Title:

Milieu matters: An *in vitro* wound milieu to recapitulate key features of, and probe new insights into, polymicrobial biofilms

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Abstract

Bacterial biofilms are a major cause of delayed wound healing. Consequently, the study of wound biofilms, particularly in host-relevant conditions, has gained importance. Most *in vitro* biofilm studies employ refined laboratory media to study biofilms, conditions that are not relevant to the infection state. To mimic the wound milieu, *in vitro* biofilm studies often incorporate serum or plasma in growth conditions, or employ clot or matrix-based biofilm models. While incorporating serum or plasma alone is a minimalistic approach, the more complex *in vitro* wound models are technically demanding, and poorly compatible with standard biofilm assays. Based on previous reports of clinical wound fluid composition, we have developed an *in vitro* wound milieu (IVWM) that includes, in addition to serum (to recapitulate wound fluid), matrix elements and biochemical factors. In comparison with Luria-Bertani broth and Fetal Bovine Serum (FBS), the IVWM was used to study planktonic growth and biofilm features, including interspecies interactions, of common wound pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. We demonstrate that the IVWM recapitulates widely reported *in vivo* biofilm features such as metabolic activity, increased antibiotic tolerance, 3D structure, and interspecies interactions for single- and co-species biofilms. Further, the IVWM is simple to formulate, uses laboratory-grade components, and is compatible with standard biofilm assays. Given this, it holds potential as a tractable approach to study wound biofilms under host-relevant conditions.

KEY WORDS: wounds, biofilms, host milieu, *Staphylococcus aureus, Pseudomonas aeruginosa*, polymicrobial, interspecies interactions

Introduction

Wound healing is mediated by several host factors, including inflammatory, immune and biochemical components [1,2]. Following injury, a protein-rich fluid leaks into the wound, which along with cellular and matrix elements, results in a characteristic wound milieu [3,4]. Interplay across various factors is reflected in this milieu, which is known to influence progression and outcome of the wound state [5–8]. Microbial infections are the single-most important cause of delayed wound healing [2,9]. In the wound bed, bacteria form biofilms, which are polymicrobial communities enmeshed in a self-produced extracellular matrix [10–12]. Biofilms in wounds are typically observed as microscopic bacterial aggregates on the surface of, or embedded in, host tissue [13–17], and in the presence of the wound milieu.

Hitherto, the study of biofilms in wounds has typically relied on *in vivo* animal systems or *in vitro* laboratory studies [18,19]. *In vivo* systems are limited by the fact that they are not widely-available, and pose technical and ethical challenges. On the other hand, the majority of *in vitro* biofilm studies employ laboratory media [20,21] (such as refined protein broths) to grow biofilms, and analyze effects of antimicrobial treatments. However, the composition of laboratory media is not relevant in the context of the wound infection state. Recognizing this, recent studies have incorporated serum or plasma in *in vitro* growth conditions, to more closely represent the host milieu [22–27]. This is relevant given that wound fluid has been shown to resemble the biochemical and nutrient profile of serum [22]. However, the wound milieu is more complex, and includes additional host factors and matrix elements [28]. To recapitulate this, clot and matrix-based *in vitro* wound biofilm models have been developed that more closely mimic *in vivo* conditions [22,27,29–31]. However, these models are technically demanding, low-throughput, and poorly compatible with standard biofilm assays.

In this study, we have developed a simple *in vitro* wound milieu (IVWM) that includes, in addition to serum (to recapitulate wound fluid), matrix elements such as collagen, fibrinogen and fibronectin, and host factors such as lactoferrin and lactic acid. The formulation of the milieu is based on the composition of clinical wound fluid, as reported across previous studies [32,33]. We employ this composite milieu to study planktonic growth, biofilm features, and interspecies interactions of common wound pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa* [34]. Using laboratory media (Luria-Bertani broth) and fetal bovine serum (FBS) for comparison, we demonstrate that the *in vitro* wound milieu recapitulates key *in vivo* biofilm features such as biomass formation, metabolic activity, antibiotic tolerance, three-dimensional structure, and interspecies interactions. While expectedly different from laboratory media, we find that these features are distinct from that observed with serum alone. Notably, the impact of the IVWM on co-species growth of the pathogens differs from that in serum, and similar to *in vivo* conditions appears to provide an advantage to *P. aeruginosa* [18,19]. Further,

the IVWM is easy to formulate, high-throughput, and is compatible with standard biofilm assays.

Materials and Methods

Bacterial strains and growth conditions

All experiments were carried out using fluorescently tagged strains of *Pseudomonas aeruginosa* (PAO1-pUCP18, mCherry [35]) and *Staphylococcus aureus* (Strain AH 133-pAH13, GFP [36]). These strains were a gift from Dr. Kendra Rumbaugh (Texas Tech University Health Science Center, Lubbock, TX). Selection for SA-GFP was done with 10 µg/ml erythromycin and for PAO1-mCherry with 100 µg/ml ampicillin on Luria-Bertani (B) agar plates and in overnight LB broth cultures. Strains were streaked onto LB agar (Sigma) and incubated overnight at 37°C. Isolated colonies were grown in Luria-Bertani (LB) broth overnight under shaking conditions at 37°C, unless otherwise stated.

Preparation of *in vitro* wound milieu

An *in vitro* host milieu (IVWM) was prepared with sterile fetal bovine serum (FBS) (Thermofisher Scientific) as the base component. Other components added included sterile rat tail collagen (Sigma, 50 µg/ml), lactoferrin (Sigma, 2 mg/mL stock prepared by dissolving in 1X PBS (pH 7.4) and filter sterilized), fibronectin (Sigma, 1 mg/ml stock solution prepared using autoclaved distilled water), fibrinogen (Sigma, 0.9% NaCl (prewarmed at 37°C) was used to prepare a stock solution of 10 mg/ml and filter sterilized) and lactic acid (Sigma, 11.4 M stock concentration). Collagen and lactoferrin were stored at 4°C. FBS, fibronectin and fibrinogen were stored at -20°C. Lactic acid was stored at room temperature. Components were either purchased sterile or filter sterilized using a 0.22 µm syringe filter. The components were combined in concentrations given in Table 1 to result in the final IVWM. The IVWM was freshly prepared each time, and was used immediately after use (not stored). The pH of the IVWM was measured using a pH probe and pH strip. Specific gravity of IVWM was calculated as the relative weight of IVWM compared to the weight of an equal volume of distilled water [37].

Planktonic growth

For growth curves in LB:

Overnight cultures of *S. aureus* and *P. aeruginosa* were grown in LB broth under shaking conditions at 37°C. The next day, cultures were quantified by measuring optical density (O.D.) at 600 nm with a multimode microplate reader (Tecan Infinite 200 PRO). To set up growth curves, overnight cultures were diluted in sterile LB broth (1:100) and 100 µL of the diluted culture (consisting of ~10^5 cells) was added per well, in replicates of three, to a sterile, transparent, round bottom, untreated 96-well polystyrene plate.

Uninoculated LB was used as a control. Plates were incubated in the multimode microplate reader (Tecan Infinite 200 PRO) at 37˚C with shaking in orbital mode (2 mm amplitude), and O.D. was measured at 600 nm every 30 minutes for 12-14 hours.

For growth curves in FBS and IVWM:

Growth curves in FBS and IVWM were done using overnight cultures set up in FBS. Briefly, each bacterial colony was inoculated in FBS and incubated overnight at 37˚C under shaking conditions. The next day, cultures were quantified by measuring O.D. at 600 nm with a multimode microplate reader (Tecan Infinite 200 PRO). To set up growth curves, each culture was diluted in sterile FBS or IVWM (1:100) and 100 µL of this diluted culture (consisting of ~10^5 cells) was added per well, in replicates of three, to a sterile, transparent, round bottom, untreated 96-well polystyrene plate. Uninoculated FBS or IVWM was used as a control. The plate reader was set to a temperature of 37˚C with shaking in orbital mode (2 mm amplitude), and absorbance was measured at 600 nm every 30 minutes for 12-14 hours.

For all growth curves, O.D. versus time was plotted and growth rates (doubling times) were calculated.

Colony Forming Units (CFUs)

In order to quantify the proportion of living cells in the planktonic cultures, alone and under co-species conditions, colony count assays were carried out. Overnight cultures in LB, FBS and IVWM were diluted to ~10^6 cells/ml in respective media. From these diluted cultures, 100 µL (containing ~10^5 cells) was added to 100 µL of the respective media in a fresh tube (for single-species cultures). For co-species cultures, 100 µL each of the diluted culture of *S. aureus* and *P. aeruginosa* (~10^5 cells of each strain) were added together in a single fresh tube. All conditions were set up in at least 3 replicates. Cultures were incubated under shaking conditions at 37˚C for 7 hours. Cultures were then diluted in LB to different dilutions and plated on selective media *Pseudomonas* isolation agar BioVeg (SRL Chemicals) and *Staphylococcus* Medium 110 (SRL Chemicals). Plates were incubated overnight for 24 hours at 37˚C. Based on protocols using *Staphylococcus* Medium 110, these plates required incubation for upto 36-48 hours to obtain visible countable colonies.

Biofilm formation

Overnight cultures *S. aureus* and *P. aeruginosa* were each diluted to ~10^6 cells/ml in LB, FBS and IVWM. From these diluted cultures, 50 µL (~10^5 cells) was added in multiple replicates (at least three) to a transparent, round bottom 96-well polystyrene plate, unless otherwise stated. To these wells, 50 µL of the media in which biofilm formation was to be tested was added, to maintain a constant volume of 100 µL.

For polymicrobial biofilms, individual cultures of *P. aeruginosa* and *S. aureus* were diluted to ~10^6 cells/ml (as above). From these diluted cultures, 50 µL (~10^5 cells) of each was added to wells in at least three replicates to make a total volume of 100 µL. Biofilms were allowed to grow under static conditions at 37˚C for 24 hours. These pre-formed biofilms were used to measure metabolic activity (XTT assay) and to visualize the biofilm (confocal microscopy).

XTT assay for biofilm metabolic activity

Pre-formed biofilms of *S. aureus* and *P. aeruginosa* grown for 24 hours under different conditions (as described above) were washed once with LB after removing suspended media. Menadione solution (7 mg/ml) was diluted 1:100 in sterile distilled water. A mixture of LB:XTT:Menadione in 79:20:1 ratio was freshly prepared [38], and 150 µL of this was added to each well. The plates were covered in aluminum foil and incubated for 4 hours at 37˚C (static). From each well, 100 µl was transferred to a new 96-well plate and absorbance was measured at 492 nm. In order to evaluate the presence or absence of any interactions in co-species biofilms, the co-species biofilm absorbance value was compared to an 'expected' value (calculated as the additive absorbance value of the single-species biofilms).

Antibiotic Susceptibility of Pre-formed Biofilms

Pre-formed biofilms of *S. aureus* and *P. aeruginosa* were grown for 24 hours as previously described. Briefly, overnight cultures grown in LB were diluted in the respective media (LB, FBS, IVWM) in which antibiotic susceptibility was to be tested. From these diluted cultures, ~10^5 cells were added (in triplicates) to each well of a 96-well plate, and the plate was incubated for 24 hours at 37˚C. After 24 hours, the suspended media was gently removed and the biofilms were washed once with LB. Antibiotics were diluted to varying concentrations (0-64 µg/mL for tobramycin and 0-512 µg/mL for vancomycin) in the respective media in which biofilm susceptibility was to be tested (LB, FBS, IVWM) and 100 µl of the antibiotic-media solution was added into the wells. Wells with untreated biofilms (no antibiotic added) were also included. Uninoculated media was used as a control. Plates were incubated at 37˚C for 24 hours. After 24 hours, the XTT assay was performed to quantify viability (as described above). The concentration resulting in 80% reduction in biofilm metabolic activity (representing living cells), compared to the untreated biofilms, was considered as the $MBEC_{80}$ value for that particular antibiotic.

Biofilm Visualization using Confocal Microscopy

To visualize *in situ* three-dimensional biofilm structure, 24-hour old undisturbed biofilms were set up as previously described in LB, FBS or IVWM (as single-species and co-species). Briefly, LB overnight cultures of *P. aeruginosa* and *S. aureus* were diluted in

the respective media in which biofilm structure was to be observed and mixed in a 1:1 ratio for co-species biofilms. After incubation at 37°C for 24 hours, the biofilms were directly examined with confocal laser scanning microscopy (Leica SP8 Spectral CLSM). To enable this direct visualization, biofilms were grown in 96-well, black polystyrene tissue-culture treated flat-bottom plates with a transparent bottom (Corning). These plate specifications allow visualization of the biofilm structure in the well and minimum interference of fluorescence signals from neighboring wells. The tissue-culture treatment imparts an overall negative charge to the surface, and results in a hydrophilic surface, and this treatment (achieved by corona discharge) is known to reduce the attachment of negatively charged bacteria (such as *P. aeruginosa* and *S. aureus*). In doing so, this enables the study of the role of the media conditions (host components in FBS and IVWM) in biofilm formation.

Wells were imaged using a 488 nm laser for excitation and a 496–551 nm emission filter for *S. aureus*-GFP, and a 561 nm laser for excitation and a 590–625 nm emission filter for *P. aeruginosa*-mCherry. To visualize the 3D architecture of the biofilms across the entire well, an 8x8 tile scan approach was used with an overlap of 30% and a Z-stack step size of 10 μm. The images were processed and reconstructed in the Leica Application Suite (LAS) software. Mean intensity measurements were carried out using the LAS software. Biofilm thickness was measured using ImageJ.

Results and Discussion

Development of an *in vitro* wound milieu (IVWM) that mimics host conditions

Following injury, the wound bed is bathed in protein-rich exudate, which along with additional host elements, results in a characteristic wound milieu [1,3,4,39]. The composition of wound fluid has been widely reported to resemble that of serum [32,33,40], with several *in vitro* wound studies using serum to mimic wound conditions [22,27,30,41]. To develop an *in vitro* wound milieu (IVWM), we used fetal bovine serum (FBS) as the base component, to which relevant host matrix and biochemical factors were added. Components were chosen based on previous reports of biochemical analyses across clinical wound fluids [32,33], as well as their identified roles in the wound bed.

Based on analysis of wound fluid composition [32], we decided to use 70% FBS as the base component of the IVWM, since at this concentration the levels of multiple components in serum fit into the range of values for that component in wound fluid [32,42]. While the concentrations of several biochemical factors in serum and wound fluid are similar [32], the wound milieu is also characterized by the presence of additional host-derived biochemical factors. In the initial inflammatory state of wound

repair, the wound microenvironment is characterized by increased levels of lactate and lactoferrin. Lactate in the wound microenvironment is a result of local tissue damage, and increased concentrations are reflected in the local milieu [43–45]. While the levels of lactoferrin in the plasma and serum of healthy individuals is typically low [46–49], in the presence of microbial infections [50], increased levels are reported in the wound milieu. In addition to possessing antimicrobial properties [51–53], lactoferrin has also been shown to be important for wound re-epithelialization [54]. Given this, lactate and lactoferrin were included in the IVWM at concentrations that mimicked host conditions (Table 1).

The IVWM composition also included relevant matrix components such as fibrinogen, fibronectin and collagen [55]. While fibrinogen is present in wound tissue [56,57] and plasma [58], it is notably absent from serum. On the other hand, the matrix protein fibronectin is typically present at high concentrations in serum or plasma [59], but undergoes degradation under inflammatory wound conditions [60–62]. To mimic this, fibronectin was added to the IVWM, to resemble lower concentrations as relevant to the wound milieu [57,62–64]. Given its critical role as an extracellular matrix protein in the wound bed [22,23,27,30,65–68], collagen was also included in the IVWM (Table 1). At the concentration of collagen added, the IVWM was in liquid form (not a gel) resembling the wound fluid milieu.

The pH of the formulated IVWM was determined to be 5.25 and specific gravity was 0.966, which is similar to that measured in wound fluid [33,69,70].

Table 1: Composition of the *in vitro* wound milieu (IVWM) and rationale for inclusion of components

Components	Final concentration in IVWM	Rationale	References
FBS	70 %	The major component and base of IVWM; at this concentration, FBS accounts for several components in wound fluid	[32,33,40,42]
Lactic acid	11-12 mM	Host biochemical factor released in response to tissue damage; based on levels detected in wounds immediately following injury	[43–45]
Lactoferrin	20-30 µg/ml	Host biochemical factor increased in the presence of microbes	[46–54]
Fibrinogen	200-400 µg/ml	Host matrix protein, notably absent from serum, present in the wound milieu	[56–58]
Fibronectin	30-60 µg/ml	Host matrix protein, present in lower concentrations in the wound milieu	[57,59–64]
Collagen	10-12 µg/ml	Host matrix protein, critical component of the	[22,23,27,30,65–

		wound bed	68]

Planktonic growth of *P. aeruginosa* and *S. aureus* in laboratory media, fetal bovine serum and the *in vitro* wound milieu (IVWM)

To determine the effects of the IVWM on the planktonic growth of *P. aeruginosa* and *S. aureus* (single-species), we performed growth curves in the IVWM, and in LB medium and FBS for comparison (Figure 1). Luria-Bertani (LB) broth is widely-used to study biofilms under *in vitro* conditions [20,38,71–73], however, its composition (refined yeast extract and tryptone) poorly mimics the infection state. To recapitulate factors relevant to the wound milieu, several biofilm models incorporate fetal bovine serum (FBS), in varying concentrations, into growth conditions [22,26,74,75]. Given that concentrations of FBS employed across different studies range widely, we chose to use the maximum possible FBS concentration (100%) for comparison.

When examined in LB medium, *P. aeruginosa* and *S. aureus* showed typical growth curves, with doubling times of 30±1 minutes and 28±1 minutes respectively, in accordance with previous reports [76,77]. In 100% FBS, *P. aeruginosa* was observed to grow slower as compared to growth in LB medium, with a doubling time of 56±7 minutes. On the other hand, *S. aureus* showed markedly impaired growth in FBS. In the IVWM, consisting of 70% FBS with additional matrix and host components, *P. aeruginosa* was observed to double every 43±7 minutes, which is faster than that seen in FBS alone, and cultures were also observed to enter exponential phase earlier. Similar to that observed in FBS, *S. aureus* demonstrated significantly impaired growth in the IVWM.

As compared with LB media, we find that the planktonic growth of *P. aeruginosa* and *S. aureus* are notably different under growth conditions that incorporate host factors. In the presence of FBS, both pathogens displayed markedly slower growth; components in serum are known to impair the growth of *S. aureus* [78–80]. However, when grown in IVWM, containing serum at a concentration that recapitulates wound fluid (70% FBS), and with additional matrix and biochemical factors, a greater difference in growth across the two species was observed. The IVWM was observed to better support the growth of *P. aeruginosa* (as compared with FBS alone), while *S. aureus* showed significantly impaired growth. This indicates that in the IVWM, *P. aeruginosa* has a distinct growth advantage in planktonic state, as compared with its co-pathogen *S. aureus.*

Interspecies interactions between planktonic *P. aeruginosa* and *S. aureus* under different conditions

We next wanted to understand the effects of the IVWM, in comparison with LB and FBS, on interspecies interactions between planktonic *P. aeruginosa* and *S. aureus*. For

this, *P. aeruginosa* and *S. aureus* were co-cultured under planktonic conditions in LB, FBS and IVWM (at a starting ratio of 1:1, with ~10^5 CFU of each strain), and after 7 hours, were plated on selective media to obtain viable counts of each bacterial species. In LB media, both *S. aureus* and *P. aeruginosa* showed a decrease in viable cells in co-species conditions (Figures 2A and B), the number of viable cells recovered for *S. aureus* was ~3% and ~50% for *P. aeruginosa*, compared to when grown alone. Notably, this inhibitory effect was more predominantly observed for *S. aureus* (Figure 2B), which is in accordance to previously published reports of *P. aeruginosa* outcompeting *S. aureus* in LB medium [18,20,21,31,81,82].

In FBS and IVWM, *S. aureus* demonstrated the same trend, with reduced recovery of viable cells under co-species conditions (Figure 2). Notably, in the IVWM, viable *S. aureus* cells recovered from planktonic co-cultures were very few, representing less than 1% of the viable *S. aureus* cells in single-species cultures. However, under these conditions, the recovery of viable *P. aeruginosa* was similar or better under co-species conditions compared to that when grown alone. This effect was more pronounced in the IVWM (Figure 2A and B), with a 46-112% increased recovery under co-species conditions. It is important to note that even when grown alone, the IVWM is observed to better support the growth of *P. aeruginosa* as compared to *S. aureus* (Figures 1 and 2A). Further, in co-species conditions, interspecies interactions could result in *P. aeruginosa* inhibiting the growth of *S. aureus*, resulting in a net effect where *P. aeruginosa* significantly outcompetes *S. aureus*. This overall effect is similar to that seen under *in vivo* conditions, where in spite of co-existence between the two common wound pathogens, *P. aeruginosa* is observed to outcompete *S. aureus* in wound infections [18,31,81,83–85].

Biofilm formation, metabolic activity and interspecies interactions of *P. aeruginosa* and *S. aureus* biofilms under different conditions

To understand the effects of the IVWM on biofilm formation, metabolic activity and interspecies interactions of *P. aeruginosa* and *S. aureus* biofilms, 24-hour biofilms (alone and as co-species) were grown in microtiter plates in IVWM, and in LB and FBS for comparison. Biofilms were then examined with the XTT assay (Figure 3), which measures bacterial metabolic activity, and is therefore a proxy for living cells in the biofilm.

As previously reported across several studies [20,71], after 24 hours of growth in LB medium, *P. aeruginosa* and *S. aureus* formed biofilms that displayed the presence of metabolic activity (Figure 3). This is also observed in the presence of host components, as in FBS and IVWM, where both *P. aeruginosa* and *S. aureus* formed metabolically-active biofilms (Figure 2). Several host components, including serum, plasma, and matrix factors such as collagen, fibrinogen, fibronectin, have been shown to support the

formation of *P. aeruginosa* [86–88] and *S. aureus* [89–97] biofilms. It is important to note that in our study, though *S. aureus* biofilms in FBS showed the presence of metabolic activity, when examined visually they were seen as a thin layer on the bottom and sides of the microtiter well (data not shown). This could possibly be the reason that certain previous studies using biomass staining protocols (and not metabolic activity) [23,80,96], report reduced or absent biofilm formation in the presence of FBS or plasma.

When *P. aeruginosa and* S. *aureus* biofilms were grown together in LB medium, the metabolic activity of the co-species biofilms is significantly reduced as compared with that expected (the additive value of single-species biofilms of both pathogens) (Figure 3), with the observed values being 50±12% of the expected value. Interspecies interactions between *P. aeruginosa* and *S. aureus* in biofilms are widely reported [18,20,21,34,98–100], and are influenced by several factors, including host matrix and chemical factors. In the presence of host components, as in FBS and the IVWM, the metabolic activity of co-species biofilms was similarly less than that expected (Figure 3), observed as 74±15% and 64±15% of the expected value respectively. This indicates that, even in the presence of host components, co-species biofilms of *P. aeruginosa* and *S. aureus* demonstrate the possible effects of interspecies interactions.

Overall, our results indicate that the IVWM supports biofilm formation and metabolic activity of *P. aeruginosa* and *S. aureus* biofilms, and indicates the presence of interspecies biofilm interactions.

Antibiotic susceptibility of *P. aeruginosa* and *S. aureus* biofilms in the *in vitro* wound milieu (IVWM)

To study the effects of the IVWM of the antibiotic susceptibility of *P. aeruginosa* and *S. aureus* biofilms, pre-formed biofilms were grown for 24 hours (also in LB and FBS), and exposed to varying concentrations of tobramycin and vancomycin respectively, and assayed for antibiotic susceptibility (Table 2). The Minimum Biofilm Eradication Concentration (MBEC) was considered to be the concentration resulting in 80% of biofilm eradication ($MBEC_{80}$).

For biofilms grown in LB media, the $MBEC_{80}$ of *P. aeruginosa* for tobramycin was similar to the previously reported value of 1 µg/mL [38]. On the other hand, the $MBEC_{80}$ of pre-formed *P. aeruginosa* biofilms grown in FBS and IVWM was determined to be 8 µg/mL, an 8-fold increase compared to LB. While the presence of serum is known to increase antimicrobial tolerance [101–103], owing to the serum-binding properties of certain antibiotics, including binding of tobramycin and serum albumin [104], this increased tolerance could also be due to the inherent properties of the biofilm formed under these conditions. The similarity of $MBEC_{80}$ in FBS and IVWM (which contains 70% FBS) could possibly indicate the dominant role of serum in influencing the antibiotic tolerance of *P. aeruginosa* biofilms.

When grown in LB media, pre-formed *S. aureus* biofilms displayed increased tolerance, even at higher concentrations of vancomycin ($MBEC_{80}$>512 µg/mL). This is in accordance with previously reported values, across different laboratory media conditions [73,101,105,106]. Interestingly, we find that biofilms grown in FBS demonstrate increased susceptibility to vancomycin, with a 32-fold reduction in the $MBEC_{80}$ (8 µg/mL) in FBS, as compared with LB. Visually, *S. aureus* biofilms in FBS were observed as a very thin layer on the round-bottom surface of the microtiter wells data not shown). Despite having a high metabolic activity, we speculate that inhibitory effects of serum on *S. aureus* [80], along with the formation of thin biofilms, leads to this increased susceptibility.

When grown in IVWM, 24-hour old *S. aureus* biofilms showed increased tolerance to vancomycin, with an $MBEC_{80}$ greater than 512 µg/mL. This value is 32-fold higher than that observed for biofilms grown in FBS alone, which is important to note given that the IVWM comprises 70% FBS, along with additional matrix and host factors. Host factors and matrix components, such as plasma [90] and fibronectin [107], have been shown to reduce the susceptibility of *S. aureus* to vancomycin. On the other hand, this effect could also be due to host components such as lactoferrin, known to have antimicrobial properties against *S. aureus* [108–112]; however, its role in the presence of multiple components such as in the IVWM, or even in serum alone, is not known.

Wound biofilms are known to display increased tolerance to antimicrobial treatments, and our results show that the host and matrix factors present in the IVWM recapitulates the increased antibiotic tolerance of both *P. aeruginosa* and *S. aureus* biofilms.

Table 2: Minimum Biofilm Eradication Concentration (MBEC)* for *P. aeruginosa* and *S. aureus* under different conditions (*The concentration resulting in 80% reduction in biofilm metabolic activity (by the XTT assay) was considered as the $MBEC_{80}$ value for that particular antibiotic)

	LB	**FBS**	**IVWM**
***P. aeruginosa* (PAO1)** Tobramycin	1 µg/mL	8 µg/mL	8 µg/mL
***S. aureus* (AH 133)** Vancomycin	> 512 µg/mL	16 µg/mL	> 512 µg/mL

3D Biofilm structure of *P. aeruginosa* and *S. aureus* biofilms under different conditions

In order to visualize the 3D structure of the biofilms under different conditions, undisturbed 24-hour old biofilms of *P. aeruginosa* (PAO1-mCherry) and *S. aureus* (AH133-GFP) were imaged (as single and mixed-species) using confocal microscopy via the tilescan approach. The tilescan approach uses an 8x8 grid set to scan the entire well, and thereby enables visualization of the 3D structure across the entire biofilm. To reduce the role of surface attachment [113], given that polystyrene is not a biotic surface, and better understand the role of the media conditions in biofilm structure, biofilms were grown in tissue-culture treated 96 well plates.

When grown alone in LB media, *S. aureus* formed dense biofilms after 24 hours, with an average thickness of 92±3 μm (Figure 4A). In FBS, *S. aureus* biofilms were observed to be thinner, as compared with LB, with an average thickness of 63±4 μm (Figure 4A and C). In the IVWM, *S. aureus* formed biofilms that were 36-56% thinner than that seen in FBS and LB, with an average thickness of 40±3 μm (Figure 4A). Notably, this indicates that studies in refined protein-based media, such as LB broth, significantly overestimate biofilm thickness.

When grown in LB media, *P. aeruginosa* displayed minimal biofilm formation after 24 hours (Figure 4B). This is possibly due to the tissue culture treatment of the wells, which is known to reduce the surface attachment of bacteria. On the other hand, in FBS and IVWM, 24-hour old *P. aeruginosa* biofilms were observed as mat-like structures; the average thickness was 41±2 μm in FBS, and 39±1 μm in the IVWM (Figure 4B). Notably, this highlights the role of host components (as in FBS and in the IVWM) in *P. aeruginosa* biofilm formation.

Despite the fact that *S. aureus* biofilms are thinner in the IVWM as compared to that in FBS (Figure 4A), the $MBEC_{80}$ in the IVWM is higher (>512 μg/mL) than that measured in FBS (16 μg/mL) (Table 2). Previous studies have correlated the thickness of biofilms to the antibiotic resistance observed, with thicker biofilms typically observed to display increased antibiotic tolerance, possibly due to reduced antibiotic penetration and the production of deeper gradients [114,115]. However, the presence of serum along with additional host components such as collagen, has been observed to increase antibiotic tolerance for *P. aeruginosa* and *S. aureus* aggregates [23,115–119].

3D Biofilm structure of *P. aeruginosa* and *S. aureus* co-species biofilms

To examine co-species biofilms, *P. aeruginosa* (PAO1-mCherry) and *S. aureus* (AH133-GFP) were inoculated in a 1:1 ratio, followed by *in situ* visualization of 24-hour biofilms (Figure 5). In LB media, under co-species conditions, *P. aeruginosa* displayed minimal biofilm formation (similar to that observed in single-species state) (Figure 5A and B). Notably, under co-species conditions, *S. aureus* was also seen to form sparse biofilms. Based on known interspecies interactions between the two pathogens, it is likely that under co-species conditions in LB media, inoculated *P. aeruginosa* results in killing of *S. aureus*, and therefore significantly reduced *S. aureus* biofilms are observed [85,120].

In FBS, the two pathogens were observed to form a robust co-species biofilm (Figure 5A and B), consisting of a thick mat of *S. aureus* (thickness 20±2 μm) and *P. aeruginosa* (thickness 27±1 μm). This indicates that presence of host components (as in FBS), enables the coexistence of *S. aureus* and *P. aeruginosa* biofilms, which is in accordance with previous work that reports the 'rescue' of *S. aureus* biofilms [81], and protection from killing by *P. aeruginosa*, in the presence of host components.

However, this is notably different in the presence of additional host components, that more closely mimic the wound milieu. In the IVWM, the co-species biofilm shows a distinct predominance of *P. aeruginosa* (average thickness 29±3 μm), with the presence of *S. aureus* significantly less as compared to that observed in FBS (Figure 5A and B). This could result from the presence of additional host factors countering the protective effect against *P. aeruginosa* killing, or that certain factors in the IVWM, such as lactoferrin exhibit antimicrobial activity [53,121,122], including against *S. aureus* biofilms [51,112,123–125].

Overall, our results indicate that the IVWM supports the formation of dense, mat-like biofilms of *P. aeruginosa* and *S. aureus* when grown alone, and under co-species conditions the polymicrobial biofilm shows a distinct predominance of *P. aeruginosa*. This is similar to *in vivo* conditions where in spite of well-established co-existence of the two pathogens, *P. aeruginosa* appears to outcompete *S. aureus* [18,19].

Conclusions

Based on previous reports of clinical wound fluid composition, we have developed an *in vitro* wound milieu (IVWM), consisting of fetal bovine serum, with additional host matrix and biochemical factors. Our results indicate that the IVWM recapitulates key *in vivo* biofilm features such as biomass formation, metabolic activity, antibiotic tolerance, three-dimensional structure, and interspecies interactions. Notably, under both planktonic and biofilm conditions, the IVWM supported a distinct predominance of *P. aeruginosa*. This is important to explore further, particularly in clinical and *in vivo* conditions, given that *P. aeruginosa*-*S. aureus* interactions have been largely studied in *in vitro* systems [20,21,120,123]. Notably, we find that this is distinct from that observed in serum alone, underscoring the role of additional matrix and biochemical factors.

While the IVWM recapitulates key factors in the wound microenvironment, it is certainly not representative of all aspects of the wound milieu. However, its ease of formulation, use of widely-available components, and compatibility with standard biofilm assays, lends itself well for further adaptations and modifications, such as the inclusion of additional factors such as commensal microbes, glucose, matrix-metalloproteinases [126–130]. Given this, the IVWM holds potential as a tractable approach to study wound biofilms under host-relevant conditions, particularly for high-throughput applications such as time-lapse biofilm studies and combination antimicrobial approaches. In doing so, it could bridge the gap between reductionist *in vitro* systems and *in vivo* models, and provide more human-relevant insights in laboratory biofilm studies.

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Author Contributions:

Snehal Kadam: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Data curation, Visualization, Writing original draft, Editing draft. **M Vandana:** Conceptualization, Methodology, Investigation, Validation. **Devyani Bhide:** Investigation, Validation. **Rutuja Ugale:** Investigation. **Utkarsha Tikhole:** Investigation. **Karishma S Kaushik:** Conceptualization, Methodology, Formal analysis, Project administration, Supervision, Writing original draft, Editing draft, Funding acquisition.

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References:

[1]   Gonzalez ACDO, Andrade ZDA, Costa TF, Medrado ARAP. Wound healing - A literature review. An Bras Dermatol 2016;91:614–20. https://doi.org/10.1590/abd1806-4841.20164741.

[2]   Guo S, DiPietro LA. Factors Affecting Wound Healing. J Dent Res 2010;89:219–29. https://doi.org/10.1177/0022034509359125.

[3]   Junker JPE, Caterson EJ, Eriksson E. The microenvironment of wound healing. J Craniofac Surg 2013;24:12–6. https://doi.org/10.1097/SCS.0b013e31827104fb.

[4]   Uluer ET, Vatansever HS, Kurt FŐ. Wound Healing and Microenvironment.

Wound Heal., Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2017, p. 67–77. https://doi.org/10.1002/9781119282518.ch5.

[5] Manuela B, Milad K, Anna-Lena S, Julian-Dario R, Ewa Klara S. Acute and Chronic Wound Fluid Inversely Influence Wound Healing in an in-Vitro 3D Wound Model. J Tissue Repair Regen 2017;1:1. https://doi.org/10.14302/issn.2640-6403.jtrr-17-1818.

[6] Drinkwater SL, Smith A, Burnand KG. What Can Wound Fluids Tell Us About the Venous Ulcer Microenvironment? Int J Low Extrem Wounds 2002;1:184–90. https://doi.org/10.1177/153473460200100307.

[7] Staiano-Coico L, Higgins P, Schwartz SB, Zimm A, Goncalves J. Wound fluids: a reflection of the state of healing. Ostomy Wound Manage 2000.

[8] Schneider LA, Korber A, Grabbe S, Dissemond J. Influence of pH on wound-healing: A new perspective for wound-therapy? Arch Dermatol Res 2007;298:413–20. https://doi.org/10.1007/s00403-006-0713-x.

[9] Edwards R, Harding KG. Bacteria and wound healing. Curr Opin Infect Dis 2004. https://doi.org/10.1097/00001432-200404000-00004.

[10] Percival SL, McCarty SM, Lipsky B. Biofilms and Wounds: An Overview of the Evidence. Adv Wound Care 2015;4:373–81. https://doi.org/10.1089/wound.2014.0557.

[11] Cooper RA, Bjarnsholt T, Alhede M. Biofilms in wounds: A review of present knowledge. J Wound Care 2014;23:570–82. https://doi.org/10.12968/jowc.2014.23.11.570.

[12] Bjarnsholt T. The role of bacterial biofilms in chronic infections. APMIS Suppl 2013;121:1–51. https://doi.org/10.1111/apm.12099.

[13] Fazli M, Bjarnsholt T, Kirketerp-Møller K, Jørgensen B, Andersen AS, Krogfelt KA, et al. Nonrandom distribution of Pseudomonas aeruginosa and Staphylococcus aureus in chronic wounds. J Clin Microbiol 2009. https://doi.org/10.1128/JCM.01395-09.

[14] Bjarnsholt T, Kirketerp-Møller K, Jensen PØ, Madsen KG, Phipps R, Krogfelt K, et al. Why chronic wounds will not heal: a novel hypothesis. Wound Repair Regen 2008;16:2–10. https://doi.org/10.1111/j.1524-475X.2007.00283.x.

[15] Johani K, Malone M, Jensen S, Gosbell I, Dickson H, Hu H, et al. Microscopy visualisation confirms multi-species biofilms are ubiquitous in diabetic foot ulcers. Int Wound J 2017;14:1160–9. https://doi.org/10.1111/iwj.12777.

[16] Davis SC, Ricotti C, Cazzaniga A, Welsh E, Eaglstein WH, Mertz PM. Microscopic and physiologic evidence for biofilm-associated wound colonization in vivo. Wound Repair Regen 2008;16:23–9. https://doi.org/10.1111/j.1524-475X.2007.00303.x.

[17] Kirketerp-Møller K, Jensen P, Fazli M, Madsen KG, Pedersen J, Moser C, et al.

Distribution, organization, and ecology of bacteria in chronic wounds. J Clin Microbiol 2008;46:2717–22. https://doi.org/10.1128/JCM.00501-08.

[18] Hotterbeekx A, Kumar-Singh S, Goossens H, Malhotra-Kumar S. In vivo and In vitro interactions between Pseudomonas aeruginosa and Staphylococcus spp. Front Cell Infect Microbiol 2017;7:106. https://doi.org/10.3389/fcimb.2017.00106.

[19] Pastar I, Nusbaum AG, Gil J, Patel SB, Chen J, Valdes J, et al. Interactions of Methicillin Resistant Staphylococcus aureus USA300 and Pseudomonas aeruginosa in Polymicrobial Wound Infection. PLoS One 2013;8:e56846. https://doi.org/10.1371/journal.pone.0056846.

[20] Wijesinghe G, Dilhari A, Gayani B, Kottegoda N, Samaranayake L, Weerasekera M. Influence of Laboratory Culture Media on in vitro Growth, Adhesion, and Biofilm Formation of Pseudomonas aeruginosa and Staphylococcus aureus. Med Princ Pract 2019;28:28–35. https://doi.org/10.1159/000494757.

[21] Cendra M del M, Blanco-Cabra N, Pedraz L, Torrents E. Optimal environmental and culture conditions allow the in vitro coexistence of Pseudomonas aeruginosa and Staphylococcus aureus in stable biofilms. Sci Rep 2019;9. https://doi.org/10.1038/s41598-019-52726-0.

[22] Slade EA, Thorn RMS, Young A, Reynolds DM. An in vitro collagen perfusion wound biofilm model; with applications for antimicrobial studies and microbial metabolomics. BMC Microbiol 2019;19:310. https://doi.org/10.1186/s12866-019-1682-5.

[23] Werthén M, Henriksson L, Jensen PØ, Sternberg C, Givskov M, Bjarnsholt T. An in vitro model of bacterial infections in wounds and other soft tissues. APMIS 2010;118:156–64. https://doi.org/10.1111/j.1600-0463.2009.02580.x.

[24] Bowler PG, Jones SA, Walker M, Parsons D. Microbicidal Properties of a Silver-Containing Hydrofiber® Dressing Against a Variety of Burn Wound Pathogens. J Burn Care Rehabil 2004;25:192–6. https://doi.org/10.1097/01.BCR.0000112331.72232.1B.

[25] Said J, Dodoo CC, Walker M, Parsons D, Stapleton P, Beezer AE, et al. An in vitro test of the efficacy of silver-containing wound dressings against Staphylococcus aureus and Pseudomonas aeruginosa in simulated wound fluid. Int J Pharm 2014;462:123–8. https://doi.org/10.1016/j.ijpharm.2013.12.037.

[26] Leonhard M, Zatorska B, Moser D, Tan Y, Schneider-Stickler B. Evaluation of combined growth media for in vitro cultivation of oropharyngeal biofilms on prosthetic silicone. J Mater Sci Mater Med 2018;29:45. https://doi.org/10.1007/s10856-018-6051-7.

[27] Price BL, Lovering AM, Bowling FL, Dobson CB. Development of a novel collagen wound model to simulate the activity and distribution of antimicrobials in soft tissue during diabetic foot infection. Antimicrob Agents Chemother 2016;60:6880–9. https://doi.org/10.1128/AAC.01064-16.

[28] Scalise A, Bianchi A, Tartaglione C, Bolletta E, Pierangeli M, Torresetti M, et al. Microenvironment and microbiology of skin wounds: The role of bacterial biofilms and related factors. Semin Vasc Surg 2015;28:151–9. https://doi.org/10.1053/j.semvascsurg.2016.01.003.

[29] Sun Y, Dowd SE, Smith E, Rhoads DD, Wolcott RD. In vitro multispecies Lubbock chronic wound biofilm model. Wound Repair Regen 2008;16:805–13. https://doi.org/10.1111/j.1524-475X.2008.00434.x.

[30] Thaarup IC, Bjarnsholt T. Current In Vitro Biofilm-Infected Chronic Wound Models for Developing New Treatment Possibilities. Adv Wound Care 2021;10:91–102. https://doi.org/10.1089/wound.2020.1176.

[31] DeLeon S, Clinton A, Fowler H, Everett J, Horswill AR, Rumbaugh KP. Synergistic interactions of Pseudomonas aeruginosa and Staphylococcus aureus in an In vitro wound model. Infect Immun 2014;82:4718–28. https://doi.org/10.1128/IAI.02198-14.

[32] Trengove NJ, Langton SR, Stacey MC. Biochemical analysis of wound fluid from nonhealing and healing chronic leg ulcers. Wound Repair Regen 1996;4:234–9. https://doi.org/10.1046/j.1524-475X.1996.40211.x.

[33] Cutting KF. Wound exudate: composition and functions. Br J Community Nurs 2003;8:S4–9. https://doi.org/10.12968/bjcn.2003.8.sup3.11577.

[34] Alves PM, Al-Badi E, Withycombe C, Jones PM, Purdy KJ, Maddocks SE. Interaction between Staphylococcus aureus and Pseudomonas aeruginosa is beneficial for colonisation and pathogenicity in a mixed biofilm. Pathog Dis 2018;76:fty003. https://doi.org/10.1093/femspd/fty003.

[35] Irie Y, Borlee BR, O'Connor JR, Hill PJ, Harwood CS, Wozniak DJ, et al. Self-produced exopolysaccharide is a signal that stimulates biofilm formation in Pseudomonas aeruginosa. Proc Natl Acad Sci U S A 2012;109:20632–6. https://doi.org/10.1073/pnas.1217993109.

[36] Malone CL, Boles BR, Lauderdale KJ, Thoendel M, Kavanaugh JS, Horswill AR. Fluorescent reporters for Staphylococcus aureus. J Microbiol Methods 2009;77:251–60. https://doi.org/10.1016/j.mimet.2009.02.011.

[37] Stauffer E, Dolan JA, Newman R. Chemistry and Physics of Fire and Liquid Fuels. Fire Debris Anal., Elsevier; 2008, p. 85–129. https://doi.org/10.1016/b978-012663971-1.50008-7.

[38] Kaushik KS, Stolhandske J, Shindell O, Smyth HD, Gordon VD. Tobramycin and bicarbonate synergise to kill planktonic Pseudomonas aeruginosa, but antagonise to promote biofilm Survival. Npj Biofilms Microbiomes 2016;2:16006. https://doi.org/10.1038/npjbiofilms.2016.6.

[39] White R, Cutting KF. Modern exudate management: a review of wound treatments. World Wide Wounds 2006.

[40] Karayiannakis AJ, Zbar A, Polychronidis A, Simopoulos C. Serum and drainage

fluid vascular endothelial growth factor levels in early surgical wounds. Eur Surg Res 2003;35:492–6. https://doi.org/10.1159/000073388.

[41] Brackman G, Coenye T. In vitro and in vivo biofilm wound models and their application. Adv Exp Med Biol 2016:15–32. https://doi.org/10.1007/5584_2015_5002.

[42] James TJ, Hughes MA, Cherry GW, Taylor RP. Simple biochemical markers to assess chronic wounds. Wound Repair Regen 2000;8:264–9. https://doi.org/10.1046/j.1524-475X.2000.00264.x.

[43] Hunt TK, Conolly WB, Aronson SB, Goldstein P. Anaerobic metabolism and wound healing: An hypothesis for the initiation and cessation of collagen synthesis in wounds. Am J Surg 1978;135:328–32. https://doi.org/10.1016/0002-9610(78)90061-2.

[44] Löffler M, Zieker D, Weinreich J, Löb S, Königsrainer I, Symons S, et al. Wound fluid lactate concentration: A helpful marker for diagnosing soft-tissue infection in diabetic foot ulcers? Preliminary findings. Diabet Med 2011;28:175–8. https://doi.org/10.1111/j.1464-5491.2010.03123.x.

[45] Britland S, Ross-Smith O, Jamil H, Smith AG, Vowden K, Vowden P. The lactate conundrum in wound healing: Clinical and experimental findings indicate the requirement for a rapid point-of-care diagnostic. Biotechnol Prog 2012;28:917–24. https://doi.org/10.1002/btpr.1561.

[46] Birgens HS. Lactoferrin in plasma measured by an ELISA technique: Evidence that plasma lactoferrin is an indicator of neutrophil turnover and bone marrow activity in acute leukaemia. Scand J Haematol 1985;34:326–31. https://doi.org/10.1111/j.1600-0609.1985.tb00757.x.

[47] Bennett RM, Kokocinski T. Lactoferrin Content of Peripheral Blood Cells. Br J Haematol 1978;39:509–21. https://doi.org/10.1111/j.1365-2141.1978.tb03620.x.

[48] Venge P, Foucard T, Henriksen J, Håkansson L, Kreuger A. Serum-levels of lactoferrin, lysozyme and myeloperoxidase in normal, infection-prone and leukemic children. Clin Chim Acta 1984;136:121–30. https://doi.org/10.1016/0009-8981(84)90283-3.

[49] Malmquist J, Thorell JI, Wollheim FA. Lactoferrin and Lysozyme in Arthritic Exudates. Acta Med Scand 1977;202:313–8. https://doi.org/10.1111/j.0954-6820.1977.tb16834.x.

[50] Takayama Y. Lactoferrin and its role in wound healing. Springer Netherlands; 2013. https://doi.org/10.1007/978-94-007-2467-9.

[51] Ammons MC, Copié V. Lactoferrin: a bioinspired, anti-biofilm therapeutic. Biofouling 2013;29:443–55. https://doi.org/10.1080/08927014.2013.773317.

[52] Quintieri L, Caputo L, Monaci L, Cavalluzzi MM, Denora N. Lactoferrin-Derived Peptides as a Control Strategy against Skinborne Staphylococcal Biofilms. Biomedicines 2020;8:323. https://doi.org/10.3390/biomedicines8090323.

[53] Singh PK, Parsek MR, Greenberg EP, Welsh MJ. A component of innate immunity prevents bacterial biofilm development. Nature 2002;417:552–5. https://doi.org/10.1038/417552a.

[54] Tang L, Wu JJ, Ma Q, Cui T, Andreopoulos FM, Gil J, et al. Human lactoferrin stimulates skin keratinocyte function and wound re-epithelialization. Br J Dermatol 2010;163:38–47. https://doi.org/10.1111/j.1365-2133.2010.09748.x.

[55] Chen ZJ, Yang JP, Wu BM, Tawil B. A novel three-dimensional wound healing model. J Dev Biol 2014;2:198–209. https://doi.org/10.3390/jdb2040198.

[56] Ko YP, Flick MJ. Fibrinogen Is at the Interface of Host Defense and Pathogen Virulence in Staphylococcus aureus Infection. Semin Thromb Hemost 2016;42:408–21. https://doi.org/10.1055/s-0036-1579635.

[57] Clark RAF, An JQ, Greiling D, Khan A, Schwarzbauer JE. Fibroblast Migration on Fibronectin Requires Three Distinct Functional Domains. J Invest Dermatol 2003;121:695–705. https://doi.org/10.1046/j.1523-1747.2003.12484.x.

[58] Kaur J, Jain A. Fibrinogen 2020.

[59] To WS, Midwood KS. Plasma and cellular fibronectin: Distinct and independent functions during tissue repair. Fibrogenes Tissue Repair 2011;4:1–17. https://doi.org/10.1186/1755-1536-4-21.

[60] Bruhn HD, Heimburger N. Factor-VIII-Related Antigen and Cold-Insoluble Globulin in Leukemias and Carcinomas. Pathophysiol Haemost Thromb 1976;5:189–92. https://doi.org/10.1159/000214134.

[61] Ai S, Kuzuya M, Iguchi A. Neutrophil elastase in pressure ulcer fluid degrades fibronectin in the exudates. Geriatr Gerontol Int 2004;4:141–5. https://doi.org/10.1111/j.1447-0594.2004.00243.x.

[62] Hayman EG, Ruoslahti E. Distribution of fetal bovine serum fibronectin and endogenous rat cell fibronectin in extracellular matrix. J Cell Biol 1979. https://doi.org/10.1083/jcb.83.1.255.

[63] Wysocki A. Wound fluids and the pathogenesis of chronic wounds. J WOCN 1996;23:283–90. https://doi.org/10.1016/s1071-5754(96)90047-9.

[64] Wysocki AB, Grinnell F. Fibronectin profiles in normal and chronic wound fluid. Lab Investig 1990.

[65] Kadam S, Nadkarni S, Lele J, Sakhalkar S, Mokashi P, Kaushik KS. Bioengineered Platforms for Chronic Wound Infection Studies: How Can We Make Them More Human-Relevant? Front Bioeng Biotechnol 2019;7. https://doi.org/10.3389/fbioe.2019.00418.

[66] Birkenhauer E, Neethirajan S, Weese JS. Collagen and hyaluronan at wound sites influence early polymicrobial biofilm adhesive events. BMC Microbiol 2014;14:191. https://doi.org/10.1186/1471-2180-14-191.

[67] Velnar T, Bailey T, Smrkolj V. The Wound Healing Process: an Overview of the Cellular and Molecular Mechanisms. vol. 37. 2009.

[68] Tarlton JF, Bailey AJ, Crawford E, Jones D, Moore K, Harding KD. Prognostic value of markers of collagen remodeling in venous ulcers. Wound Repair Regen 1999;7:347–55. https://doi.org/10.1046/j.1524-475X.1999.00347.x.

[69] Nagoba BS, Suryawanshi NM, Wadher B, Selkar S. Acidic environment and wound healing: A review. Wounds 2015.

[70] Metcalf DG, Haalboom M, Bowler PG, Gamerith C, Sigl E, Heinzle A, et al. Elevated wound fluid pH correlates with increased risk of wound infection. Wound Med 2019;26:100166. https://doi.org/10.1016/j.wndm.2019.100166.

[71] She P, Wang Y, Liu Y, Tan F, Chen L, Luo Z, et al. Effects of exogenous glucose on Pseudomonas aeruginosa biofilm formation and antibiotic resistance. Microbiologyopen 2019;8. https://doi.org/10.1002/mbo3.933.

[72] Seidl K, Goerke C, Wolz C, Mack D, Berger-Bächi B, Bischoff M. Staphylococcus aureus CcpA affects biofilm formation. Infect Immun 2008;76:2044–50. https://doi.org/10.1128/IAI.00035-08.

[73] Chen X, Thomsen TR, Winkler H, Xu Y. Influence of biofilm growth age, media, antibiotic concentration and exposure time on Staphylococcus aureus and Pseudomonas aeruginosa biofilm removal in vitro. BMC Microbiol 2020;20:264. https://doi.org/10.1186/s12866-020-01947-9.

[74] Gries CM, Biddle T, Bose JL, Kielian T, Lo DD. Staphylococcus aureus fibronectin binding protein a mediates biofilm development and infection. Infect Immun 2020;88. https://doi.org/10.1128/IAI.00859-19.

[75] Crabbé A, Liu Y, Matthijs N, Rigole P, De La Fuente-Nùñez C, Davis R, et al. Antimicrobial efficacy against Pseudomonas aeruginosa biofilm formation in a three-dimensional lung epithelial model and the influence of fetal bovine serum. Sci Rep 2017;7. https://doi.org/10.1038/srep43321.

[76] Langevin PB, Gravenstein N, Doyle TJ, Roberts SA, Skinner S, Langevin SO, et al. Growth of Staphylococcus aureus in Diprivan and Intralipid: Implications on the pathogenesis of infections. Anesthesiology 1999;91:1394–400. https://doi.org/10.1097/00000542-199911000-00032.

[77] LaBauve AE, Wargo MJ. Growth and laboratory maintenance of Pseudomonas aeruginosa. Curr Protoc Microbiol 2012;0 6:Unit. https://doi.org/10.1002/9780471729259.mc06e01s25.

[78] Harriott MM, Noverr MC. Candida albicans and Staphylococcus aureus form polymicrobial biofilms: Effects on antimicrobial resistance. Antimicrob Agents Chemother 2009;53:3914–22. https://doi.org/10.1128/AAC.00657-09.

[79] Ehrenkranz NJ, Elllott DF, Zarco R. Serum Bacteriostasis of Staphylococcus aureus. 1971.

[80] Abraham NM, Jefferson KK. A low molecular weight component of serum inhibits biofilm formation in staphylococcus aureus. Microb Pathog 2010;49:388–91. https://doi.org/10.1016/j.micpath.2010.07.005.

[81] Smith AC, Rice A, Sutton B, Gabrilska R, Wessel AK, Whiteley M, et al. Albumin inhibits Pseudomonas aeruginosa quorum sensing and alters polymicrobial interactions. Infect Immun 2017;85. https://doi.org/10.1128/IAI.00116-17.

[82] Hutchison JB, Rodesney CA, Kaushik KS, Le HH, Hurwitz DA, Irie Y, et al. Single-cell control of initial spatial structure in biofilm development using laser trapping. Langmuir 2014;30:4522–30. https://doi.org/10.1021/la500128y.

[83] Korgaonkar A, Trivedi U, Rumbaugh KP, Whiteley M. Community surveillance enhances Pseudomonas aeruginosa virulence during polymicrobial infection. Proc Natl Acad Sci U S A 2013;110:1059–64. https://doi.org/10.1073/pnas.1214550110.

[84] Dalton T, Dowd SE, Wolcott RD, Sun Y, Watters C, Griswold JA, et al. An In Vivo Polymicrobial Biofilm Wound Infection Model to Study Interspecies Interactions. PLoS One 2011;6:e27317. https://doi.org/10.1371/journal.pone.0027317.

[85] Filkins LM, Graber JA, Olson DG, Dolben EL, Lynd LR, Bhuju S, et al. Coculture of Staphylococcus aureus with Pseudomonas aeruginosa drives S. aureus towards fermentative metabolism and reduced viability in a cystic fibrosis model. J Bacteriol 2015;197:2252–64. https://doi.org/10.1128/JB.00059-15.

[86] Di Martino P. Effects of antibiotics on adherence of Pseudomonas aeruginosa and Pseudomonas fluorescens to human fibronectin. Chemotherapy 2001;47:344–9. https://doi.org/10.1159/000048541.

[87] Gagnière H, Di Martino P. Effects of antibiotics on Pseudomonas aeruginosa NK125502 and Pseudomonas fluorescens MF0 biofilm formation on immobilized fibronectin. J Chemother 2004;16:244–7. https://doi.org/10.1179/joc.2004.16.3.244.

[88] Rebiere-Huët J, Di Martino P, Hulen C. Inhibition of Pseudomonas aeruginosa adhesion to fibronectin by PA-IL and monosaccharides: Involvement of a lectin-like process. Can J Microbiol 2004;50:303–12. https://doi.org/10.1139/w04-015.

[89] Kwiecinski J, Kahlmeter G, Jin T. Biofilm formation by staphylococcus aureus isolates from skin and soft tissue infections. Curr Microbiol 2015;70:698–703. https://doi.org/10.1007/s00284-014-0770-x.

[90] Cardile AP, Sanchez CJ, Samberg ME, Romano DR, Hardy SK, Wenke JC, et al. Human plasma enhances the expression of Staphylococcal microbial surface components recognizing adhesive matrix molecules promoting biofilm formation and increases antimicrobial tolerance in Vitro. BMC Res Notes 2014;7:457. https://doi.org/10.1186/1756-0500-7-457.

[91] Mccourt J, O'Halloran DP, Mccarthy H, O'Gara JP, Geoghegan JA. Fibronectin-binding proteins are required for biofilm formation by community-associated

methicillin-resistant Staphylococcus aureus strain LAC. FEMS Microbiol Lett 2014;353:157–64. https://doi.org/10.1111/1574-6968.12424.

[92] Herrmann M, Vaudaux PE, Pittet D, Auckenthaler R, Lew PD, Perdreau FS, et al. Fibronectin, Fibrinogen, and Laminin Act as Mediators of Adherence of Clinical Staphylococcal Isolates to Foreign Material. J Infect Dis 1988;158:693–701. https://doi.org/10.1093/infdis/158.4.693.

[93] Akiyama H, Ueda M, Kanzaki H, Tada J, Jirô Arata. Biofilm formation of Staphylococcus aureus strains isolated from impetigo and furuncle: Role of fibrinogen and fibrin. J Dermatol Sci 1997;16:2–10. https://doi.org/10.1016/S0923-1811(97)00611-7.

[94] Herman-Bausier P, El-Kirat-Chatel S, Foster TJ, Geoghegan JA, Dufrêne YF. Staphylococcus aureus fibronectin-binding protein a mediates cell- cell adhesion through low-affinity homophilic bonds. MBio 2015;6:1–10. https://doi.org/10.1128/mBio.00413-15.

[95] Thompson KM, Abraham N, Jefferson KK. Staphylococcus aureus extracellular adherence protein contributes to biofilm formation in the presence of serum. FEMS Microbiol Lett 2010;305:143–7. https://doi.org/10.1111/j.1574-6968.2010.01918.x.

[96] Millenbaugh N, Watters C, Burton T, Kirui D. Enzymatic degradation of in vitro Staphylococcus aureus biofilms supplemented with human plasma. Infect Drug Resist 2016;9:71. https://doi.org/10.2147/IDR.S103101.

[97] Proctor RA, Mosherj DF, Olbrantzj PJ. Fibronectin Binding to Staphylococcus aureus. vol. 257. 1982.

[98] Magalhães AP, Jorge P, Pereira MO. Pseudomonas aeruginosa and Staphylococcus aureus communication in biofilm infections: insights through network and database construction. Crit Rev Microbiol 2019;45:712–28. https://doi.org/10.1080/1040841X.2019.1700209.

[99] Beaudoin T, Yau YCW, Stapleton PJ, Gong Y, Wang PW, Guttman DS, et al. Staphylococcus aureus interaction with Pseudomonas aeruginosa biofilm enhances tobramycin resistance. Npj Biofilms Microbiomes 2017;3. https://doi.org/10.1038/s41522-017-0035-0.

[100] Armbruster CR, Wolter DJ, Mishra M, Hayden HS, Radey MC, Merrihew G, et al. Staphylococcus aureus protein a mediates interspecies interactions at the cell surface of Pseudomonas aeruginosa. MBio 2016;7. https://doi.org/10.1128/mBio.00538-16.

[101] Li J, Xie S, Ahmed S, Wang F, Gu Y, Zhang C, et al. Antimicrobial activity and resistance: Influencing factors. Front Pharmacol 2017;8:364. https://doi.org/10.3389/fphar.2017.00364.

[102] Morrison JM, Chojnacki M, Fadrowski JJ, Bauza C, Dunman PM, Dudas RA, et al. Serum-Associated Antibiotic Tolerance in Pediatric Clinical Isolates of

Pseudomonas aeruginosa. J Pediatric Infect Dis Soc 2019;XX:1–9. https://doi.org/10.1093/jpids/piz094.

[103] Blanchard C, Barnett P, Perlmutter J, Dunman PM. Identification of acinetobacter baumannii serum-associated antibiotic efflux pump inhibitors. Antimicrob Agents Chemother 2014;58:6360–70. https://doi.org/10.1128/AAC.03535-14.

[104] Raza M, Wei Y, Jiang Y, Ahmad A, Raza S, Ullah S, et al. Molecular mechanism of tobramycin with human serum albumin for probing binding interactions: Multi-spectroscopic and computational approaches. New J Chem 2017;41:8203–13. https://doi.org/10.1039/c7nj02054f.

[105] Mottola C, Matias CS, Mendes JJ, Melo-Cristino J, Tavares L, Cavaco-Silva P, et al. Susceptibility patterns of Staphylococcus aureus biofilms in diabetic foot infections. BMC Microbiol 2016;16. https://doi.org/10.1186/s12866-016-0737-0.

[106] Antunes ALS, Bonfanti JW, Perez LRR, Pinto CCF, de Freitas ALP, Macedo AJ, et al. High vancomycin resistance among biofilms produced by Staphylococcus species isolated from central venous catheters. Mem Inst Oswaldo Cruz 2011;106:51–5. https://doi.org/10.1590/S0074-02762011000100008.

[107] Chuard C. Decreased susceptibility to antibiotic killing of a stable small colony variant of Staphylococcus aureus in fluid phase and on fibronectin- coated surfaces. J Antimicrob Chemother 1997;39:603–8. https://doi.org/10.1093/jac/39.5.603.

[108] Aguila A, Herrera AG, Morrison D, Cosgrove B, Perojo A, Montesinos I, et al. Bacteriostatic activity of human lactoferrin against Staphylococcus aureus is a function of its iron-binding properties and is not influenced by antibiotic resistance . FEMS Immunol Med Microbiol 2001;31:145–52. https://doi.org/10.1111/j.1574-695x.2001.tb00511.x.

[109] Jenssen H, Hancock REW. Antimicrobial properties of lactoferrin. Biochimie 2009;91:19–29. https://doi.org/10.1016/j.biochi.2008.05.015.

[110] Sinha M, Kaushik S, Kaur P, Sharma S, Singh TP. Antimicrobial lactoferrin peptides: The hidden players in the protective function of a multifunctional protein. Int J Pept 2013;2013. https://doi.org/10.1155/2013/390230.

[111] Stallmann HP, Faber C, Bronckers ALJJ, De Blieck-Hogervorst JMA, Brouwer CPJM, Amerongen AVN, et al. Histatin and lactoferrin derived peptides: Antimicrobial properties and effects on mammalian cells. Peptides 2005;26:2355–9. https://doi.org/10.1016/j.peptides.2005.05.014.

[112] Flores-Villaseñor H, Canizalez-Román A, Reyes-Lopez M, Nazmi K, De La Garza M, Zazueta-Beltrán J, et al. Bactericidal effect of bovine lactoferrin, LFcin, LFampin and LFchimera on antibiotic-resistant Staphylococcus aureus and Escherichia coli. BioMetals, vol. 23, Biometals; 2010, p. 569–78. https://doi.org/10.1007/s10534-010-9306-4.

[113] Gross M, Cramton SE, Go¨tz F, Go¨tz G, Peschel A. Key Role of Teichoic Acid

Net Charge in Staphylococcus aureus Colonization of Artificial Surfaces. Infect Immun 2001;69:3423–6. https://doi.org/10.1128/IAI.69.5.3423-3426.2001.

[114] Hall CW, Mah T-F. Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. FEMS Microbiol Rev 2017;41:276–301. https://doi.org/10.1093/femsre/fux010.

[115] Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. Antibiotic resistance of bacterial biofilms. Int J Antimicrob Agents 2010;35:322–32. https://doi.org/10.1016/j.ijantimicag.2009.12.011.

[116] Lebeaux D, Ghigo J-M, Beloin C. Biofilm-Related Infections: Bridging the Gap between Clinical Management and Fundamental Aspects of Recalcitrance toward Antibiotics. Microbiol Mol Biol Rev 2014;78:510–43. https://doi.org/10.1128/mmbr.00013-14.

[117] Tewari A, Jain B, Dhamannapatil PS, Saxena MK. Biofilm Resistance to Antimicrobial Agents and Novel Approaches to Combat Biofilm Mediated Resistance in Bacteria. vol. 14. 2018.

[118] Batoni G, Maisetta G, Esin S. Antimicrobial peptides and their interaction with biofilms of medically relevant bacteria. Biochim Biophys Acta - Biomembr 2016;1858:1044–60. https://doi.org/10.1016/j.bbamem.2015.10.013.

[119] Sharma D, Misba L, Khan AU. Antibiotics versus biofilm: An emerging battleground in microbial communities. Antimicrob Resist Infect Control 2019;8:1–10. https://doi.org/10.1186/s13756-019-0533-3.

[120] Tognon M, Köhler T, Luscher A, Van Delden C. Transcriptional profiling of Pseudomonas aeruginosa and Staphylococcus aureus during in vitro co-culture. BMC Genomics 2019;20:30. https://doi.org/10.1186/s12864-018-5398-y.

[121] Sharbafi R, Moradian F, Rafeiei A. Evaluation of antimicrobial activity of lactoferrin against P. aeruginosa and E. coli growth. J Babol Univ Med Sci 2016. https://doi.org/10.22088/jbums.18.7.19.

[122] Kamiya H, Ehara T, Matsumoto T. Inhibitory effects of lactoferrin on biofilm formation in clinical isolates of Pseudomonas aeruginosa. J Infect Chemother 2012;18:47–52. https://doi.org/10.1007/s10156-011-0287-1.

[123] Gounani Z, Şen Karaman D, Venu AP, Cheng F, Rosenholm JM. Coculture of P. aeruginosa and S. aureus on cell derived matrix - An in vitro model of biofilms in infected wounds. J Microbiol Methods 2020;175:105994. https://doi.org/10.1016/j.mimet.2020.105994.

[124] Bruni N, Capucchio MT, Biasibetti E, Pessione E, Cirrincione S, Giraudo L, et al. Antimicrobial activity of lactoferrin-related peptides and applications in human and veterinary medicine. Molecules 2016;21:752. https://doi.org/10.3390/molecules21060752.

[125] Nibbering PH, Ravensbergen E, Welling MM, Van Berkel LA, Van Berkel PHC, Pauwels EKJ, et al. Human Lactoferrin and Peptides Derived from Its N Terminus

Are Highly Effective against Infections with Antibiotic-Resistant Bacteria. Infect Immun 2001;69:1469–76. https://doi.org/10.1128/IAI.69.3.1469-1476.2001.

[126] Armstrong DG, Jude EB. The role of matrix metalloproteinases in wound healing. J Am Podiatr Med Assoc 2002;92:12–8.

[127] Ravanti L, Kähäri VM. Matrix metalloproteinases in wound repair (review). Int J Mol Med 2000;6:391–407.

[128] Ayuk SM, Abrahamse H, Houreld NN. The Role of Matrix Metalloproteinases in Diabetic Wound Healing in relation to Photobiomodulation. J Diabetes Res 2016;2016:2897656. https://doi.org/10.1155/2016/2897656.

[129] Brem H, Tomic-Canic M. Cellular and molecular basis of wound healing in diabetes. J Clin Invest 2007. https://doi.org/10.1172/JCI32169.

[130] Tomic-Canic M, Burgess JL, O'Neill KE, Strbo N, Pastar I. Skin Microbiota and its Interplay with Wound Healing. Am J Clin Dermatol 2020;21:36–43. https://doi.org/10.1007/s40257-020-00536-w.

Figure Legends

Figure 1: IVWM supports the planktonic growth of *P. aeruginosa*, but not of *S. aureus*. Planktonic growth curves of *Pseudomonas aeruginosa* (PAO1) and *Staphylococcus aureus* (AH133) were performed in Luria-Bertani broth (LB), Fetal Bovine Serum (FBS) and the *in vitro* host milieu (IVWM). Optical density (OD 600) was measured at intervals of 30 minutes for 12 hours. Error bars represent SEM, n=3.
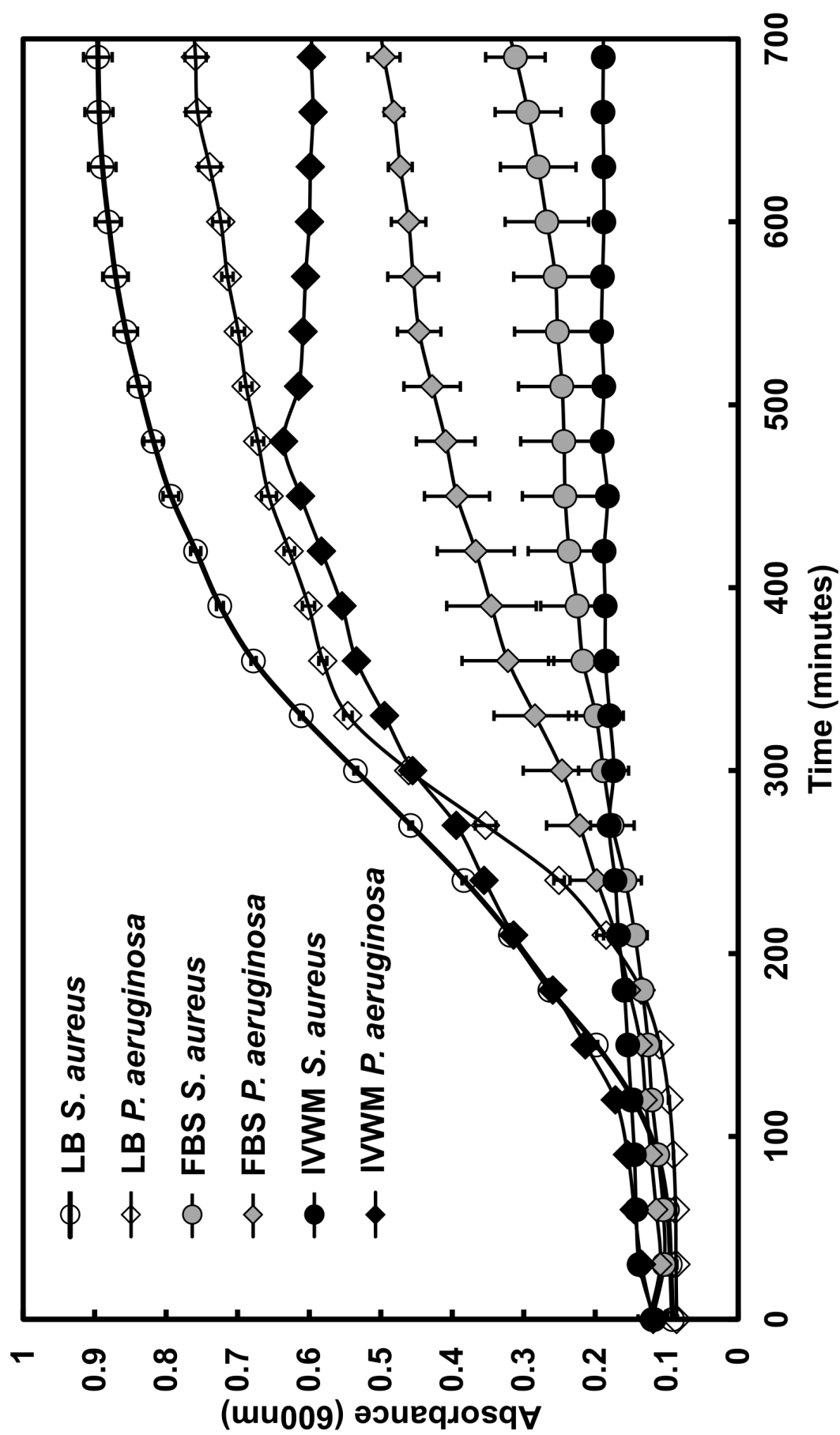
Figure 2: In the IVWM, interspecies interactions between planktonic *P. aeruginosa* and *S. aureus* result in *P. aeruginosa* significantly outcompeting *S. aureus*. Viable counts of planktonic *P. aeruginosa* and *S. aureus* grown under co-species conditions in Luria-Bertani broth (LB), Fetal Bovine Serum (FBS) and the *in vitro* wound milieu (IVWM) were quantified using the Colony Forming Units (CFU) technique. (A) CFUs of planktonic single-species and co-species cultures of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in LB, FBS and IVWM (B) Ratio of CFU/mL of the colony counts in co-species to that in single-species planktonic cultures of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in LB, FBS and IVWM. Error bars represent SEM, n=4.
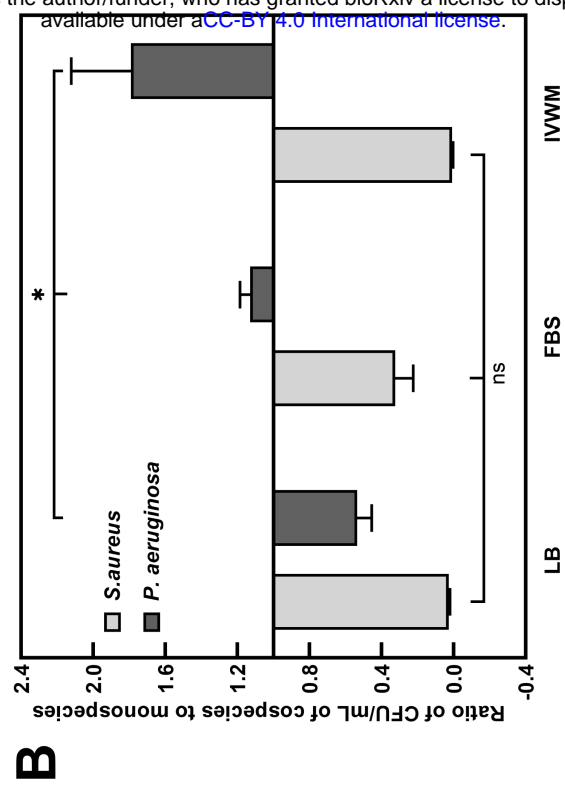
Figure 3: IVWM supports the formation of metabolically-active biofilms of *P. aeruginosa* and *S. aureus*, and indicates the presence of interspecies interactions. Pre-formed 24-hour biofilms of *Pseudomonas aeruginosa* and *Staphylococcus aureus* were quantified for metabolic activity, in single-species and co-species state, in LB, FBS and IVWM, by the XTT assay. Error bars represent SEM, n=3.

Figure 4: 3D biofilm structure of *P. aeruginosa* and *S. aureus* single-species biofilms in IVWM are distinct from that in LB and FBS. Tile-scan confocal microscopy showing 3D structure of **(A)** *P. aeruginosa* (PAO1-mCherry) and **(B)** *S. aureus* (AH133-GFP) biofilms in LB, FBS and IVWM. To reduce the role of surface attachment, and better explore the role of the different media conditions, biofilms were grown in tissue-culture treated microtiter plates.
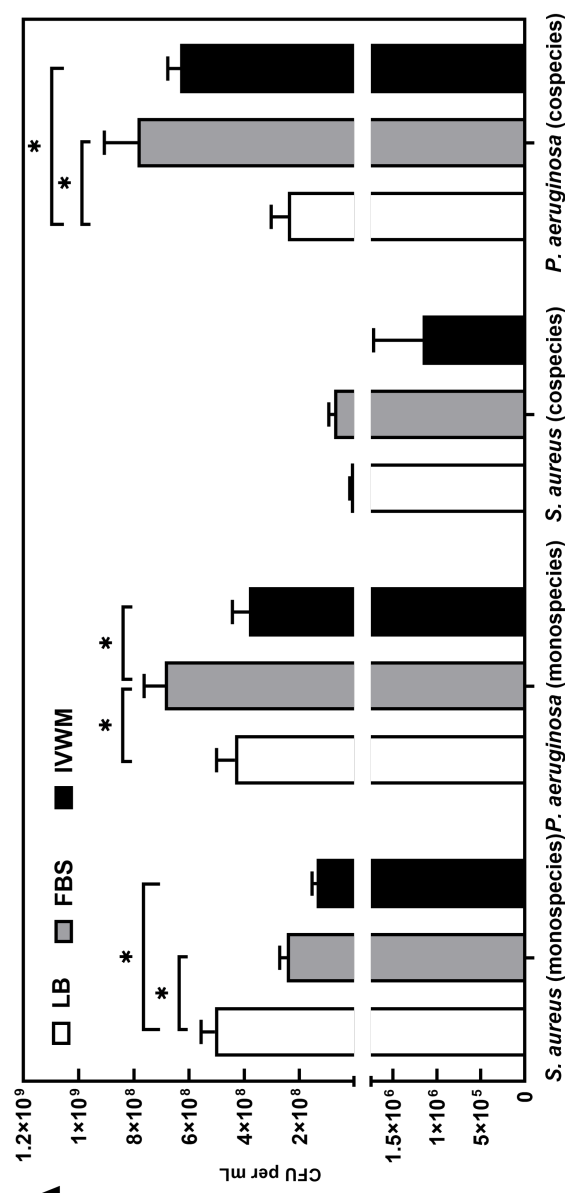
Figure 5: 3D biofilm structure of co-species *P. aeruginosa* and *S. aureus* biofilms in IVWM shows distinct predominance of *P. aeruginosa*. (A) Tile-scan confocal microscopy of *P. aeruginosa* (PAO1-mCherry) and *S. aureus* (AH133-GFP) co-species biofilms in LB, FBS and IVWM. **(B)** Mean intensity of fluorescence (representing PAO1-mCherry and SA-GFP) across the Z-height of the biofilm (with the bottom as Z = 0). Error bars represent SEM, n=3.

A

B