Polymicrobial Conditions Affect Antibiotic Susceptibility in Clinically Relevant Bacterial Species

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Abstract

Chronic wounds, defined as those which remain open and inflamed for greater than six weeks, are a major area of clinical concern. Resulting in thousands of amputations per year and billions of dollars spent globally in treatment, chronic wounds are notoriously difficult to successfully treat. Two hallmarks of chronic wounds are that they are thought to harbor biofilm-associated bacteria and tend to be polymicrobial. While the research literature has repeatedly demonstrated the effects of biofilms on wound persistence and the changes to the efficacy of antibiotics, few studies have demonstrated what effect the polymicrobial condition has on the antibiotic tolerance of bacteria. To further explore this, four species of clinically relevant wound pathogens (*Pseudomonas aeruginosa, Acinetobacter baumanii, Staphylococcus aureus, and Enterococcus faecalis*) were tested in mono- and polymicrobial conditions using the current gold-standard methods for determining antibiotic susceptibility. Noticeable differences in antibiotic tolerance were observed in the polymicrobial condition, including both increased and decreased susceptibility, depending on the antibiotic used. Our data demonstrate that the current clinical methods used for testing antibiotic susceptibility can generate results that are not representative of the infection environment, which may contribute to treatment failure and persistence of polymicrobial infections.

Keywords: Minimum inhibotry concentration (MIC), antimicrobial susceptibility testing (AST), wound, diagnostics, synergism

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Introduction

While a variety of chronic conditions affect Americans each year, less prominent, yet still clinically-impactful conditions manage to slip under the radar of the popular consciousness; one of these is chronic wounds. Often occurring in diabetic patients, the bed-ridden, and those with vascular diseases, chronic wounds are currently an area of major clinical concern- costing billions of dollars every year to treat and resulting in thousands of amputations in the United States alone.[1, 2] The most common definition of these wounds is those which remain open and in a prolonged state of inflammation for greater than six weeks.[3] Standard treatment protocols do exist for the management of chronic wounds, including the use of oral antibiotics, cleaning of the wound via physical means (commonly called debridement), and even the use of strong, topical antibiotics, yet as seen in the treatment costs listed above, those protocols remain of mixed efficacy.[4] To illustrate how these wounds occur, the case of a proto-typical diabetic patient will be used. For patients with diabetes, a gradual loss of sensation in the limbs, termed diabetic peripheral neuropathy, can often occur.[5] When combined with decreased circulation to the extremities, such a patient may receive a wound on their foot, and due to the lack of sensation, the patient will remain largely unaware of the existence of the damage, continuing with their normal activities of daily life. [5,6] As the wound progresses in its infection and tissue necrosis, it may become apparent to the patient or to caregivers who are assisting them, and the patient will be brought in for treatment. The open wound will be treated with antibiotics, but the wound will often persist, and refuse to heal or reduce its inflammatory condition.[5] At that point, debridement, specialized wound dressings, and further antibiotic treatment will be attempted, but the wound will often continue to remain infected, and amputation of the affected digit, appendage, or limb may be necessary to ensure the patient's survival.[5] As mentioned above, many thousands of patients receive amputations on the basis of chronic wound treatment failure every year, and so this area of study is a critical one to improving health outcomes in the United States.[1, 2] A question remains, though. Why do the current clinical best-practices in the treatment of these wounds fail so often? While it may be obvious that there exists some gap in the understanding of these wounds, it is by no means clear *where* that gap exists. In order to elucidate these gaps, it is imperative to first understand what is currently known by both the scientific and clinical communities around the structure and composition of these wounds.

It is widely understood that two major features characterize chronic wounds.[4] The first is that the bacteria within the wounds form biofilms. Biofilms are defined by the secreted extracellular polysaccharide (EPS) matrices that bacteria form to protect against dislodgement, to better control their microenvironment, and to serve as a mechanical barrier to external conditions.[7] More simply put, biofilms are a structure by which bacteria protect themselves and adhere to their infection site.[7, 8] For example, the necessity of brushing one's teeth with an abrasive compound such as a toothbrush arises from the biofilms that oral bacteria form as they grow; this can be observed from the off-white

plaque that is physically removed from teeth during this cleaning. Without the abrasive removal of biofilm, the bacteria would continue to adhere to the surface of the teeth, with poor outcomes for one's oral health.[9] The second well known characteristic of chronic wounds is that they tend to be polymicrobial- meaning that an infected wound is colonized by multiple species of bacteria, and almost without exception contain multiple species of pathogenic bacteria.[10, 11] It is more commonly thought that infections consist of a single species of bacteria or infectious agent (fulfilling Koch's postulates), but the research literature has shown extensively that, once within a biofilm, numerous species of bacteria can happily coexist. Next-generation sequencing (an advanced method of genetic analysis) of wound samples has revealed that up to several hundred different species of bacteria may infect chronic wounds, though not necessarily all simultaneously (demonstrating a dynamic and ever-changing nature of a chronic wound).[12] This polymicrobial environment can also promote synergistic interactions among bacteria within the wound. Synergistic interactions in bacteria are those in which multiple bacterial species inhabiting the same environment creates differences in behavior from a single species condition.[13, 14] This often occurs by the upregulation of virulence factors among bacteria in those communities, and is associated with poorer patient outcomes.[15] The synergistic interactions within the polymicrobial biofilm environment are also known to increase both the antimicrobial tolerance and resistance in constituent microbes through increased rates of horizontal gene transfer and differential gene expression.[8, 16-19] Tolerance to antibiotics is characterized by transient changes in a bacterial population in response to their environment, usually via differential gene expression (DGE), which allows them to survive when exposed to antibiotics (Fig. 1).[20] This change in metabolism as a result of the synergistic interaction-caused DGE often decreases the efficacy of antibiotics by changing the availability of target sites or processes, which leads to bacteria surviving a normally lethal treatment.[20] Resistance, on the other hand, involves the acquisition of specific genes that allow the bacteria to counter the effect of the antibiotic (Fig. 1), and is a 'permanent' change in their genetic makeup, often associated with horizontal gene transfer (HGT).[21] HGT is the method by which antimicrobial resistance genes are shared within a mature bacterial population, and the polymicrobial environment favors these interactions.[12] Using *in vitro* research models, biofilms have also been shown to play a major role in the antibiotic tolerance of pathogens via the mechanical barrier that the biofilm forms, as well as the increased DGE that affects the efficacy of therapeutic compounds of the bacteria within the biofilm. The effect of biofilm formation alone can result in a one thousand fold decrease in antibiotic susceptibility due to the conditions within that microenvironment.[20, 22, 23] While this has been extensively demonstrated in the literature, most studies investigating the contribution of a biofilm to changes in susceptibility have focused on monomicrobial, or single-species, bacterial suspensions.[24-32] Because of this, our aim was to determine what effects a polymicrobial condition has on the antimicrobial susceptibility of a group of bacteria- an effect independent of biofilm formation- and one that is currently a gap in the research knowledge, especially

when compared to the large body of research on the effects of biofilm formation on antibiotic susceptibility.

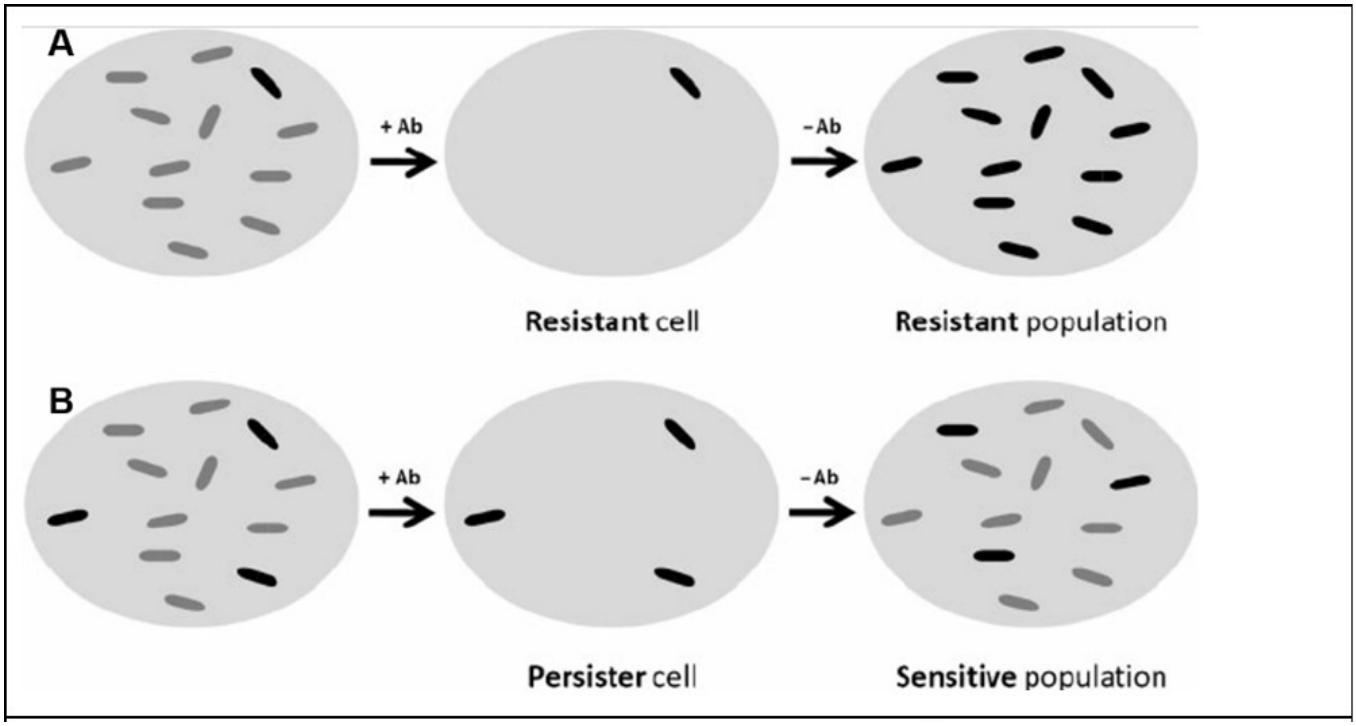
Methods and Materials

Species and Strains

Four species were chosen for use in the polymicrobial culture: *Acinetobacter baumanii* (AB), *Pseudomonas aeruginosa* (PA), *Staphylococcus aureus* (SA), and *Enterococcus faecalis* (EF). Each of these species is both a common infector of chronic wounds and a member of the ESKAPE pathogen family, which are a leading cause of nosocomial infections.[17,37] Clinical and Laboratory Standards Institute (CLSI) -recommended quality control strains of *A. baumanii* (ATCC® 19606), *P. aeruginosa* (ATCC® 27853), *E. faecalis* (ATCC® 29212), and *S. aureus* (ATCC® 29213) were used.[38] These strains are pan-susceptible and were used to ensure the reliability of the established MIC breakpoints provided by CLSI while eliminating any possibility of antibiotic resistance genes affecting the results of the experiments.

Figure 1

Antibiotic Resistance Versus Tolerance. (Taken from [21])



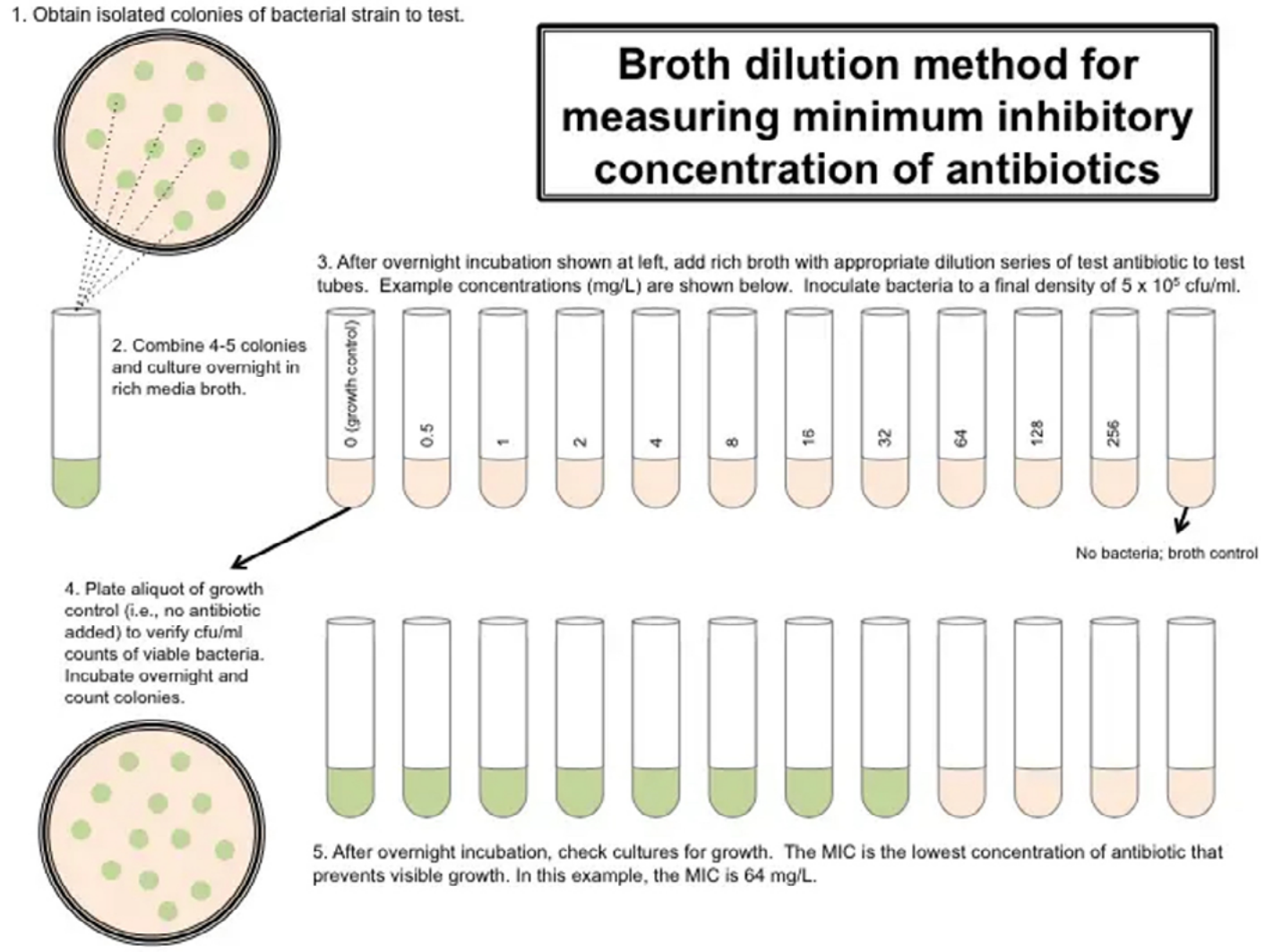
Note. A microbial population (confined by a light-grey ellipse) initially consists of mainly antibiotic-sensitive cells (dark-grey). (A) In addition, the population may also contain resistant cells (black), resulting from a permanent change at the genetic level. After antibiotic treatment (+Ab), only resistant cells remain. Upon regrowth (-Ab), the entire population is composed of resistant individuals. (B) Alternatively, the population may contain persister cells (black), resulting from a reversible phenotypic switch to a tolerant state. After antibiotic treatment, only persister cells remain. Upon regrowth, the population will exhibit the same sensitivity as the original population.

Minimum Inhibitory Concentration (MIC)

Our experiments were conducted in accordance with the CLSI M100 and M7 guidelines for determining antibiotic susceptibility.[38, 39] MICs were determined by first conducting mono-species MICs in 96-well plates and the results were cross-checked with the CLSI's established MIC breakpoints for each species used stratified by drug. Bacterial cryo-stocks were grown overnight in lysogeny (LB) broth (Thermo Fisher Scientific, Hampton, NH) , then the above CLSI protocol was followed. Mono-species MICs were conducted first to ensure that the results were in accordance with published guidelines as an internal control. Then, each of the four species was grown in lysogeny (LB) broth (Thermo Fisher Scientific, Hampton, NH) overnight separately, then combined in a 1:1:1:1 ratio inoculating dose, which ensured that total colony forming units (CFUs) of bacteria and volume added was equivalent between the mono and poly-microbial conditions. Antibiotic concentration was diluted across the 96-well

Figure 2

An Example of Broth Microdilution Technique as Directed by CLSI.



Note. A brief visual outline of the current diagnostic MIC protocol, as used via CLSI guidelines. Taken from [42]

plate in accordance with CLSI M7 and M100 guidelines, with the highest concentration at 128ug/ml; concentrations were serially halved across the 12 wells in a row down to 0.06ug/mL. Cation-Adjusted Mueller Hinton Broth (CAMHB) (Thermo Fisher Scientific, Hampton, NH) was used as the media, and after inoculation with the bacterial dose, the plate was sealed and incubated at 37^0C. After 18-24 hours of incubation, the plates were removed and MICs were determined visually via turbidity, as described as best-practice by the CLSI (Fig. 2).[39] All experiments were conducted in triplicate with biological replicates. The results were recorded and compared with the mono-species results for that antibiotic to determine if any notable differences were observed.

It has previously been observed that polymicrobial cultures gave results not in accordance with CLSI breakpoint guidelines, but since those assays were conducted with the disk diffusion method, their results cannot directly compared to the broth microdilution methods commonly used in US clinical laboratories.[40, 41] Given the lack of consistency in experimental conditions from previous in vitro research, we aimed to use the current clinical method of broth microdilution to determine what effect a polymicrobial community had on individual MICs among the species in a polymicrobial suspension. Using the current clinical model allowed for effective comparison both across antibiotics and species in the polymicrobial environment and ensured the validity of results in the current clinical standards. Limitations to this method do exist, however. Given that the bacteria in a polymicrobial condition cannot be differentiated visually, it is not possible to establish changes for individual species' MIC in this condition on the basis of turbidity alone. Because of this, an additional viability method was used to assess changes in antibiotic tolerance for each individual species (described below).

Viability

Two antibiotics - penicillin and ceftazidime - were tested with a modification of the MIC protocol. After following the previously described MIC protocol, the bacterial suspensions were extracted from the wells, diluted to the first order in phosphate buffered saline (1XPBS), then 10µl volume was spot-plated on selective and differential media for each bacteria (*Pseudomonas* Isolation Agar for the recovery of PA (Thermo Fisher Scientific, Hampton, NH), Mannitol Salt Agar (Thermo Fisher Scientific, Hampton, NH) for the recovery of SA, Bile Esculin Agar for the recovery of EF, and Leeds Agar (Thermo Fisher Scientific, Hampton, NH) for the recovery of AB, to observe any possible differences in viability of those cells in mono- and polymicrobial conditions. Selective and differential agars were used to allow for the differentiation of individual species within the polymicrobial condition, though the results of these assays are not directly comparable to the MICs for either of those antibiotics as they do not rely on the turbidity assessment used by the CLSI. In addition, the scientific literature has demonstrated that the visual assessment of turbidity corresponds only to an approximately 50 percent decrease in OD reading, so viable bacteria will still be present in wells above the visually assessed MIC.[43]

Results

Our results are demonstrated in the following figures, which are divided into two parts. The first covers our MIC data, and the second shows our viability data. For the MIC charts, the values listed show the individual MICs for each bacterium per antibiotic treatment, then the MIC polymicrobial value from our data, when read in accordance with CLSI guidelines. The viability data shows the comparison of spot plated viability values between the individual and polymicrobial conditions for a particular antibiotic treatment.

MIC Values

These data reflect comparisons of results obtained following the CLSI gold standard broth microdilution method for individual suspensions versus a polymicrobial planktonic suspension. MIC results were obtained for gentamicin, tobramycin, penicillin, tetracycline, doxycycline, and ceftazidime (Fig. 3). For gentamicin (Fig. 3A) and penicillin (Fig. 3C) the polymicrobial MIC was the same as the highest individual MIC result. This demonstrates that there was no observable change in the polymicrobial community MIC determinable by turbidity alone. For tetracycline (Fig. 3B), the highest observable individual MIC was *E. faecalis* at 32 μg/mL; however, the polymicrobial MIC result was 128 μg/mL. This demonstrates that mixing the four species together changes antimicrobial susceptibility of the population by 4-fold for at least one species in the suspension. For ceftazidime (Fig. 3D), the highest individual MIC value was *E. faecalis* at >128 μg/mL; however, the polymicrobial MIC result was 16 μg/mL. This demonstrates that the polymicrobial condition increases the susceptibility of *E. faecalis* by 4-fold. It is worth noting that any changes to individual MICs within the predetermined minimum and maximum ranges cannot be evaluated via turbidity and MIC alone in a polymicrobial condition.

Viability Values

The viability assays were run in order to determine changes in viability of the individual and polymicrobial conditions following antimicrobial challenge. All viability assays were conducted in triplicate with biological replicates. These results are not comparable to conventional MICs because the turbidity reduction observed in MICs correlates to an estimated 50% reduction in viable cells, whereas these data relate to the total elimination of viable bacteria.[43] For penicillin (Fig. 4A), both SA and AB observed decreases to antimicrobial susceptibility, at 64 and 8-fold respectively, in the polymicrobial versus individual conditions. For ceftazidime (Fig. 4B), AB observed a reduction in susceptibility by 2.86-fold, while SA observed an increase in susceptibility by 3-fold in the polymicrobial versus individual conditions. PA and EF do not exhibit any observable changes in susceptibility.

Discussion

Our data across the two methods - MIC and viability - demonstrate several notable results. In the first case, gentamicin, tobramycin, penicillin, and doxycycline did not show a difference in the observable MIC in the polymicrobial condition over the given values for the individual. In other words, the range of possible MIC values of the individual condition overlapped with that of the polymicrobial.

Because of this, and because the samples were assessed visually, it is not possible to determine the contribution of each individual species to the turbidity observed in the polymicrobial condition. For instance, in the case of gentamicin, the polymicrobial MIC is 8μg/ml. Given that the MIC value in the individual condition for *A. baumannii* is 4μg/mL, it is not possible for us to determine if any changes in MIC for that species occurred, because the individual MIC result for *E. faecalis* at 8μg/mL is the same as the polymicrobial result. Put another way, in the case of gentamicin, the polymicrobial condition having the same MIC as one of the individual MICs (in this case *E. faecalis*) might represent that the individual species had no

Figure 3

Comparison of Minimum Inhibitory Concentrations (MIC) in Mono- and Polymicrobial Conditions.

(A) Gentamicin

Species	MIC Value (μg/mL)
P. aeruginosa	0.5±0†
S. aureus	0.5±0†
A. baumannii	4±0†
E. faecalis	8±0†
Polymicrobial	8±0

(B) Tetracycline

Species	MIC Value (μg/mL)
P. aeruginosa	32±0†
S. aureus	0.5±0†
A. baumannii	4±0†
E. faecalis	32±0†
Polymicrobial	128±0

(C) Penicillin

Species	MIC Value (μg/mL)
P. aeruginosa	>128±0
S. aureus	2±0†
A. baumannii	16±0
E. faecalis	4±0†
Polymicrobial	>128

(D) Ceftazidime

Species	MIC Value (μg/mL)
P. aeruginosa	2±0†
S. aureus	16±0†
A. baumannii	1±0†
E. faecalis	>128±0
Polymicrobial	16±0

Note. Monomicrobial versus Polymicrobial MIC results for gentamicin (A), tetracycline (B), penicillin (C), and ceftazidime (D). † denotes that the value is within the published guidelines from the Clinical Laboratory Science Institute's M7 and M100 manuals for MIC breakpoints (CLSI, 2018a, 2018b). MIC values greater than 128 μL have been calculated as that number. CLSI does not publish established breakpoint values for *P. aeruginosa* and *A. baumanii* when treated with penicillin and for *E. faecalis* when treated with ceftazidime. n=3.

change in MIC, or it could represent that one or more of the species increased in MIC up to the polymicrobial MIC, but that change cannot be determined because the contribution of each individual species in the polymicrobial condition is not observable via an assessment of turbidity.

Two notable differences in MIC were observed in the polymicrobial condition. With tetracycline, a decrease in susceptibility was observed in the polymicrobial condition, which correlates to a decreased antibiotic efficacy, presumably via tolerance as all strains of bacteria used are pan-susceptible. As the polymicrobial MIC is substantially greater than any of the component individual MICs, it is not possible to determine which bacterial species' tolerance was increased, or which combination of those species was affected. With ceftazidime, however, an opposite effect was observed, where a sensitization interaction occurred. In this case, *E. faecalis* can be identified as the bacteria whose tolerance decreased, as its individual MIC is substantially higher than the polymicrobial value, whereas *P. aeruginosa*, *S. aureus*, and *A. baumanii*'s individual MIC values are equal to or lower than the polymicrobial MIC. As tetracycline and ceftazidime are both clinically important antibiotics, these results demonstrate a notable and potentially clinically significant change.[44, 45]

For the viability experiments, bacteria treated with penicillin and ceftazidime both showed notable differences in antibiotic susceptibility in the polymicrobial condition. When treated with penicillin in the polymicrobial environment, both *S. aureus* and *A. baumanii* showed notable increases in tolerance to the antibi-

Figure 4

Comparison of Viability in Mono- and Polymicrobial Conditions.

(A) Penicillin

Species	Individual Viability Value (µg/mL)	Polymicrobial Viability Value (µg/mL)
P. aeruginosa	>128±0	>128±0
S. aureus	2±0†	128±0
A. baumannii	16±0	>128±0
E. faecalis	4±0†	4±0

(B) Ceftazidime

Species	Individual Viability Value (µg/mL)	Polymicrobial Viability Value (µg/mL)
P. aeruginosa	>128±0†	>128±0
S. aureus	96±55.43†	32±27.71
A. baumannii	37.33±24.44†	106.67±37.0
E. faecalis	>128±0	>128±0

Note. Monomicrobial versus Polymicrobial Viability results for penicillin (A) and ceftazidime (B). † denotes that the value is within the published guidelines from the Clinical Laboratory Science Institute's M7 and M100 manuals for MIC breakpoints (CLSI, 2018a, 2018b). Viability values greater than 128 µL have been calculated as that number. CLSI does not publish established breakpoint values for *P. aeruginosa* and *A. baumanii* when treated with penicillin and for *E. faecalis* when treated with ceftazidime. n=3.

otic challenge. For *S. aureus,* this difference amounts to a 64-fold increase in concentration necessary to successfully kill the bacteria, and for *A. baumanii*, an 8-fold increase. However, when treated with ceftazidime, *S. aureus* demonstrated a decreased tolerance of 3-fold, but *A. baumanii* showed an almost three-fold increase in tolerance to the compound. As penicillin and ceftazidime are both clinically important, oft-prescribed antibiotics, this shift in tolerances is highly relevant for care practitioners in the wound and lab environments.

Given that our results are consistent with both the existing literature around the polymicrobial effect on antibiotic susceptibility and uses the current clinical model, these data demonstrate that there exists a gap in the current clinical diagnostic schema for determining the antimicrobial susceptibility of polymicrobial infections. As it has also been repeatedly demonstrated in the literature that synergistic interactions among bacteria within a wound produce more negative outcomes clinically, and that chronic wounds harbor polymicrobial infections, our data is consistent both internally and externally with the observations found in clinical practice.[10, 11, 15] Since both tolerance and resistance can play important roles in the success of infection treatment, and as our data demonstrates, tolerance alone can notably change the susceptibility of bacteria to antibiotic treatments, it is critical that the clinical models be adapted to allow for the presence of polymicrobial cultures during the assessment process. This change could potentially result in more accurate assessments of antibiotic susceptibility across the clinical spectrum, and may be of particular benefit to the treatment of the notoriously intractable chronic wounds. Though the rise of sequencing technologies in the clinical laboratory is no doubt of great value for clinical microbiologists and provides valuable data in the diagnostic process (particularly for microbial identification), it is important to note that because the above data assesses transient changes to antibiotic tolerance rather than antibiotic resistance, the former of which is the result of phenotypic rather than genotypic differences, methods such as 16S next-generation sequencing (NGS) or rapid qPCR will not be able to determine these changes in tolerance, since those technologies rely on and assess for the presence of antibiotic resistance genes.[46]

It is noteworthy that the differing combinations of bacterial species and antimicrobial drug yield different results. In some instances, the susceptibility is decreased, and in other instances the susceptibility of one or more species is increased. This implies that there is no 'one size fits all' approach to changes in susceptibility due to the polymicrobial condition, and that more research is necessary to understand why different bacterial consortia respond differently to antimicrobial challenges in clinical settings. It is also noteworthy that most of the existing literature, including this study, focus on either the contribution of either the polymicrobial or biofilm condition to changes in susceptibility, when in the clinical setting both of these conditions commonly exist simultaneously. This implies that the effects seen in these studies might be compounded when combined in the clinical setting, and more research needs to be done to understand both the cumulative effect of those conditions in the clinical wound setting and how the entirety of the microbial

environment can be taken into account when considering antimicrobial susceptibility in clinical diagnostic procedures.

In conclusion, antibiotic susceptibility testing is a crucial part of the chronic wound treatment process. Healthcare practitioners rely on the information that clinical microbiologists provide to determine the most appropriate antibiotic treatment, and it is critical that the information the clinical laboratory generates is representative of the infection, accurate, and applicable to the patient. Using clinically-relevant models based upon the current gold-standard guidelines, our results show that the polymicrobial condition of wounds may be modifying their response to antimicrobial chemotherapy, and therefore the results from the current method may not be an accurate reflection of susceptibility in the chronic wound infection environment. In adapting the method of assessment to better reflect the wound environment by including polymicrobial cultures, it is entirely possible that the costs, both physical and economic, of chronic wound care might be minimized, leading to improved patient care and outcomes.

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