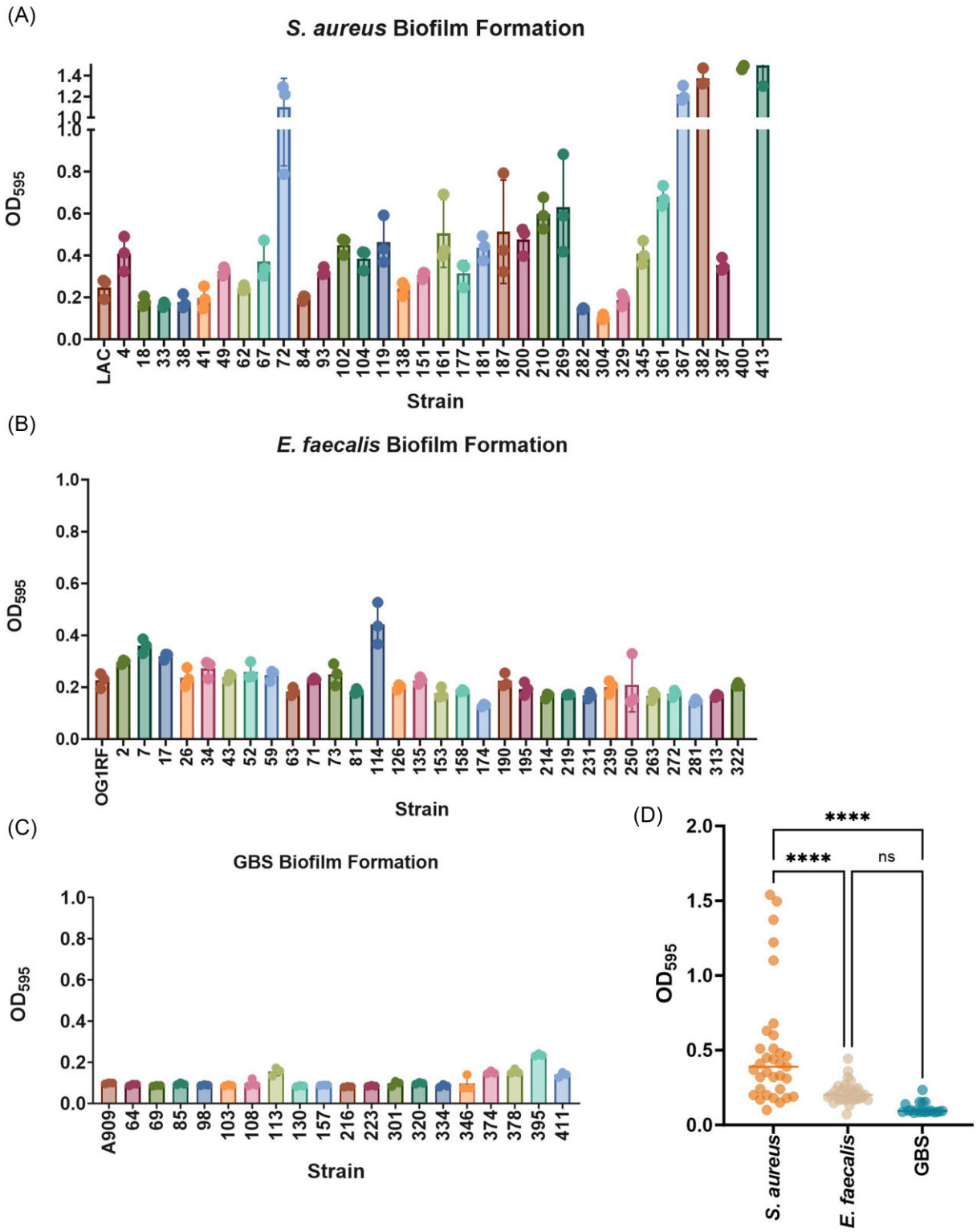

Figure 2. Biofilm formation across clinical isolates. (A) Biofilm formation amongst *S. aureus* isolates measured by OD_{595}. (B) *E. faecalis* biofilm formation, (C) GBS biofilm formation. (D) Average biofilm formation of each clinical isolate. Significance determined by one-way ANOVA comparing each column to the average of the other column; *P*-value **** =<.0001.

faecalis is often absent from 16S studies on the DFU microbiome, but commonly identified in culture based and metagenomic studies (Citron et al. 2007, Loesche et al. 2017, Shettigar et al. 2018, Mudrik-Zohar et al. 2022). An additional 23 species were recovered in at least two unique patients including *Corynebacterium striatum*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, commensal *Staphylococci* and *Candida* species (Fig. 1A).

Analysis of wound tissues further revealed that most samples were polymicrobial with 70/96 (72.9%) having more than one unique species recovered (Table S2). Interestingly, DFUs contain-

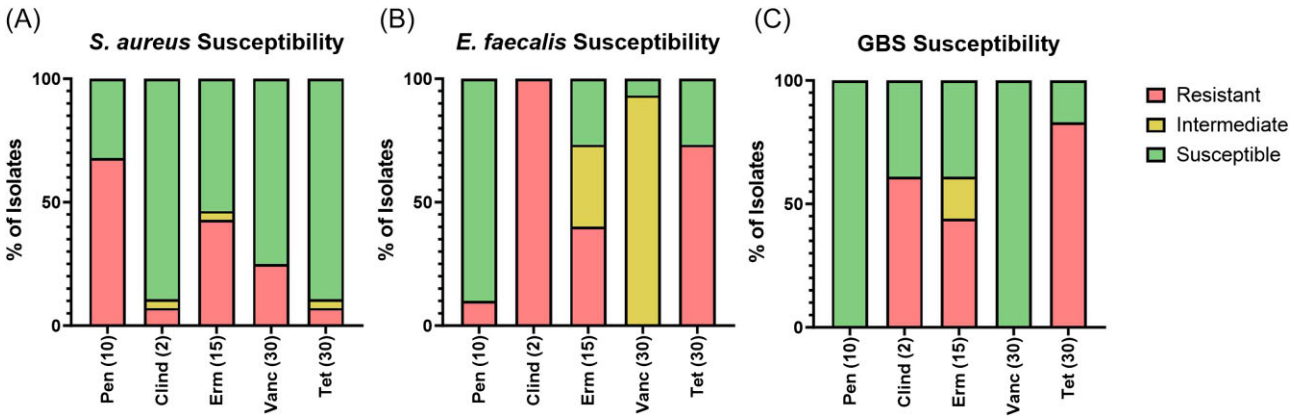

Figure 3. Antibiotic susceptibility amongst clinical isolates. (A) Susceptibility of *S. aureus* (B) *E. faecalis* and (C) GBS isolates collected from diabetic debridement tissue.

Table 2. Susceptibility of *S. aureus* isolates to antibiotics. Numbers reflect the zone of inhibition with red cells resistant, yellow intermediate and green susceptible to the antibiotic indicated.

Strain	Pen (10)	Clind (2)	Erm (15)	Vanc (30)	Tet (30)
4	0	0	0	18	11
18	14	29	12	17	35
33	14	28	12	17	36
38	12	28	30	18	32
41	0	0	0	19	30
49	0	15	10	18	34
62	42	30	34	19	36
67	10	30	10	17	35
72	15	30	33	18	18
84	13	29	11	18	35
93	38	29	30	18	32
102	0	28	10	17	34
104	40	28	30	17	35
119	39	27	29	17	32
138	37	26	30	16	30
151	14	26	27	16	30
161	15	29	30	18	32
177	34	28	30	16	33
181	13	30	11	19	35
187	40	30	31	18	38
200	14	29	11	18	35
210	12	28	31	18	34
269	15	27	29	18	31
282	16	27	28	16	31
304	41	28	16	18	32
329	28	27	30	15	31
345	13	25	0	16	30
367	36	26	0	15	0

ing either *E. faecalis*, or GBS were frequently co-isolated with *S. aureus* with 42.4% of *E. faecalis* positive wounds and 52.6% of GBS wounds having *S. aureus* respectively (Fig. 1B, C). We therefore chose to characterize *E. faecalis*, *S. aureus* and GBS strains moving forward as each of these species has been implicated in diabetic wound infection (Citron et al. 2007, ALbeloushi et al. 2019, Kalan et al. 2019, Keogh et al. 2022).

Biofilm formation is highly correlated with species

In chronic wound infections, bacterial species often live in biofilm communities as opposed to planktonic (Davis et al. 2008, James et al. 2008). We utilized clinical isolates of *E. faecalis*, *S. aureus* and GBS to test their ability to form static biofilms in comparison to laboratory isolates LAC (*S. aureus*), OG1RF (*E. faecalis*) and A909 (GBS). We determined a wide range of biofilm capacity based on biomass after 24 h. Of all isolates, *S. aureus* was the best biofilm forming species with an average OD_{595} (biomass) of 0.50 (Fig. 2A). *E. faecalis* isolates had the next highest biofilm formation with an average OD_{595} of 0.22 (Fig. 2B). GBS isolates exhibited little to no biofilm formation under our tested conditions average OD_{595} of 0.11 (Fig. 2C). Comparison of the average biofilm formation of each clinical isolate shows *S. aureus* isolates have significantly higher biofilm formation than any of the *E. faecalis* or GBS strains (Fig. 2D).

Table 3. Susceptibility of *E. faecalis* isolates to antibiotics.

Strain	Pen (10)	Clind (2)	Erm (15)	Vanc (30)	Tet (30)
2	18	0	0	15	8
7	18	0	22	15	7
17	19	0	22	16	8
26	20	0	0	16	8
34	20	0	23	16	8
43	23	0	0	16	8
52	22	0	21	15	11
59	20	0	21	15	10
63	22	0	25	15	7
71	19	0	21	16	8
73	13	0	25	15	25
81	18	0	0	16	10
114	20	0	19	15	28
126	20	0	19	15	7
135	20	0	0	16	8
153	22	0	23	16	27
158	22	0	23	16	27
174	19	0	18	15	29
190	23	0	20	15	7
195	22	0	21	15	26
214	25	0	0	16	0
219	21	0	24	17	10
231	20	0	0	16	7
239	15	0	0	17	8
250	18	0	0	15	7
263	19	0	0	16	7
272	16	0	0	16	28
281	14	0	25	15	29
313	15	0	23	16	8
322	12	0	0	15	10

Strains from DFUs have high levels of antibiotic resistance

We next tested our panel of clinical isolates for antibiotic resistance to penicillin, clindamycin, erythromycin, vancomycin and tetracycline as described in materials and methods. Analysis of antibiotic susceptibility demonstrated that *S. aureus* strains were highly resistant to penicillin (68%) and erythromycin (43%). However, the majority of S. aureus isolates were susceptible to clindamycin (89%), vancomycin (75%) and tetracycline (89%) (Fig. 3A, Table 2). Still, 25% of all *S. aureus* isolates were determined to be vancomycin resistant based on the Performance Standards for Antimicrobial Susceptibility Testing cutoffs (Weinstein and Lewis 2020a). To determine whether these are true vancomycin resistant *S. aureus*, follow-up work looking for the presence of absence of the Van cassette should be performed.

E. faecalis strains were highly susceptible to penicillin (90%), but the majority were resistant to tetracycline (73.3%) and (93.3%) had intermediate resistance to vancomycin. These data are concerning; however, no true vancomycin resistant strains were recovered therefore these strains are not likely VRE (Fig. 3B, Table 3). Of note is that most *E. faecalis* strains have intrinsic resistance to clindamycin, which was reflected in our experiment (Rams et al. 2013). GBS strains had high levels of resistance to tetracycline (83%) which is well documented almost human adapted GBS isolates (Da Cunha et al. 2014). GBS isolates also had high levels of resistance to clindamycin (61%) but were all susceptible to penicillin and vancomycin (Fig. 3C, Table 4). These data are important as clindamycin is frequently used to treat neonatal and maternal GBS infection in the case of penicillin allergy, and the Center for Disease Control (CDC) has identified clindamycin resistant GBS as a concerning threat (Frieden 2013).

Co-infection with *S. aureus* promotes *E. faecalis* and GBS persistence in diabetic wounds

Due to the high co-incidence of *E. faecalis* and GBS recovered with *S. aureus* in diabetic wound tissues, we sought to determine the consequence of polymicrobial infection *in vivo*. We utilized a murine excision wound model first described by Chong et al. for *E. faecalis* chronic wound infection and induced diabetes in mice via low-dose streptozotocin injections as previously described (Chong et al. 2017, Keogh et al. 2022, Akbari et al. 2023). Diabetic mice were wounded and infected with either *S. aureus* (LAC), *E. faecalis*, (OG1RF) or GBS (A909) alone, or co-infected with *S. aureus*. Four days post infection mice were sacrificed, and wounds were harvested for CFU enumeration. All tissues had high bacterial burden with *S. aureus* (9.0-$\log_{10}$ CFU/g), GBS at (6.9-$\log_{10}$ CFU/g) and *E. faecalis* (6.7-$\log_{10}$ CFU/g). Interestingly, the presence of *S. aureus* led to a significant increase in recovered GBS (8.9-$\log_{10}$ CFU/g) and *E. faecalis* (8.0-$\log_{10}$ CFU/g) than either species had in mono-infection. *S. aureus* burdens were slightly reduced in co-infection with GBS but remained high regardless of the presence of *E. faecalis* (Fig. 4). Collectively, these data suggest that *S. aureus* may enhance the survival of other pathogens in DFU.

Discussion

Herein, we characterized 85 Gram-positive bacterial isolates (33, *E. faecalis*, 33 *S. aureus* and 19 GBS) cultured from diabetic debridement tissues at the University of Colorado, Anschutz Med-

Table 4. Susceptibility of GBS isolates to antibiotics.

Strain	Pen (10)	Clind (2)	Erm (15)	Vanc (30)	Tet (30)
64	42	36	36	20	35
69	42	0	0	21	14
85	36	0	12	18	11
98	34	0	17	20	0
103	40	0	0	21	10
108	40	24	25	22	10
113	38	31	32	20	13
130	36	0	0	21	0
157	38	0	0	21	0
216	40	10	15	21	12
223	40	0	16	20	10
301	36	0	17	19	10
320	39	31	36	20	18
334	40	7	11	19	12
346	37	24	27	20	28
374	45	30	26	23	40
378	38	0	0	20	13
395	36	28	30	20	16

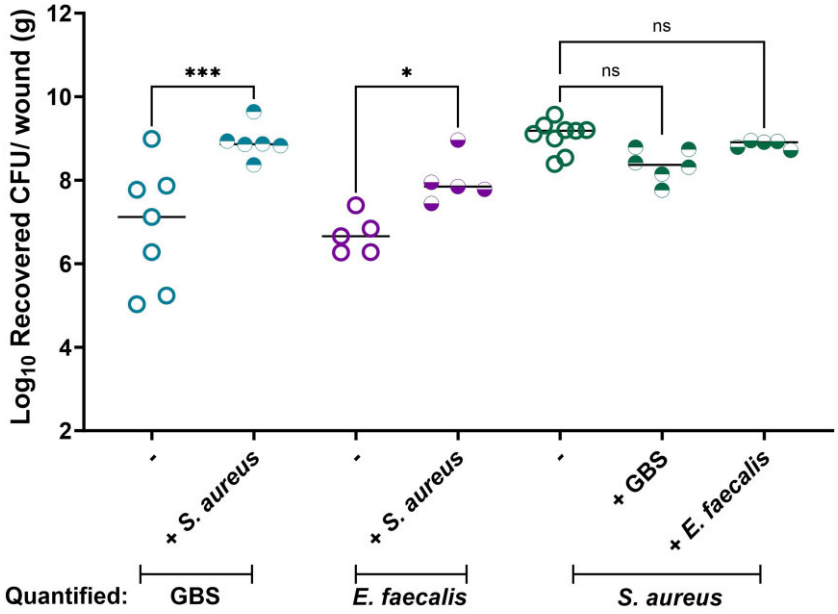

Figure 4. *S. aureus* promotes *E. faecalis* and GBS in Diabetic Wound Infection. CFU recovered from diabetic wound tissues of mice infected with bacterial strains. All animal infections proceeded for 4 days with 3 days under adhesive and sacrifice 24 hours after adhesive removal. Species quantified is indicated under the column along with whether the species was from mono-infected (-) or co-infected wounds. Significance determined by One-way ANOVA with Sidak's multiple comparisons; $^{*}P < 0.05$, and $^{***}P < 0.001$.

ical Campus. Compared to other studies, we found high levels of *E. faecalis* in debridement tissues with 33.7% of all patients having *E. faecalis* recovered (Fig. 1A). Many 16S studies show relatively low abundance of *E. faecalis* in diabetic wounds, yet other studies using culture-based methods and emerging studies on the DFU microbiome using metagenomics find *E. faecalis* is very prevalent in wound tissues (James et al. 2008, Grice et al. 2010, Gardner et al. 2013, Wolcott et al. 2016, ALbeloushi et al. 2019). It has been previously speculated by James et al. (James et al. 2008) that this species is often mis-classified in 16S analyses, although it is also possible that *E. faecalis* abundance is specific to geographic locations. *S. aureus* was also present in 33.7% of our samples, confirming findings from numerous groups that *S. aureus* is a prominent pathogen in DFU (Citron et al. 2007, Cohen et al. 2019, Kalan et al. 2019, Thurlow et al. 2020, Lavigne et al. 2021). The next two most frequently isolated species were *S. epidermidis* and GBS. We chose to focus on GBS in this paper as GBS is highly pathogenic in immunocompromised populations and has been implicated in diabetic wound infections (Sendi et al. 2008, Wolcott et al. 2016). In addition, GBS infections are increasing in adult populations, and diabetes it the most common co-morbidity associated with GBS adult disease, making adult GBS clinical isolates of high interest (Farley 2001, Sendi et al. 2008, Francois Watkins et al. 2019). While we did not focus on *S. epidermidis* isolates for this paper, it would be interesting to determine whether the *S. epidermidis* present in debridement tissue was from surrounding healthy skin, or if *S. epidermidis* has the capacity to be pathogenic in this niche (Galkowska et al. 2009, Perim et al. 2015, Wolcott et

al. 2016, Severn and Horswill 2023). It is also tempting to speculate that *S. epidermidis* may be contributing to the polymicrobial community in DFU possibly via enhancing multi-species biofilm formation (Holt et al. 2017, Severn and Horswill 2023). The most frequent Gram-negative species identified in our collection was *Pseudomonas aeruginosa* (12.2%). This frequency reflects work by Wolcott et al. which utilized 16S DNA pyrosequencing from almost 3 000 chronic wound samples and found *P. aeruginosa* in 14% of diabetic wounds (Wolcott et al. 2016). Of note is that we did not specifically focus on anaerobes in this work as all isolates were grown under aerobic conditions. However, it would be interesting and relevant to incubate plates under anaerobic conditions in a further study.

We next chose to test the antibiotic resistance profiles of our isolates, as antibiotic resistance is on the rise, and treatment failure is common in DFU (Watters et al. 2014, Rahim et al. 2017, ALbeloushi et al. 2019, Heravi et al. 2020). We utilized a range of antibiotics including antibiotics targeting cell wall synthesis (penicillin and vancomycin), protein synthesis inhibitors clindamycin, and erythromycin. *E. faecalis* isolates are intrinsically resistant to clindamycin (Rams et al. 2013), but also had high resistance to tetracycline and intermediate resistance to vancomycin (Table 3). Vancomycin resistant *Enterococci* are considered a serious threat by the Center for Disease Control (CDC) (Frieden 2013), and *Enterococci* are known to transfer antibiotic resistance genes to other species during polymicrobial infection (Noble et al. 1992, Arthur et al. 1993, Périchon and Courvalin 2009). GBS isolates were susceptible to penicillin, which is important as the identification of a penicillin resistant GBS strain should be reported to the CDC (Weinstein and Lewis 2020b). However, clindamycin resistance was found in 61% of all GBS isolates (Fig. 3, Table 4), which is a major concern as clindamycin is a last-resort antibiotic administered to pregnant adults and now considered a concerning threat by the CDC (Frieden 2013). Whether these strains emerged in individuals receiving clindamycin is unknown, as patient antibiotic history was not released for this study. Regardless, emergent clindamycin resistant GBS strains should be monitored in adult populations (Murdoch and Barth Reller 2001). *S. aureus* isolates had high resistance to penicillin and erythromycin (Fig.3, Table 2). However, the majority of isolates were susceptible to vancomycin, clindamycin and tetracycline (Fig. 3, Table 2). Of note, is that *S. aureus* strains have additional mechanisms of surviving antibiotic treatment such as antibiotic tolerance and biofilm formation (Moormeier et al. 2014, Radlinski et al. 2017, Kalan et al. 2019, Rowe et al. 2019). Finally, another consideration is that all strains were grown in liquid and solid BHI for standardization in Kirby-Bauer assays, however, it is known that antibiotic susceptibility can change dramatically with growth condition (Traub and Leonhard 1994, Olson et al. 2002). It is therefore possible that *S. aureus* strains would have high resistance to antibiotics in biofilm or another growth condition.

Here, we found that *S. aureus* was the best biofilm producing species in static biofilm formation assays (Fig. 2D). *S. aureus* biofilms have previously been implicated in antibiotic failure in chronic infection, and we find high biofilm formation amongst recovered *S. aureus* isolates (Fig. 2A, D) (Kuehl et al. 2020, Gimza and Cassat 2021). Our *E. faecalis* clinical isolates had moderate biofilm formation, which was strain dependent (Fig. 2B). Of note our recovered biomass was slightly lower than what has been shown for other *E. faecalis* chronic wound isolates (Ch'ng et al. 2022). We speculate these differences may be due to *in vitro* conditions such as use of Brain-Heart Infusion (BHI) for growth, as growth media has previously been found to influence *E. faecalis* biomass (Kristich et al. 2004). GBS isolates had virtually no biofilm forming capacity in our tested conditions (Fig. 2C). Work by D'Urzo et al. found that GBS growth at an acidic pH can enhance biofilm formation (D'Urzo et al. 2014). It is therefore possible that under different growth conditions our GBS isolates would exhibit enhanced biofilm capacity (Rosini and Margarit 2015).

Finally, we found that *S. aureus* was able to promote both *E. faecalis* and GBS persistence in diabetic wound infection *in vivo* (Fig. 4). One possible mechanism of protection is that *S. aureus* is enhancing *E. faecalis* and GBS biofilm formation *in vivo*. Interestingly, Ch'ng et al. found that *S. aureus* heme augmentation supplements *E. faecalis* biofilm formation *in vitro* (Ch'ng et al. 2022). *E. faecalis* and GBS are heme auxotrophs and require exogenous heme to undergo aerobic respiration (Lechardeur et al. 2010, Joubert et al. 2017). Therefore, it is possible that *S. aureus* derived heme drives both *E. faecalis* and GBS persistence in this niche. However, the diabetic wound microenvironment has high levels of host derived heme, which may be enough to promote *E. faecalis* and GBS aerobic respiration even without *S. aureus* present (Wagener et al. 2003, Leal and Carvalho 2022). If this hypothesis were true, further work on determining how *E. faecalis* circumvents heme toxicity or survives reactive oxygen species intermediates would be warranted (Anzaldi and Skaar 2010, Saillant et al. 2021).

An alternative hypothesis is that *S. aureus* immune evasion proteins such as the major endonuclease Nuc may be promoting the degradation of extracellular DNA released by neutrophils in a process called NETosis (Thammavongsa et al. 2013, Kavanaugh et al. 2019). NET formation is significantly greater in diabetic individuals compared to non-diabetic, and Nuc contributes to *S. aureus* virulence (Berends et al. 2010, Dowey et al. 2021). Whether *S. aureus* Nuc can assist in *E. faecalis* and GBS immune evasion is unknown, however Hsien-Neng Kao et al. found that *E. faecalis* can suppress *S. aureus* induced NET formation, suggesting that somehow these species each assist the other in immune evasion (Kao et al. 2023). Continued work on the mechanism(s) of *S. aureus* protection of these species is extremely important in trying to resolve polymicrobial wound infections.

In conclusion, our data has found that *E. faecalis*, *S. aureus* and GBS are important species in diabetic wound infections from patients in Colorado. We find that antibiotic resistant *E. faecalis* and GBS is high, and that clindamycin resistant GBS is emerging in adult infections. High biofilm formation amongst *S. aureus* clinical isolates may be contributing to *S. aureus* survival to antibiotic treatment and debridement practices. Finally, our work strongly supports *S. aureus* promotion of *E. faecalis* in polymicrobial infection and is the first to investigate polymicrobial interactions between *S. aureus* and GBS in this clinically relevant niche.

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Supplementary data

Supplementary data is available at *FEMSMC Journal* online.

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