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Title: Implications of antimicrobial combinations in complex wound biofilms containing fungi

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Abstract (75 word limit)

Diabetic foot ulcer treatment currently focuses on targeting bacterial biofilms, while dismissing fungi. To investigate this we used an in vitro biofilm model containing bacteria and fungi, reflective of the wound environment, to test the impact of antimicrobials. Here we showed that while mono-treatment approaches influenced biofilm composition it had no discernible effect on overall quantity. Only by combining bacterial and fungal specific antibiotics were we able to decrease the biofilm bioburden, irrespective of composition.

Diabetic foot ulcers (DFU) are an increasing healthcare burden, and cause excessive amounts of patient morbidity and mortality. It is known that infection both impairs healing and is linked to the recurrence of ulcers (1). It has been shown that chronic wounds often harbour pathogenic biofilms of polymicrobial nature, and are commonly recalcitrant to treatment (2-5). Guidelines suggest the initial use of oral antibiotics for empirical therapy with activity against Gram positive organisms, especially penicillins such as flucloxacillin (6, 7). Further coverage is provided by other antibiotics, such as the fluoroquinolone ciprofloxacin (7). Despite these chemotherapeutic approaches, resolution of infection is often hindered because of the failure to account for co-infecting pathogenic fungi (8). Indeed, fungal infection in DFU is under recognised, though recently some studies are beginning to shed light on the significant involvement of the wound mycobiome (9). While antifungal treatment have been shown to improve DFU outcome, they are only used routinely to treat superficial fungal infections in diabetics, such as onychomycosis (10, 11). Therefore, it is not a great leap to suspect fungal components as a key contributor to pathogenic DFU biofilms.

Characterised laboratory strains were used to create biofilms in standard 96-well plates, including the bacteria *Pseudomonas aeruginosa* PA14 and *Staphylococcus aureus* ATCC 13420, and the yeast *Candida albicans* SC5314 (12). Biofilm antimicrobial susceptibility testing was carried out as described previously by our group to determine the sessile minimum inhibitory concentrations (MICs) (13). Briefly, mono-species and co-culture biofilms were grown in Mueller Hinton (bacterial cultures) or RPMI broth (fungal cultures) before being treated with the flucloxacillin, ciprofloxacin, or fluconazole (Sigma, Dorset, UK) which are common clinically at a range of concentrations (0.125 to 128 mg/L) (6, 7, 11). Viability post-treatment was assessed using alamar Blue™ metabolic dye. Here it was shown that an increase in species diversity leads to elevated viability following treatment (Figure 1, $p<0.01$), as evidenced by *S. aureus* co-culture and triadic biofilms after ciprofloxacin therapy. A similar, but less dramatic, effect was observed with flucloxacillin, though for fluconazole no discernible effect was observed (Figure S1). This indicates that increased complexity of a biofilm community and physical structure provided by

C. albicans leads to enhanced resistance as has been shown elsewhere (14, 15).

Our group has recently created and described an *in vitro* interkingdom biofilm model that reflects a chronic wound environment, a model formed within a three-dimensional cellulose matrix (12). We therefore aimed to use this model to characterise *in vitro* responses to antibiotic pressure in a triadic interkingdom biofilm model that is reflective of the chronic wound environment. Triadic biofilms in the cellulose model were created by standardising all three microorganisms to 1 x 10^6 CFU/mL in PBS, and incubating with 1.25 cm^2 sections of cellulose matrix for 2 h at 37°C with agitation. The matrix was then placed on top of a 50% serum hydrogel surface and incubated at 37°C for 24 h (12). Following biofilm development, these were treated with 128 mg/mL flucloxacillin, ciprofloxacin and fluconazole, either alone or in combination, for a further 24 h at 37°C alongside untreated controls. All testing was carried out in triplicate, on three separate occasions. To differentiate between live and dead cells, a qPCR based assay (16-19) was used to assess the viable composition of the biofilms with species specific primers (12, 19). This assay utilises propidium monoazide, a DNA intercalating dye, which binds to DNA in cells with a compromised membrane preventing this DNA from being amplified in downstream PCR. Therefore only DNA from viable cells with intact membranes is detected. MasterPure™ Yeast DNA extraction kits were used as per manufacturer's instructions (Epicentre, Cambio, Cambridge, UK) to process all samples. For qPCR a Fast SYBR® Green Master Mix (Life Technologies, Paisley, UK) was used with primer sequences and thermal profile previously defined (12). Each sample was analysed in duplicate using Step One Real-Time PCR system and software (Life Technologies, Paisley, UK). Samples were quantified to calculate the colony forming equivalent (CFE) based upon a standard curve per reaction performed. Results were also confirmed by colony forming unit (CFU) counts using the Miles and Misra technique (20).

The cellulose matrix model has already been shown to create more resilient biofilms (12), and when treated with elevated levels of antibiotics (128 mg/L) there was little impact on the viable cells within the matrix, though

compositionally the biofilms were affected (Figure 2A). This implies that eliminating one microbial component of the biofilm through targeted therapy creates a niche for the other species to thrive. For example, fluconazole treatment significantly decreased *C. albicans* by approximately 1 x $\log_{10}$ ($p<0.05$). In contrast, a combined treatment of flucloxacillin/ciprofloxacin resulted in a three-fold increase in *C. albicans* (Figure 2B). In addition to consideration of the biofilm composition, the biovolume is equally important given that a *C. albicans* cell is approximately ten times the size of a *S. aureus* cell. Consequently, even in biofilms where *C*. *albicans* in low abundance, the yeast and hyphal cells still provide physical structure and support to biofilm through its spatial dominance. The only treatment observed to cause a substantial decrease in all three microbial components, and an overall reduction in viable cell composition, was the combination all three antimicrobials (Figure 3). Hierarchical clustering analysis also shows that the *C. albicans* population is very closely linked to the total CFE present, suggesting it is a key driving force within the biofilm community (Figure 3).

The data from this investigation suggests that antifungal drugs should be included in empirical therapy options alongside antibiotics. It has been recently shown that the hyphal structure and extracellular matrix of *C. albicans* mycofilms support bacterial growth, leading to a more resilient biofilms in terms of antibiotics and antifungals (14, 15). These key findings indicate that disrupting and/or impeding this supportive mycofilm structure could lead to a physical collapse of the polymicrobial community. Fungi are increasingly recognised as a key contributor to biofilms in DFU, and we have shown here that *C. albicans* appears to be an important element behind the recalcitrant nature of these biofilms. Therefore, it is imperative to consider a treatment covering these major pathogens, rather than consider them as having a supporting role. Inclusion of antifungals into routine treatment strategies could allow for easier disruption of the biofilm, decreasing microbial load in DFU, and ultimately improving patient outcomes.

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133 model.

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Figure 1: Increased microbial complexity of biofilms leads to reduced susceptibility to antimicrobial agents. Biofilm (sessile) MIC values were calculated using the alamar blue® viability test. All tests were carried out in quadruplicates on three separate occasions as described in the methods. Monoculture (Sa) biofilms were compared to co-culture biofilms (Sa + Ca, Sa + Pa) and triadic biofilms (Sa + Ca + Pa). Data was analysed using a two-way ANOVA with Tukey's multiple comparison test to compare each mono- or co-culture at each antimicrobial concentration.

Figure 2: Triadic biofilm composition is influenced by antimicrobial treatment. Antibiotics, flucloxacillin (FLX) and ciprofloxacin (CIPX), and antifungal, fluconazole (FLC), were used to treat the biofilms, either alone or in combination as described in the methods. Biofilm percentage composition is shown in the bar graphs **(A)**, while absolute numbers of viable colony forming equivalents (CFEs) present is shown below **(B)**. Treated biofilms were compared to untreated controls using a two tailed unpaired t-test (* $p<0.05$).

Figure 3: Triple antimicrobial treatment elicits the largest impact on biofilms. The heat map shows a fold-change increase in viable CFE (red), decrease (blue), or no change (white) after treatment with flucloxacillin (FLX), ciprofloxacin (CIPX), and fluconazole (FLC), either alone or in combination. Hierarchical clustering analysis (left) shows that the total bioburden is closely related to *C. albicans*, suggesting this is the component of the biofilm that is integral to infection.

Figure 1

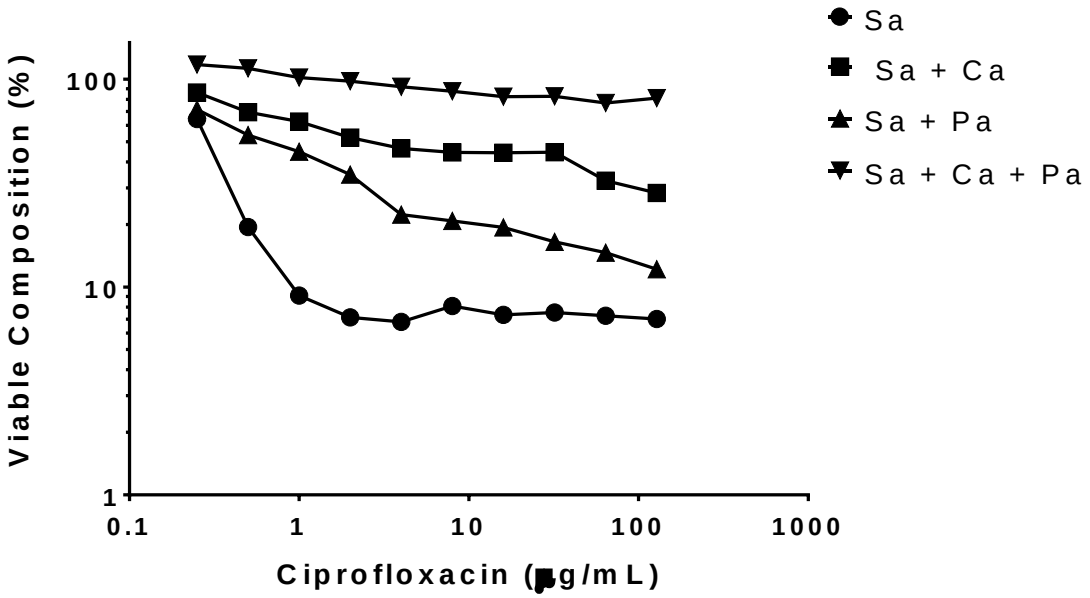


Figure 2

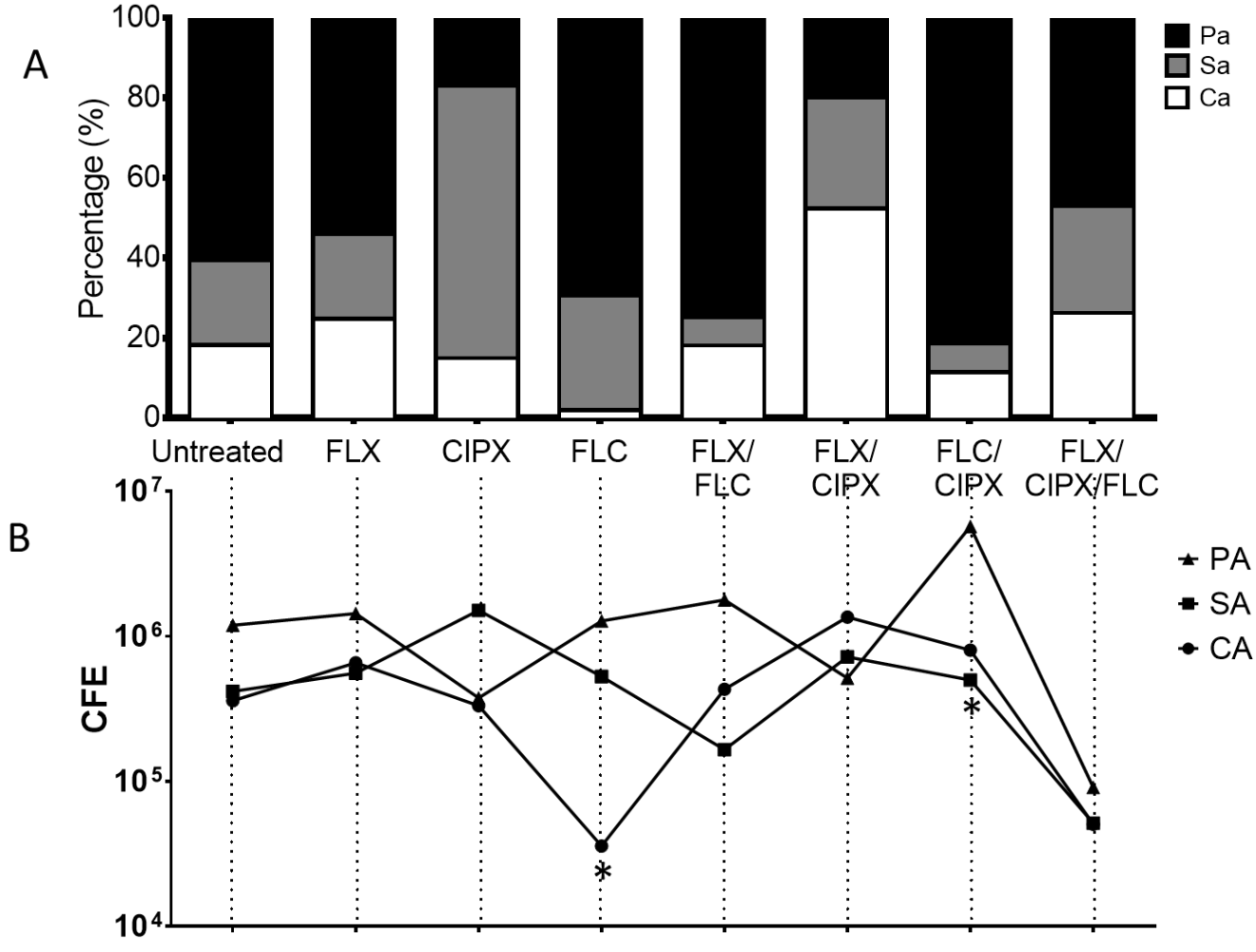


Figure 3

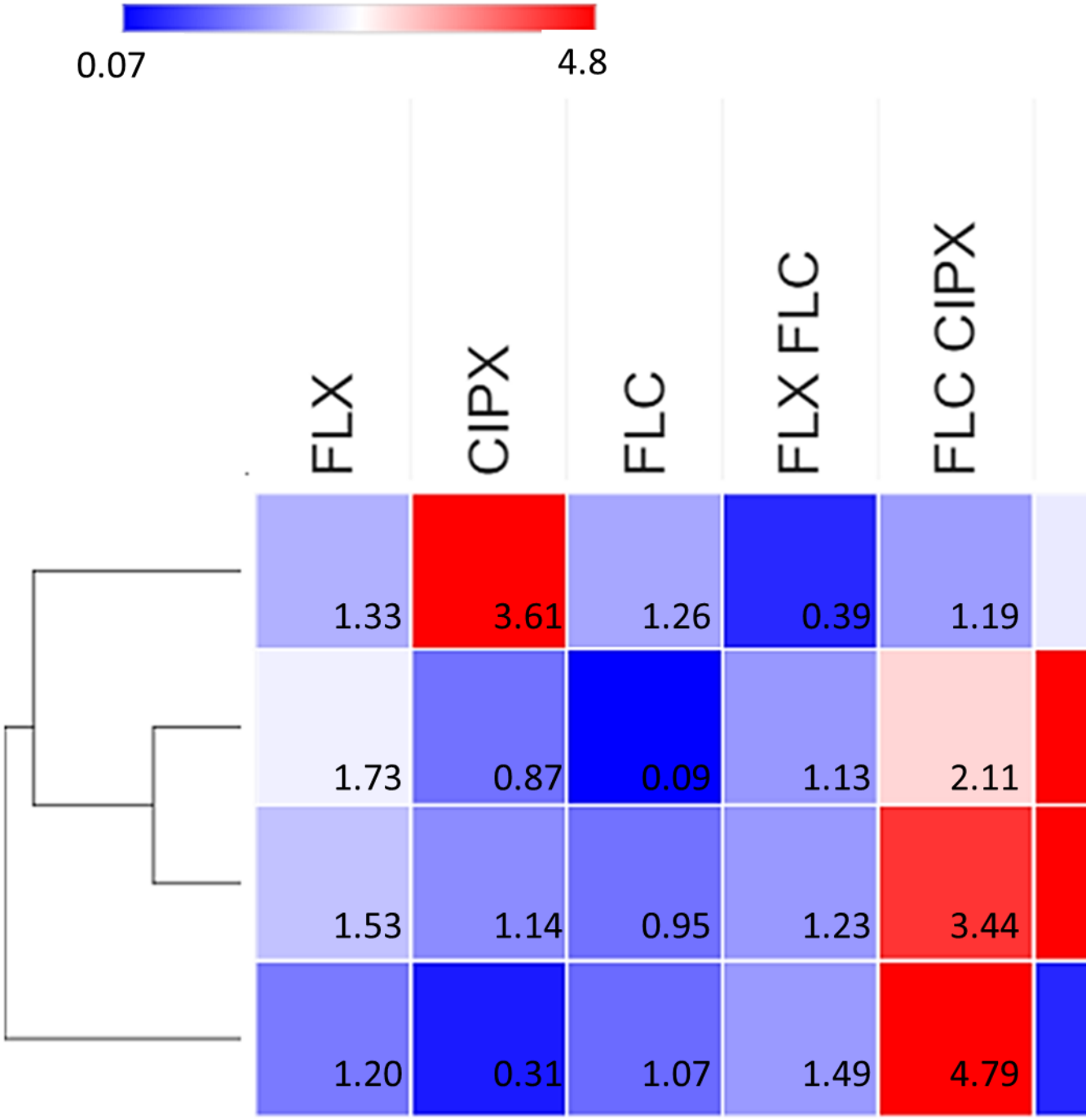

0.07
4.8
FLX
CIPX
FLC
FLX FLC
FLC CIPX
1.33
3.61
1.26
0.39
1.19
1.73
0.87
0.09
1.13
2.11
1.53
1.14
0.95
1.23
3.44
1.20
0.31
1.07
1.49
4.79

Fig S1

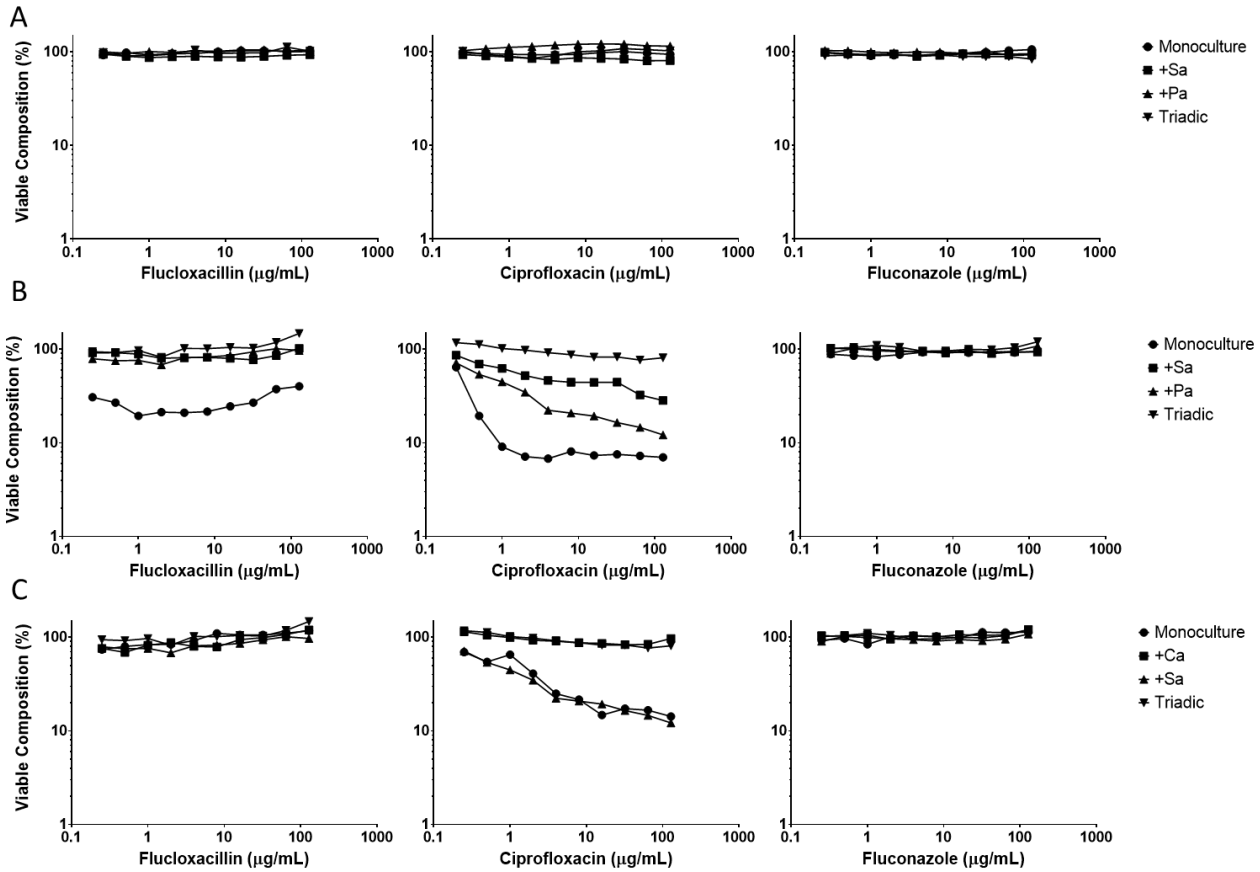
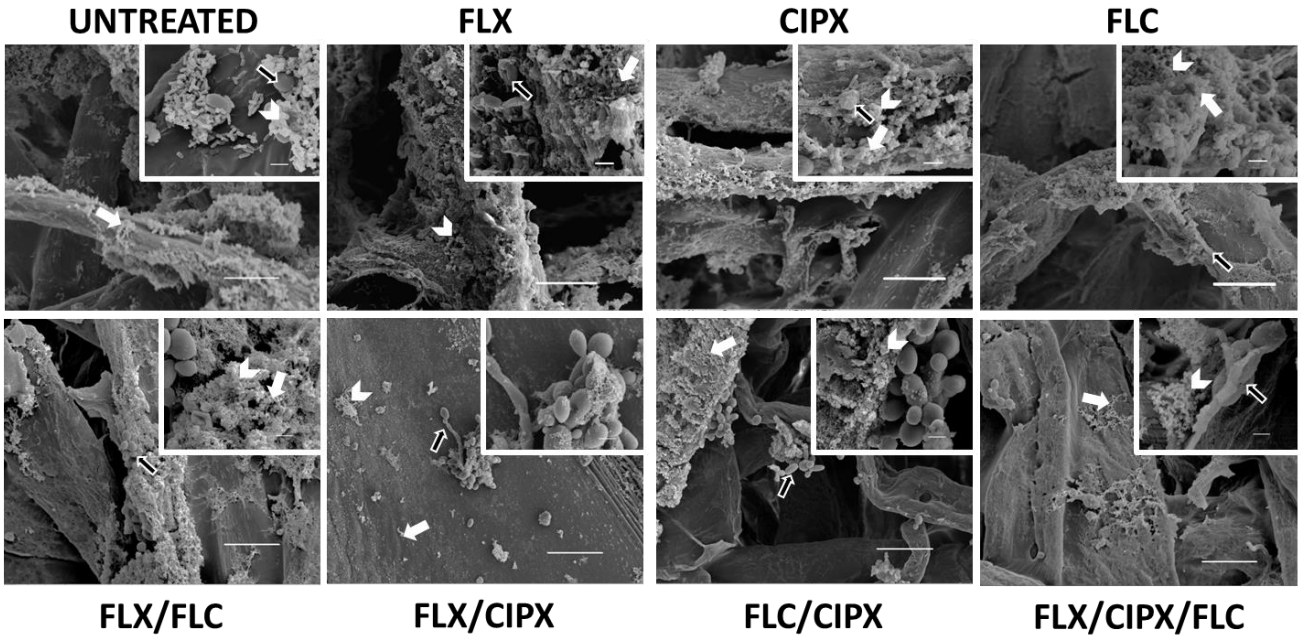

A
B
C
Viable Composition (%)
Flucloxacillin (μg/mL)
Ciprofloxacin (μg/mL)
Fluconazole (μg/mL)
Monoculture
+Sa
+Pa
Triadic
+Ca

Fig S2


UNTREATED
FLX
CIPX
FLC
FLX/FLC
FLX/CIPX
FLC/CIPX
FLX/CIPX/FLC