

# Development, characterization, and evaluation of a simple polymicrobial colony biofilm model for testing of antimicrobial wound dressings

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## Abstract

Chronic wound infections are generally of polymicrobial nature with aerobic and anaerobic bacteria, as well as fungi frequently observed in them. Wound treatment involves a series of steps, including debridement of the wound, flushing, and often the use of multiple wound dressings many of which are antimicrobial. Yet, many wound dressings are tested versus single species of planktonic microbes, which fails to mirror the real-life presence of biofilms.

**Aims:** Simple biofilm models are the first step to testing of any antimicrobial and wound dressing; therefore, the aim of this study was to develop and validate a simple polymicrobial colony biofilm wound model comprised of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans* on RPMI-1640 agar. The model was then used to evaluate the topical disinfectant chlorhexidine and four commercially available wound dressings using the polymicrobial model. The model used was as a starting point to mimic debridement in clinical care of wounds and the effectiveness of wound dressings evaluated afterwards.

**Methods and Results:** Planktonic assessment using AATCC100-2004 demonstrated that all antimicrobial wound dressings reduced the planktonic microbial burden below the limit of detection; however, when challenged with polymicrobial colony biofilms, silver wound dressings showed limited effectiveness (1–2 log CFU reductions). In contrast, a single iodine releasing wound dressing showed potent antibiofilm activity reducing all species CFUs below the limit of detection (>6–10 log) depending on the species. A disrupted biofilm model challenge was performed to represent the debridement of a wound and wound silver-based wound dressings were found to be marginally more effective than in whole colony biofilm challenges while the iodine containing wound dressing reduced microbial recovery below the limit of detection.

**Conclusions:** In this model, silver dressings were ineffective versus the whole colony biofilms but showed some recovery of activity versus the disrupted colony biofilm. The iodine wound dressing reduced the viability of all species below the level of detection. This suggests that mode of action of wound dressing should be considered for the type of biofilm challenge as should the clinical use, e.g. debridement.

## Impact Statement

These results demonstrate that biofilm management capabilities vary considerably among the wound dressings when tested in this *in vitro* model. The results from this study may have clinical implications for wound management.

**Keywords:** colony biofilm model; *Pseudomonas aeruginosa*; *Staphylococcus aureus*; *Candida albicans*; wound dressing testing

## Introduction

The burden of wounds in the UK has been estimated to affect up to 3.8 million patients, within the study year of 2017/18, of which overall 70% healed. Chronic wounds (49%) had a significantly lower healing rate compared to acute wounds (89%) (Guest et al. 2020). Suspected infection is a main factor in the chronicity of wounds with an average of eight different types of dressings prescribed per patient. This resulted in an estimated 262.5 million wound dressings used over the 2017/18 period, in addition to varying combinations of nonsteroidal anti-inflammatory drugs (NSAIDs), analgesics, and antimicrobials (Guest et al. 2020). The estimated financial burden to

the NHS from the study was £8.3 billion (95% CI £7.9–8.5 billion) (Guest et al. 2020). In the USA, the estimated Medicare cost for acute and chronic wounds is \$28.1 to \$96.8 billion (Sen 2019). A recent economic impact assessment of biofilms has estimated that the global wound biofilm related cost is \$281 billion (Cámara et al. 2022). It is therefore evident that chronic wounds are a significant burden in terms of quality of life, mortality, and financial impact on the patient and healthcare services. Biofilm structures were initially observed in over 60% of chronic wound biopsies compared to only 6% of acute wound biopsies (James et al. 2008). With advances in combined molecular biology and microscopy methods, an es-

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estimated 78.2% prevalence of biofilms have now been shown in chronic wounds (Malone *et al.* 2017a). Chronic wounds are typified by the formation of microbial biofilms and aggregates, which are encompassed within a self-derived matrix formed from extracellular polymeric substances (EPS) (Attinger and Wolcott 2012), which provides resilience to environmental conditions and up to 1000-fold increased tolerance to antimicrobial treatment (Mah 2012). This increased survival to external challenges is a result of the slow growth of microbes present, differential oxygenation, and the EPS, which has been shown to bind or reduce the effective concentration of antimicrobials reaching the microbial cells (del Pozo and Patel 2007). The complex biofilm structure can also incorporate host proteins, which further reduce the efficacy of treatments.

Next generation sequencing of *rrn* genes for encoding the 16S rRNA in chronic wound biofilms has shown that many genera of anaerobic, e.g. *Peptoniphilus*, *Anaerococcus*, and *Finegoldia* and aerobic bacteria, e.g. *Corynebacterium*, *Staphylococcus*, and *Pseudomonas* can be identified in wounds (Smith *et al.* 2016, Malone *et al.* 2017b), which culture-based methods often fail to identify. The reason for failure to identify anaerobic bacteria in culture, stems from a view that anaerobes are not detrimental to wound healing, and that isolation, culture, and identification is more time consuming, labor intensive, and expensive than aerobic and facultative culture (Bowler *et al.* 2001). The use of 16S sequencing, however, gives a bacteriocentric view of the wound environment, with culture and targeted PCR studies demonstrating the presence of fungi such as *Candida albicans* (Dowd *et al.* 2011). The appreciation of the implications of bacterial and fungal interactions has grown over the recent years and are the subject of many reviews (Peleg *et al.* 2010, Krüger *et al.* 2019, Lapiere and Richard 2022, Short *et al.* 2023). Interactions between *P. aeruginosa*, *S. aureus*, and *C. albicans* have significant clinical relevance. Systemic *S. aureus* infection can be mediated by attachment to *C. albicans* hyphae mediated through the interaction with Als3 adhesins. *S. aureus* is unable to adhere to *C. albicans* mutants of Als3 but this function is restored in the complemented mutant (Schlecht *et al.* 2015). *P. aeruginosa* has been shown to suppress and kill *S. aureus* through a variety of mechanisms (Pastar *et al.* 2013), but can also act synergistically *in vivo*, which can be replicated *in vitro* (DeLeon *et al.* 2014). *P. aeruginosa* can promote small-colony variant formation in *S. aureus* by the production of cyanide and phenazines (Biswas *et al.* 2009) and 4-hydroxy-2-heptylquinoline *N*-oxide (HQNO) (Mitchell *et al.* 2010). *C. albicans* interactions with *P. aeruginosa* are likewise complex with both physical and chemical interactions occurring. *P. aeruginosa* can attach to *C. albicans* hyphae and secrete hydrolytic enzymes, such as haemolytic phospholipase C (PlcH) and phenazines resulting in hyphal killing (Fourie *et al.* 2016). Farnesol produced by *C. albicans* has been shown to inhibit *P. aeruginosa* quorum sensing and also provide protection for *S. aureus* to vancomycin through a postulated increase in the production of reactive oxygen species (Kong *et al.* 2017). These illustrative examples of microbial interactions between clinically relevant microbes, while not exhaustive, highlight the importance of testing antimicrobial interventions using an appropriate microbial complexity that is clinically relevant.

Chronic wound treatment involves debridement and cleansing of the wound coupled with the use of topical antiseptics and/or antimicrobial dressings. Many antimicrobial wound dressing technologies are available to clinicians, with

silver-based technologies being the most dominant and iodine-based antimicrobial wound dressings becoming widespread in recent years. It is important to note that antimicrobial testing for wound dressings is often performed either with planktonic suspensions of microbes such as the AATCC100-2004 (100-2004 2008) method, which is essentially a planktonic suspension challenge versus the antimicrobial material, or using suspensions of the antimicrobial components of a wound dressing, which have significantly different biochemical and biophysical interactions with the microbes in both planktonic and biofilm testing. A recent shift has seen wound dressing manufacturers testing antimicrobial products versus biofilms formed on coupons in a CDC bioreactor ASTM E2871-19 (Percival *et al.* 2017), or a drip flow model. Biofilms models of increased complexity, such as the Lubbock (Sun *et al.* 2008), modified Lubbock (Kucera *et al.* 2014), wound milieu (Sun *et al.* 2008), and polymicrobial hydrogel (Townsend *et al.* 2016) have been used to assess the efficacy of topical antimicrobials and antimicrobial wound dressings. It is therefore paramount that for research to have a translational impact, appropriate models for different stages of testing are developed and standardized (Highmore *et al.* 2022).

Despite the antagonistic interactions between *P. aeruginosa*, *S. aureus*, and *C. albicans* in *in vitro* culture systems, in this study, a standardized simple polymicrobial colony biofilm model comprised of these three organisms was developed and confocal laser scanning microscopy was used to visually that the biofilm features of EPS, eDNA, and spatial interactions between the three species were observed, indicating that a biofilm was formed in this model. Challenge of the model with the antiseptic chlorhexidine demonstrates that microorganisms are more resistant to killing in the three-species biofilm community than in a monospecies biofilms. Assessment of four commercially available antimicrobial dressings was conducted in this model and we show that the silver-based wound dressings were ineffective in the whole biofilm model and minimally effective in a physically disrupted version of the model, which is in keeping with the clinical observation of some wounds failing to respond to silver wound dressings. The iodine containing wound dressing was effective in eradicating the polymicrobial biofilm in both the whole and the disrupted model, mirroring their clinical effectiveness. Therefore, this multikingdom biofilm will be useful for the examination of antimicrobial wound care products in a semihigh throughput manner and represents a valuable tool to investigate interspecies and interkingdom interactions in a simple model system that has stability for up to 72 h.

## Materials and methods

### Culture conditions and standardization

*Pseudomonas aeruginosa* PAO1-L (*P. aeruginosa* PAO1-L), *Staphylococcus aureus* SH1000 (*S. aureus* SH1000), and *Candida albicans* SC5314 (*C. albicans* SC5314) were used for all work in this study, except *S. aureus* HMC 6–1, which was used for growth of biofilms for electron microscopy studies (Stoffel *et al.* 2020). All working stocks of *P. aeruginosa* PAO1-L, *S. aureus* SH1000, and *C. albicans* SC5314 were maintained at 4°C on Lysogeny broth agar [LB (Oxoid, Cambridge, UK)] for bacteria and Sabouraud agar [SAB (Oxoid, Cambridge, UK)] for *C. albicans* SC5314. *P. aeruginosa* PAO1-L and *S. aureus* were propagated in LB for 16 h at 37°C

with shaking at 200 rpm and *C. albicans* SC5314 was propagated in yeast peptone dextrose broth [YPD (Oxoid, Cambridge, UK)] for 16 h at 30°C with shaking at 200 rpm. Cultures were washed twice by centrifugation (10 000 × g), resuspended in 1x phosphate buffered saline (PBS) (Oxoid, Cambridge, UK) then adjusted to  $1 \times 10^8$  CFU ml<sup>-1</sup> by optical density (0.05 and 0.1 OD<sub>600nm</sub>, respectively) for bacteria and use of haemocytometer (Brand, Wertheim, Germany) for *C. albicans* SC5314. Fluorescent protein expressing strains were grown in appropriate media with the following antimicrobial selection, *P. aeruginosa* PAO1-L mTurquoise2 [125 µg ml<sup>-1</sup> Tetracycline (TC)] (this study), *S. aureus* SH1000 pTK005 YFP [40 µg ml<sup>-1</sup> chloramphenicol (CM)], and *C. albicans* CAF2-1 yCherry (Brothers et al. 2011).

Fluorescent *P. aeruginosa* PAO1-L was constructed using vector pSW002-PpsbA-mTurquoise2, a kind gift from Rosemarie Wilton (Addgene plasmid #108237) (Wilton et al. 2017). *P. aeruginosa* PAO1-L was transformed with pSW002-PpsbA-mTurquoise2 according to the procedure outlined by Choi and colleagues, yielding PAO1-L constitutively expressing mTurquoise2 (Choi et al. 2006).

### Assessment of growth in liquid culture and on agar

The capacity of *P. aeruginosa* PAO1-L, *S. aureus* SH1000, and *C. albicans* SC5314 to grow in broth and agar culture was assessed with the following media, LB, trypticase soy broth (TSB), brain heart infusion (BHI), Mueller–Hinton (MH), YPD, and Roswell Park Memorial Institute (RPMI)-1640 (Sigma–Aldrich, Haverhill, UK) without sodium bicarbonate and phenol red, buffered with 0.165 mol l<sup>-1</sup> MOPS (Sigma–Aldrich, Haverhill, UK). In addition, for selective isolation of microbes, *Pseudomonas* isolation agar [PIA (Oxoid, Cambridge, UK)] and mannitol salt agar [MSA (Oxoid, Cambridge, UK)] were utilized. To prevent growth of bacteria during enumeration of *C. albicans* SC5314 TC (Sigma–Aldrich, Haverhill, UK) was added to a final concentration of 125 µg ml<sup>-1</sup> in SAB agar. To prevent growth of *C. albicans* SC5314 during MSA and PIA agar enumeration, nystatin (Sigma–Aldrich, Haverhill, UK) was added to a final concentration of 10 µg ml<sup>-1</sup>.

### Biofilm model development

Polycarbonate (PC) discs [Isopore membrane filter (Sigma–Aldrich, Haverhill, UK)], with diameter of 13 mm and 0.2 µm pore size were used to support microbial biofilm growth. Briefly, the PC discs were short-wavelength ultraviolet (UV-C) treated for 10 min on each side in a benchtop UV cabinet (Spectrolinker™ XL-1000 Series UV Crosslinker, town country). Sterilization of the PC discs was confirmed by placing UV-C treated PC discs in 5 ml of LB broth O/N at 37°C, with no growth indicating UV-C treatment was sufficient to sterilize the PC discs.

To prevent cross interference of biofilms on PC discs, 5 ml of the desired growth agar was added to the wells of a 6-well CELLSTAR® Cell Culture Multiwell Plates (Greiner, Stonehouse, UK). RPMI-1640 agar was formulated by combined prewarmed 2x RPMI-1640 with an equal volume of 3% (w/v) agar (Oxoid, Cambridge, UK), then dispensing 5 ml per well. Sterilized PC discs were then placed by sterile forceps on the growth agar and 10 µl of each desired microbial inoculum added in the centre of the disc. Inoculated discs on agar were

then incubated at 37°C for 72 h and transferred to fresh growth agar every 24 h interval.

### Selection of wound dressings

Wound dressings were selected in discussion between academic and industrial partners, these represent the most commonly used wound dressings clinically in the UK and USA. Silver containing wound dressings are more commonly used than iodine wound dressings; therefore, three silver wound dressings were selected and one iodine-containing wound dressing selected. The three selected silver wound dressings were Aquacel® Ag + Advantage (ConvaTec), Acticoat™ Flex 7 (Smith & Nephew, Inc.), Exufiber® Ag+ (Mölnlycke Healthcare), and the iodine containing wound dressing was Iodoflex™ (Smith & Nephew, Inc.).

### Modified antimicrobial wound dressing planktonic testing

A modified method of AATCC100-2004 (100-2004 2008) was used to determine the antimicrobial activity of the selected wound dressings. Microbial inoculum was standardized to  $1 \times 10^5$  CFU ml<sup>-1</sup> in RPMI-1640 w/MOPS. For combined polymicrobial planktonic challenge, overnight cultures were standardized to  $1 \times 10^5$  CFU ml<sup>-1</sup> in the same volume of RPMI-1640 w/MOPS. Identical size (15 × 15 mm) wound dressing swatches and wetted filter paper (control) were placed aseptically in a 5 ml bijou vial and inoculated with 1 ml of the respective microbial inoculum for single species and a combination of all three microbial species for a polymicrobial planktonic challenge (1000 *S. aureus*: 100 *C. albicans*: 10 *P. aeruginosa*). Microbial enumerations were performed at the  $t = 0$  time point in line with the AATCC100-2004 guidelines. The bijou vials were incubated at 37°C for 24 h in a static incubator. Following incubation, 4 ml of Dey–Engley neutralizing broth (Sigma–Aldrich, Haverhill, UK) was added to each bijou vial, and the vials were vortexed and placed in a sonicating water bath [Fisherbrand FB15050 (Fisher Scientific Ltd., Loughborough, UK)] 37 kHz for 15 min. Ten-fold serial dilutions were then performed in 1x PBS from the sonicated samples and 20 µl of each dilution were plated in triplicate on the desired selection agar [*P. aeruginosa* PAO1-L = PIA, *S. aureus* SH1000 = MSA + 10 µg ml<sup>-1</sup> nystatin, and *C. albicans* SC5314 = SAB + 125 µg ml<sup>-1</sup> of tetracycline (TC)].

### Enumeration of microbes in mono and polymicrobial biofilms

At endpoint, PC discs were removed by sterile forceps to a 1 ml microcentrifuge tube containing 1 ml Dey–Engley neutralizing broth and 5 × 2.6 mm zirconium ceramic oxide beads (Fisherbrand, Loughborough, UK). The samples were then vortexed to remove the biofilm from the PC disc. The PC discs were then removed using sterile forceps and the biofilm was subjected to bead beating for 30 s using a FastPrep-24™ 5 G Homogenizer (MP Biomedicals, Loughborough, UK). The 1 mL volume was then transferred to a 5 ml Bijou containing 4 ml Dey–Engley neutralizing broth. Bijous were then floated in a sonicating water bath and sonicated for 15 min at 37 kHz. Ten-fold serial dilutions were then performed in 1x PBS from the sonicated samples and 20 µl of each dilution was plated in triplicate on the desired selection agar (PAO1-L = PIA + 4 µg ml<sup>-1</sup> nystatin, *S. aureus* SH1000 = MSA + 4 µg ml<sup>-1</sup> nystatin, and *C. albicans* SC5314 = SAB + 125 µg ml<sup>-1</sup> TC).

### Polymicrobial colony wound biofilm model

Briefly, UV-C sterilized PC discs were placed on 1x RPMI-1640 agar in 6-well plates. To each PC disc, 20  $\mu$ l of a combined final inoculum of  $1 \times 10^6$  CFU ml<sup>-1</sup> *S. aureus* SH1000 and  $1 \times 10^5$  CFU ml<sup>-1</sup> *C. albicans* SC5314 was dispensed by pipette. The multiwell plates were then incubated statically for 24 h at 37°C. The PC disc with biofilms were then transferred to new RPMI-1640 agar in 6-well plates. Then, 10  $\mu$ l of  $1 \times 10^4$  CFU ml<sup>-1</sup> of *P. aeruginosa* PAO1-L was added to each colony biofilm. The multiwell plates were then incubated statically for a further 24 h at 37°C. After 48 h cumulative incubation the colony biofilms were mature and ready for subsequent imaging, enumeration, or wound dressing testing.

### Imaging of microbial localization and matrix components in polymicrobial colony biofilm model

Microbial localization was determined by utilizing fluorescent protein (FP) expressing strains, briefly, the strains used were *P. aeruginosa* PAO1-L mTurquoise2 (125  $\mu$ g ml<sup>-1</sup> TC), *S. aureus* SH1000 YFP (20  $\mu$ g ml<sup>-1</sup> CM), and *C. albicans* CAF2-1 yCherry. To assess the viability of the microbial population in the polymicrobial biofilms wild-type strains (no FP) were stained with the LIVE/DEAD BacLight bacterial viability kit [L7007 (Thermo Fisher Scientific, Loughborough, UK)] comprised of the active components SYTO9 and Propidium Iodide (PI). Staining was performed as per the manufacturer's instructions. Assessment of the biofilm matrix composition was carried out using Concanavalin A conjugated to Alexa Fluor 633 exopolysaccharide (Thermo Fisher Scientific, Loughborough, UK) and YOYO-1 eDNA (Thermo Fisher Scientific, Loughborough, UK). Briefly, 20  $\mu$ l of a 1/100 dilution of ConA-633 was added to each colony biofilm for 1 h at RT in the dark. Following this incubation, 20  $\mu$ l of YOYO-1 to final concentration of 4 nmol l<sup>-1</sup> was added and incubated for 15 min. Following staining, PC disks were rinsed with sterile water and imaged using a LSM 700 laser scanning confocal microscope (CLSM) (Carl Zeiss, Germany).

### Scanning electron microscopy of polymicrobial colony biofilms

Polymicrobial biofilms were grown as described above for 24 h after adding *P. aeruginosa* except that *S. aureus* HCMC 6-1 (Stoffel *et al.* 2020) was used in place of *S. aureus* SH1000. Polycarbonate membranes with attached biofilms were immersed in 2.5% (w/v) glutaraldehyde and 0.1% (w/v) alcian blue (Sigma-Aldrich, St. Louis, MO, USA) in deionized water and fixed for 24 h at 4°C. Biofilms were dehydrated by immersion in increasing concentrations of ethanol (15%, 25%, 50%, 75%, 90%) for 15 to 30 min with two successive final immersions in 100% ethanol for 30 min. A critical point dryer (Samdri 795, Tousimis Research Corporation, Rockville, MD, USA) was used to dry the biofilms prior to gold sputtering (Desk V Thin Film Deposition System, Denton, Moorestown, NJ, USA). Samples were imaged using a Jeol JCM-5000 scanning electron microscope using 10 kV beam acceleration and secondary electron detection under high vacuum (Jeol U.S.A, Peabody, MA, USA).

### Assessment of wound dressing treatment by quantitative culture

The following wound dressings were evaluated: Aquacel® Ag + Advantage (ConvaTec), Acticoat™ Flex 7 (Smith & Nephew, Inc.), Exufiber® Ag+ (Mölnlycke Healthcare), and Iodoflex™ (Smith & Nephew, Inc.). Dressings were sectioned to 15 × 15 mm<sup>2</sup>. Colony biofilms on PC discs were transferred to fresh RPMI-1640 agar in 6-well plates. To apply wound dressing sections to the colony biofilms, sufficient dH<sub>2</sub>O was added onto the dressing to ~50% saturation, this equated to 450  $\mu$ l for Aquacel® Ag Advantage, 450  $\mu$ l for Exufiber® Ag+, 100  $\mu$ l for Acticoat™ Flex 7, and 200  $\mu$ l for Iodoflex™. Wound dressings were then placed on top of the colony biofilm to replicate the clinical challenge of a wound dressing being applied over the wound bed. Full saturation was deemed undesirable as wound dressings would be changed at this point. Control polymicrobial biofilms had wetted filter paper, dimensions 15 × 15 mm<sup>2</sup> applied. Following application of the wound dressing sections, multiwell plates were incubated statically for 24 h at 37°C. Microbial enumeration was then performed as previously described.

To mimic debridement of the biofilm, polycarbonate discs were removed at 48 h and placed in a 1.5 ml Eppendorf tube with 200  $\mu$ l of 1x PBS and vortexed for 1 min. The polycarbonate disc was removed and the 200  $\mu$ l volume of disrupted biofilm was pipetted onto the relevant wound dressing and placed inoculation side facedown on top of a sterile polycarbonate disc on 1x RPMI-1640 agar w/MOPS. Multiwell plates were incubated statically for 24 h at 37°C. Microbial enumeration was then performed as previously described.

### Statistical analysis

All data were assessed for normality using a Shapiro–Wilk test. To assess the statistical significance of the wound dressing tests in planktonic cultures, a one-way ANOVA with Tukey *post hoc* test was performed, which adjusts the *P*-values for multiple testing when pairwise comparisons between group means are made when the sample sizes for each group are equal. Polymicrobial biofilm wound dressing assessment was performed with two-way ANOVA using Šidák multiple comparison correction for nondisrupted, which adjusts the significance level for multiple comparisons and provides tighter bounds than Bonferroni. A two-way ANOVA with Dunnett's multiple comparison correction was used for disrupted polymicrobial biofilms, which creates a confidence interval for differences between the mean of each factor level and the mean of a control group. Statistical significance was achieved when *P* < .05. Data were input to Microsoft Excel 365 (Microsoft, USA). Assessment of normality, statistical analysis, and plotting of data were performed in GraphPad Prism 9.5.1 (GraphPad Software Inc, USA).

## Results

### Development of polymicrobial colony model

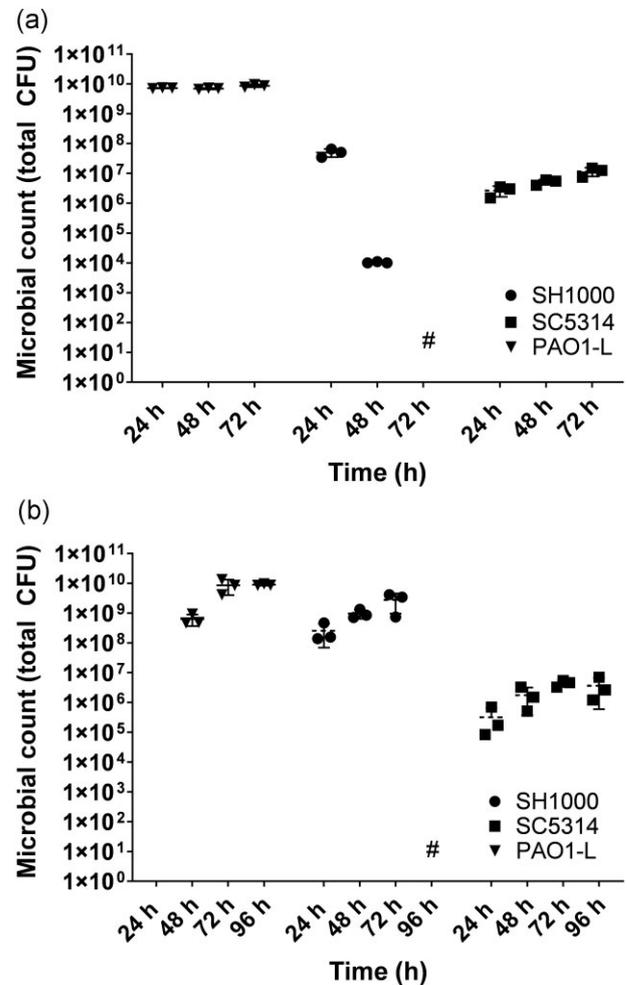
A number of media have been devised to mimic the wound environment, including chronic wound medium (Pouget *et al.* 2022); however, we were unable to establish a stable polymicrobial biofilm for the desired testing period (72 h) and *P. aeruginosa* swarmed off the polycarbonate discs after 48 h. RPMI-1640 has some similarities to the wound environment. RPMI-1640 is routinely used for tissue culture and shares

some similarities with the wound environment, including a neutral pH (chronic wounds range from neutral to alkaline pH), combinations of vitamins, amino acids, and inorganic compounds but lacks serum. RPMI-1640 contains 2 g l<sup>-1</sup> of glucose, which is a high level of blood glucose. High blood glucose is of concern in diabetic patients and has been implicated in poor phagocytosis of wound pathogens by neutrophils and nonhealing of wounds in diabetic foot ulcers (Spampinato et al. 2020). RPMI-1640 media can induce a hyphal phenotype in *C. albicans* compared to other standard media like LB, BHI, and TSB, which were unable to induce this hyphal phenotype. For these combined reasons, RPMI-1640 was investigated as the media of choice for this model alongside its ease of use and being relatively cheap.

To establish the suitability of RPMI-1640 agar for the colony biofilm model, monospecies biofilms were grown for 72 h for *S. aureus* and *C. albicans*, and 48 h for *P. aeruginosa*. All microbial species formed confluent biofilms on the PC discs under these conditions. Initial development of the polymicrobial colony biofilm model focused on the addition of all microbial species concurrently at varying inoculum size: *C. albicans* SC5314 ( $1 \times 10^4$ – $1 \times 10^6$  CFU ml<sup>-1</sup>), *S. aureus* SH1000 ( $1 \times 10^4$ – $1 \times 10^6$  CFU ml<sup>-1</sup>), and  $1 \times 10^4$  CFU ml<sup>-1</sup> for *P. aeruginosa* PAO1-L, as *P. aeruginosa* is known to overgrow other microbes. After 72 h, an average of  $1 \times 10^9$  CFU ml<sup>-1</sup> of *P. aeruginosa* PAO1-L was recovered from all colony biofilms. *S. aureus* SH1000 was not recovered after 72 h and *C. albicans* SC5314 recovery was variable between  $1 \times 10^5$ – $1 \times 10^6$  CFU ml<sup>-1</sup> range (Fig. 1a). Modification was made to the time of addition of microbes with *S. aureus* and *C. albicans* added concurrently at 0 h and incubated for 24 h, following which PAO1-L 24 h was added at 24 h. After 72 h, total incubation time (48 h total for *P. aeruginosa*), *S. aureus* was recovered in contrast to concurrent addition of all three species. However, after 72 h, the stability of the model was lost, with no recovery of *S. aureus* SH1000 at 96 h (Fig. 1b). It was also observed at 96 h that *P. aeruginosa* PAO1-L was beginning to swarm off the polycarbonate disc. Collectively, this result and observation limit the use of the model to 72 h total. Therefore, in all further experiments, an inoculum of 10 µl of  $1 \times 10^5$  CFU ml<sup>-1</sup> *C. albicans*,  $1 \times 10^6$  CFU ml<sup>-1</sup> *S. aureus*, and  $1 \times 10^4$  CFU ml<sup>-1</sup> *P. aeruginosa* was used.

### Determination of the polymicrobial biofilm architecture in the colony biofilm model

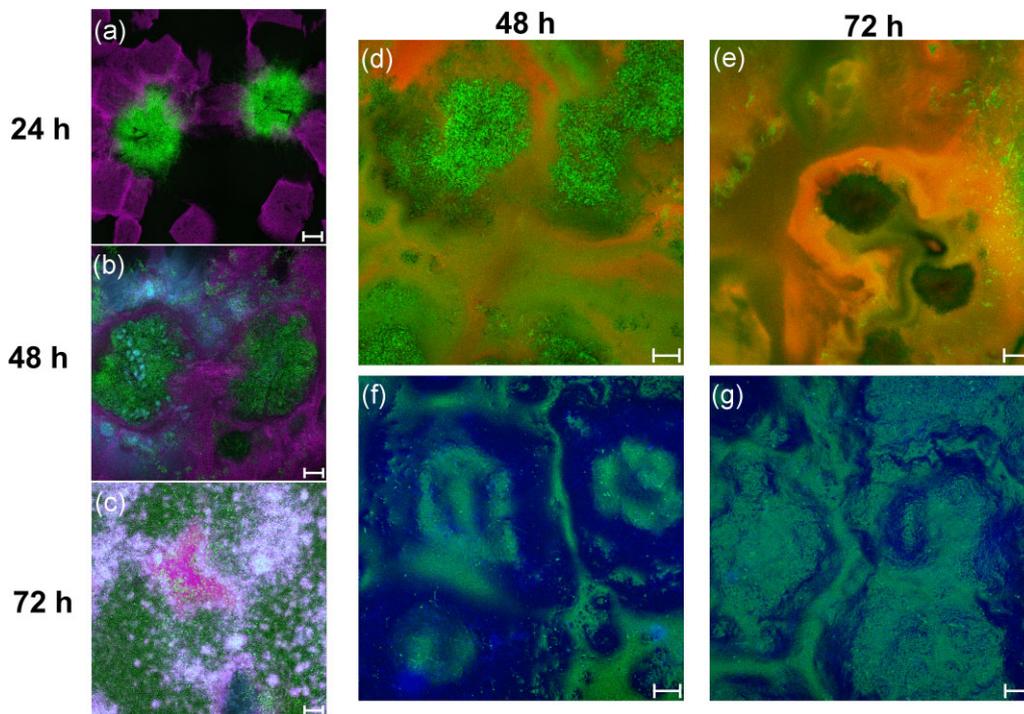
Localization of microbes in relation to each other is important in polymicrobial interactions (Wolcott et al. 2013), particularly, so between *P. aeruginosa*, *S. aureus*, and *C. albicans* where quorum sensing signalling molecules are known to interact across species and kingdom taxa (Hotterbeekx et al. 2017, Carolus et al. 2019, Grainha et al. 2020). Using strains modified to express fluorescent proteins, the spatiotemporal localization was investigated at 24 h to 72 h to determine the spatial orientation of the microorganisms in the colony biofilms over time. Representative micrographs are shown in Fig. 2. It was noted that *C. albicans* formed large budding yeast structures with hyphal projections emanating from the periphery and *S. aureus* localized around and between these structures in layers (Fig. 2a). This observation was maintained at 48 h and 72 h, with *P. aeruginosa* being initially distributed in areas surrounding but distinctly separate from regions where *S. aureus* was present (Fig. 2b). These regions of *P.*



**Figure 1.** Microbial population stability with concurrent and delayed addition of *P. aeruginosa* PAO1-L. (a) Concurrent addition of all three species led to the population of *S. aureus* SH1000 being unstable and nonrecoverable at 72 h. (b) Delayed addition of *P. aeruginosa* PAO1-L at 24 h led to stable microbial populations between 24 and 72 h for all three species. At 96 h, there was a small reduction in recovered *C. albicans* SC5314 and no recovery of *S. aureus* SH1000.  $N = 3$ . #—recovery below LOD.

*aeruginosa* did not co-localize with *S. aureus* earlier in the establishment within the biofilm, but at 72 h, it was co-localizing with *C. albicans* hyphae and *S. aureus* (Fig. 2c).

Biofilms are typically characterized by the production of a self-derived matrix of which polysaccharides and eDNA are key components. To establish, if matrix is formed in the colony biofilm, model Concanavalin A conjugated to Alexa Fluor 633 and YOYO-1 were used to visualize the PAO1-L exopolysaccharide and eDNA, respectively. In addition, live/dead staining was performed to determine the viability of the microbial population. At 48 h, there was minimal evidence of dead cells (Fig. 2d) with exopolysaccharide and eDNA present (Fig. 2f). At 72 h, there was visual evidence of an increased number of dead cells, likely attributed to the proliferation of *P. aeruginosa* (Fig. 2e) and its known impact on *S. aureus*. A complex structure of exopolysaccharide was observed (Fig. 2g), alongside an increased eDNA quantity compared to 48 h. There was some visible correlation between ConA and *C. albicans* especially the dense pockets of yeast morphology with protruding hyphae. eDNA is especially present where *P. aeruginosa*



**Figure 2.** Spatiotemporal localization of three species in a polymicrobial colony biofilm and matrix distribution. (a) Twenty-four hours with *C. albicans* (green) mixed structure of yeast and hyphal morphology visible, *S. aureus* (magenta) is observed growing around and attached to the hyphal network. (b) At 48 h, *P. aeruginosa* (cyan) is observed growing around the *C. albicans* hyphae and *S. aureus*. (c) At 72 h, a greater localization of the three species is observed where *P. aeruginosa* is visually more prevalent throughout the field of view. (d) Live/Dead (Syto 9/PI) staining of polymicrobial colony biofilms at 48 h shows minimal red staining indicating low cell death, note also the clearly visible *C. albicans* structures comprised of yeast and hyphal morphologies. (e) Live/Dead staining of polymicrobial colony biofilms at 72 h shows increased red staining indicating either increased cell death or staining of eDNA; it may also indicate a combination of both. (f) At 48 h, ConA-AF633 (green) was used to stain for exopolysaccharide and YOYO-1 (blue) was used to stain eDNA. (g) Matrix and eDNA staining at 72 h shows a complex distribution of these macromolecules. Scale bars = 100  $\mu\text{m}$ .

is localized. The representative micrographs provide clear indication that matrix components are being produced in the colony biofilm model strongly indicating a biofilm phenotype. Visualization of the polymicrobial biofilms (Fig. 3) with SEM indicates discrete regions containing *S. aureus* and *Ps. aeruginosa*. *Staphylococcus aureus* cells are observed attaching to *C. albicans* hyphae as described in the literature (Schlecht *et al.* 2015). *C. albicans* are present in both the hyphal and yeast morphology. The observed clustering of *S. aureus* in this model is similar to that seen from an *S. aureus* infected wound SEM (Li *et al.* 2016), which showed large amounts of cocci-shaped bacteria encased within an extracellular matrix; however, the representative micrographs shown only showed the presence of *S. aureus*. *P. aeruginosa* grown in an *ex-vivo* human skin wound model and visualized by SEM (Li *et al.* 2023) demonstrated cluster biofilm growth and encapsulation within an extracellular matrix. While *P. aeruginosa* is observed in this model, it is not observed to form the same clustering as seen in the mono-species *ex-vivo* human skin model. There are insufficient studies visualizing *C. albicans* by SEM in wounds, therefore, a comparison to morphology and clustering and the polymicrobial interaction.

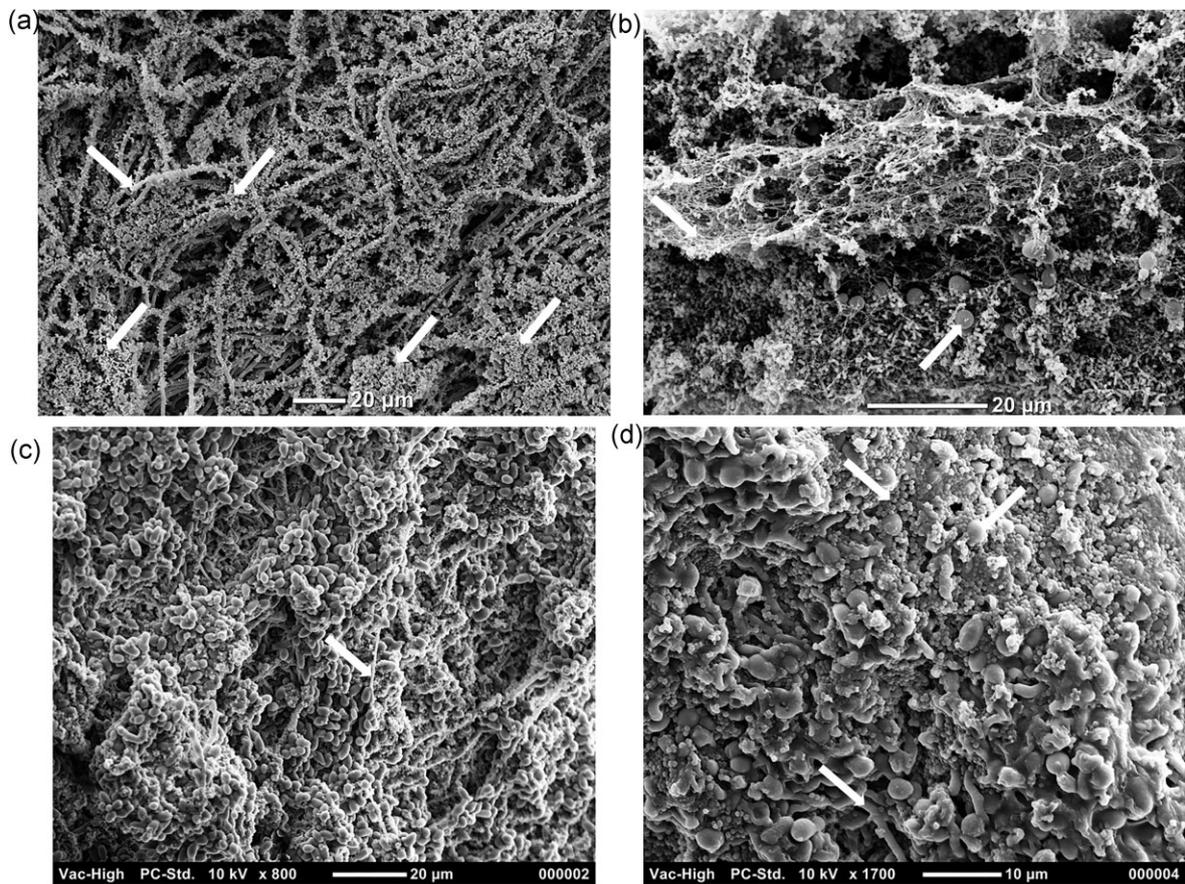
### Evaluation of antimicrobial susceptibility differences between monospecies and polymicrobial colony biofilm models

To investigate whether there are differences in antimicrobial-susceptibility between microorganisms grown as single species biofilms and as polymicrobial communities using the colony

biofilm model, biofilms were subjected to treatment with 1% chlorhexidine [CHX (Sigma–Aldrich, UK)], an antiseptic widely used to decolonize the skin of patients before medical procedures and in antimicrobial oral rinses. The colony biofilm of *P. aeruginosa* PAO1-L was not susceptible to 1% CHX treatment ( $P = .1127$ ) (Fig. 4a). Colony biofilms of *S. aureus* SH1000 and *C. albicans* SC5314 were susceptible with a 5-log and 2-log reduction, respectively ( $P = .0056$  and  $P = .0072$ ) (Fig. 4b and c). Treatment of the polymicrobial biofilms with 1% CHX resulted in no log reduction for *P. aeruginosa* ( $P > .9999$ ) and *C. albicans* ( $P > .9999$ ) and a 2-log reduction in *S. aureus* that was not significant ( $P = .1799$ ) (Fig. 4d) with some small colony variants observed on the MSA agar plates.

### Antimicrobial wound dressing testing using monospecies and polymicrobial planktonic suspensions

Prior to investigating the effectiveness of the wound dressings in the colony biofilm model, an assessment of antimicrobial wound dressings against planktonic cultures was performed. To do this, a modified AATCC100-2004 bacterial reduction method in single species and polymicrobial cultures was used as indicated in the ‘Materials and methods’ section. As expected, all wound dressings (Fig. 5) were effective in reducing the planktonic microbial burden below the limit of detection (LOD = 50 CFU  $\text{ml}^{-1}$ ) of the microbial enumeration in both single and polymicrobial cultures.



**Figure 3.** SEM visualization of 48 h polymicrobial colony biofilm model. Representative micrographs of polymicrobial biofilms (a) *C. albicans* hyphae visible (arrow), with adhered *S. aureus* along the length of the hyphae. *C. albicans* yeast morphology is visible in aggregated clusters through the visualized structure. (b) Dehydrated biofilm matrix (arrow), an artefact created through the fixation process and multiple rounds of dehydration and critical drying. *C. albicans* in yeast morphology amongst *P. aeruginosa* indicated by lower white arrow. (c) 800× magnification biofilm showing enhanced resolution of the *C. albicans* yeast and hyphae morphology with *S. aureus* adhered to the hyphae only. (d) 1700× magnification micrograph showing clear *C. albicans* yeast and hyphae morphology (arrows) and aggregated *S. aureus*. Biofilm matrix components are also visible. All SEM images captured using electron beam at 10 kV.

### Testing the effectiveness of the antimicrobial wound dressings versus monospecies and polymicrobial biofilms

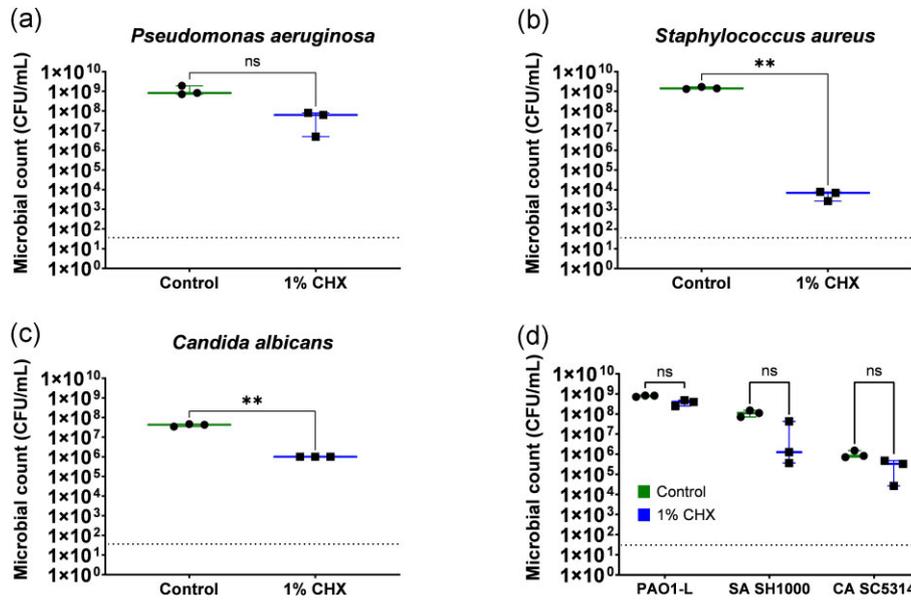
Polymicrobial biofilms were grown on the polycarbonate disks and wetted wound dressing sections placed on top of the biofilms with sterile forceps. The Acticoat™ Flex 7 wound dressing was difficult to wet due to its high hydrophobicity. Aquacel® Ag advantage did not significantly reduce CFUs in the three monospecies colony biofilms of *P. aeruginosa* PAO1-L, *S. aureus* SH1000, and *C. albicans* S5314 (Fig. 6a–c). Acticoat™ Flex 7 was effective in reducing the microbial burden by 4-log versus *P. aeruginosa* PAO1-L, 1.5 log versus *S. aureus* SH1000, and no significant reduction was observed versus *C. albicans* SC5314. Treatment with Exufiber® Ag + reduced microbial burden of PAO1-L, *S. aureus* SH1000, and *C. albicans* SC5314 by less than one log. Iodoflex reduced the recoverable microorganisms in all of the monospecies colony biofilms to levels below the LOD of the assay.

*P. aeruginosa* and *S. aureus* showed reduced susceptibility against the wound dressings Aquacel™ Ag + advantage, Acticoat™ Flex 7 and Exufiber® Ag + in the polymicrobial compared the single species colony biofilms (Fig. 6d).

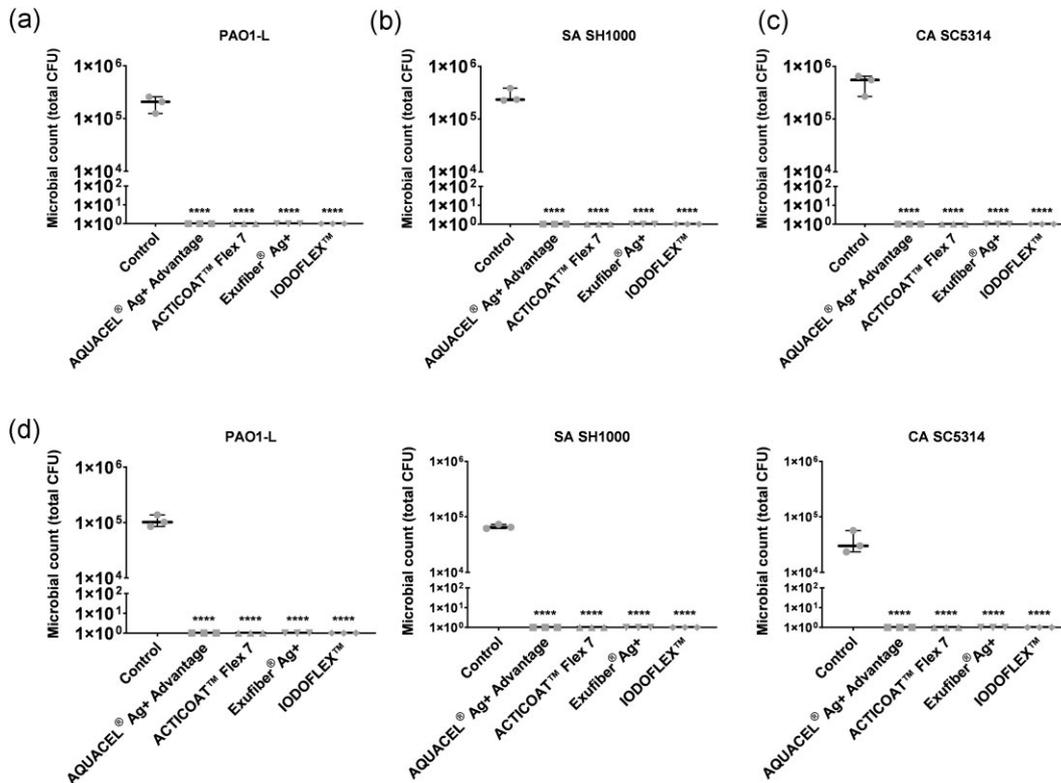
Iodoflex™ showed no recovery (below the LOD of the assay) of *C. albicans* and *S. aureus* in both types of biofilms.

Initial assessment using the colony biofilm model showed silver based wound dressings were generally ineffective against mature biofilm structures. In the clinical management of biofilms, sharp surgical debridement is still seen as a gold standard of wound care (Sen et al. 2021), where established biofilms are disrupted and in part resuspended for synergistic antimicrobial approaches. To partially replicate this clinical approach, colony biofilms were established as previously described then disrupted by vortexing only prior to wound dressing challenge, resulting in disrupted aggregates and a slightly cloudy suspension of microbial cells.

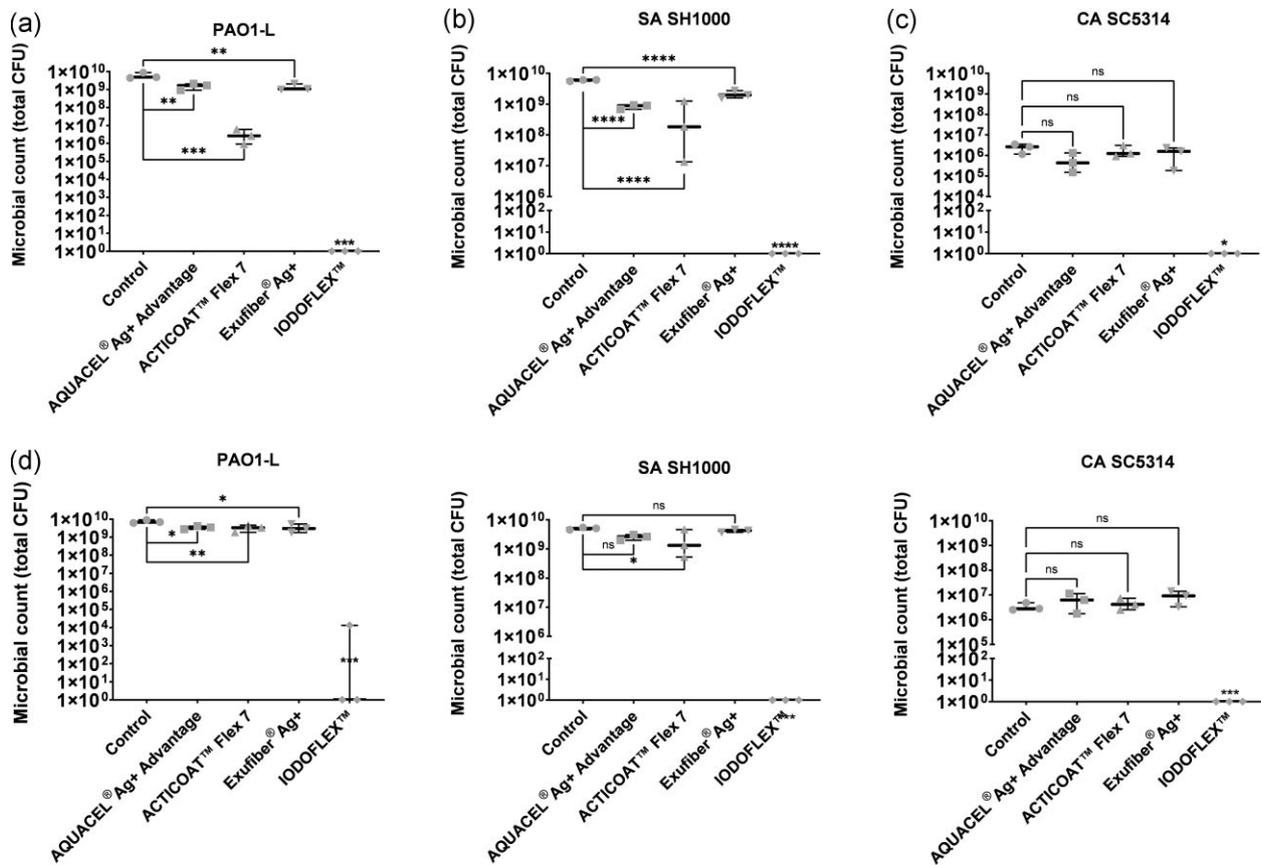
All biofilm dressings showed activity against the disrupted polymicrobial biofilm (Fig. 7). Aquacel® Ag + advantage demonstrating a 2-log reduction for *P. aeruginosa*, 2-log reduction for *S. aureus*, and 1-log reduction in *C. albicans*. Acticoat™ Flex 7 showed a 3-log reduction for *P. aeruginosa*, 3-log reduction for *S. aureus*, and 3-log reduction for *C. albicans*. Exufiber® Ag + caused a 2-log reduction for *P. aeruginosa*, a 1-log reduction for *S. aureus*, and 0.5-log reduction for *C. albicans*. Iodoflex™ was effective in reducing the microbial burden below LOD.



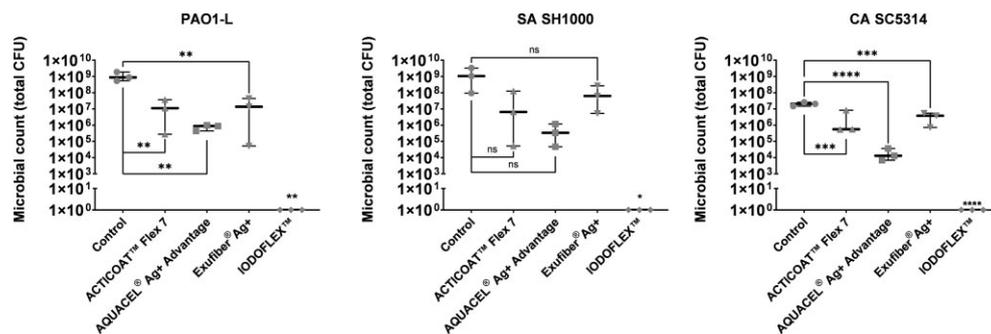
**Figure 4.** Treatment of monospecies and polymicrobial biofilms with 1% chlorhexidine gluconate. (a) *P. aeruginosa* PAO1-L was not susceptible to 1% CHX treatment. (b) *S. aureus* SH1000 was susceptible to 1% CHX treatment. (c) *C. albicans* SC5314 was susceptible to 1% CHX. (d) *P. aeruginosa* and *C. albicans* in the polymicrobial biofilms were not susceptible to 1% CHX with no significant log reduction. (a)–(c) Student *t*-test, (d) 1% CHX challenge of polymicrobial biofilm versus control microbial recovery. Two-way ANOVA with Šidák multiple comparison correction. \*\*\**P* < .01, LOD 50 CFU ml<sup>-1</sup>, *n* = 3.



**Figure 5.** Antimicrobial textile microbial reduction test. Microbial enumeration was performed at 24 h endpoint where wound dressings were sonicated in Dey–Engley neutralizing broth, and 10-fold serial dilutions performed on appropriate selective media. All wound dressing reduced planktonic counts below of the limit of detection of (a) *P. aeruginosa* PAO1-L, (b) *S. aureus* SH1000, and (c) *C. albicans* SC5314. (d) All three species were grown separately overnight and pooled using the same inoculum size as in (a)–(c), all wound dressings reduced microbial counts below the limit of detection. Limit of detection was 50 CFU. (a)–(c) One-way Anova with Dunnett multiple comparison correction. (d) Two-way ANOVA with Šidák multiple comparison correction. \*\*\*\**P* < .0001, *n* = 3. LOD 250 CFU. *n* = 3.



**Figure 6.** Microbial enumeration of polymicrobial biofilms treated with four wound dressings. Microbial enumeration was performed at 72 h endpoint biofilms were disaggregated, sonicated in Dey–Engley neutralizing broth and 10-fold serial dilutions performed on appropriate selective media. Monospecies colony biofilms of (a) *P. aeruginosa* PAO1-L, (b) *S. aureus* SH1000, and (c) *C. albicans* SC5314. (d) Polymicrobial colony biofilm. The names of the dressings used are specified in the axis. Controls are untreated wetted filter paper. Two-way ANOVA with Dunnett's multiple comparison correction was used. LOD 250 CFU.  $n = 3$ .



**Figure 7.** Microbial enumeration of dispersed polymicrobial biofilms treated with four wound dressings. At 48 h, biofilms were disaggregated by vortexing and challenged with each wound dressing or wetted filter paper for control for 24 h. Dey–Engley neutralizing broth was added to each Bijou prior to sonication and microbial enumeration was performed as described with 10-fold serial dilutions performed on appropriate selective media. Limit of detection was 50 CFU ml<sup>-1</sup>,  $n = 3$ . Two-way ANOVA with Dunnett's multiple comparison correction.

## Discussion

The management of infected wounds is complex with a combination of debridement, systemic antibiotics, and wound dressings utilized. As with antibiotic use to treat specific microbial infections, there are an array of wound dressings available for the treatment of infected wounds. Dressing formats may be appropriate for the volume of exudate produced, e.g. low, moderate, or heavy. *P. aeruginosa* and *S. aureus* are the most commonly isolated and studied microbes in relation to chronic

wounds (Puca et al. 2021). It is well documented that their presence in wounds causes significant delays in wound healing and have significant risk association with morbidity (Han and Ceilley 2017). There is growing evidence that fungi can be part of the wound microbiome; however, the role these play in chronic wound pathogenesis is poorly understood (Ge and Wang 2022, Short et al. 2023). It is however abundantly clear that chronic wounds often contain cross-interacting polymicrobial communities. Although some of these interactions are known, there are still many that remain to be identified par-

ticularly those involving anaerobic microorganism. *P. aeruginosa* exoproducts have been shown to have both antimicrobial sensitizing and protective properties. *P. aeruginosa* rhamnolipids increase tobramycin uptake and efficacy against *S. aureus* (Radlinski *et al.* 2017). *P. aeruginosa* 2-n-heptyl-4-hydroxyquinoline *N*-oxide (HQNO), HCN, and pyocyanin can increase *S. aureus* tolerance to killing by ciprofloxacin, tobramycin, and vancomycin through the inhibition of respiration and depletion of ATP (Radlinski *et al.* 2017). Conversely, HQNO has been shown to increase *S. aureus* sensitivity to chloroxylonol (Orazi *et al.* 2019). *P. aeruginosa* has also been observed to protect *S. aureus* in mixed biofilms from tobramycin.

Dual species *C. albicans* and *S. aureus* biofilms have been shown *in vivo* and *in vitro* to reduce sensitivity of *C. albicans* to miconazole (Kean *et al.* 2017). Protection of *S. aureus* by the secreted  $\beta$ -1,3-glucan cell wall component can result in enhanced tolerance of *S. aureus* to vancomycin *in vitro*, which could be overcome by addition of the antifungal caspofungin (Kong *et al.* 2016).

Three silver dressings were selected for this study as representative of those used in clinical settings. An iodine-containing wound dressing was included due to the broad-spectrum antimicrobial action of the active ingredient versus Gram-negative and positive bacteria, fungi, viruses, and protozoa (Bigliardi *et al.* 2017). There has been a wide range of literature demonstrating wound healing promotion with silver nitrate wound dressings however excessive loading of silver nitrate can inhibit wound healing (Atiyeh *et al.* 2007, Asha-Rani *et al.* 2009), and increased host tissue toxicity (Burd *et al.* 2007, Hiro *et al.* 2012). With advances in nanotechnology, formulation of silver nanoparticles (AgNPs) (Paladini and Pollini 2019) incorporation into wound dressings is desirable. The topic of antimicrobial resistance to silver has been debated over the last two decades (Percival *et al.* 2005) with sporadic evidence presented for silver resistance; however, plasmid-mediated silver resistance has been clinically observed in *P. aeruginosa*, *Acinetobacter baumannii*, and *S. aureus* isolates (Hosny *et al.* 2019). Repeated exposure to subinhibitory concentrations of silver nanoparticles has been shown to induce rapid resistance in *E. coli* and *P. aeruginosa* *in vitro* (Panáček *et al.* 2018), which may be a cause for concern particularly as there is emerging evidence that silver and other heavy metals can co-select for antibiotic resistance (McNeilly *et al.* 2021). Iodine-containing wound dressings have contraindications for some patient groups but have demonstrated that they do not interfere with wound healing or have cytotoxic effects at clinically relevant concentrations (Vermeulen *et al.* 2010). It is also of note that there is evidence of wound healing promotion by Povidone-iodine in mice through increased expression of TGF- $\beta$ , and promotion of neovascularisation and re-epithelialisation (Wang *et al.* 2017). Microbial resistance development to iodine has not been documented to date (Barreto *et al.* 2020).

The format of this polymicrobial biofilm model makes it amenable to the investigation of antimicrobial wound care products because wound dressings can be laid on top of the biofilm. This is in contrast to three-dimensional biofilm models like the CDC reactor or nonsupported biofilm models like the Lubbock biofilm model. Also, microbes are easily recovered because the entire membrane can be placed into neutralizing or recovery buffer. Furthermore, the membrane supports transfer of the biofilm without disruption of

the structure, making the model amenable to microscopic examination. This simplified wound model tested provides a low cost, medium throughput assay that can be assessed using traditional microbiological methodologies by performing colony forming units counts. Microscopy-based methodologies can also be performed with live/dead visualization and visualization of the matrix components produced by the monospecies and polymicrobial biofilms. Additional quantification methodologies that could be conducted but were not studied here include measurement of ratios of fluorescent expressing proteins and investigation of matrix components.

The difference in antimicrobial effectiveness of chlorhexidine in monospecies and polymicrobial biofilms in this study demonstrates that living in a polymicrobial environment changes antimicrobial susceptibility. This is consistent with literature demonstrating differences in antibiotic and broad-spectrum antiseptic susceptibility in polymicrobial systems (Stoffel *et al.* 2020). When monospecies biofilms were treated with commercially available antimicrobial wound care products, the *P. aeruginosa* biofilms were susceptible to killing by one of the silver-containing products (Acticoat™ Flex 7). In contrast, *P. aeruginosa* living in complex, polymicrobial biofilms was not susceptible to killing by this product. Other silver-containing wound care products, however, were effective at significantly reducing the log (CFU biofilm<sup>-1</sup>) of *S. aureus* and *C. albicans* in monospecies and polymicrobial biofilms. This result suggests that *P. aeruginosa* in polymicrobial biofilms is protected against killing by some antimicrobial formulations. The iodine-containing wound care product, Iodoflex, killed microorganisms to a level below the limit of detection in monospecies and polymicrobial biofilms except for one replicate of a polymicrobial biofilm, where there was some surviving *P. aeruginosa* detected. This result indicates that the polymicrobial biofilm system as grown here does not protect against the oxidative killing mechanism of iodine. This is consistent with published literature where Cadexomer iodine was effective in reducing the bacterial biofilm in porcine *ex vivo* model while silver carboxymethylcellulose dressings had minimal impact on biofilms (Roche *et al.* 2019). IoPlex® with I-Plexomer is a controlled release iodine foam dressing and has been shown to have broad-spectrum biofilm management in CDC ASTM 2871–19, drip flow bioreactor, and direct contact testing (Salisbury *et al.* 2022), which included anaerobic bacteria but not fungi. In contrast, silver wound dressings showed some activity in comparison to the whole direct contact biofilm method demonstrated here.

With regards to mimicking the wound debridement and subsequent application of antimicrobial dressing, an increased killing was observed compared to the intact colony biofilm model with the silver wound dressings. Debridement and combined wound debridement is often associated with better resolution of a wound and can be combined with antimicrobial wound dressings where appropriate (Nowak *et al.* 2022). It should be noted though that a review by NICE in 2016 (NICE 2016) concluded that ‘there was no evidence that silver dressings are effective for complete wound healing and measures of infection were not assessed’. It is not possible from this study to conclude that silver dressings are ineffective for wound management given the lack of measurement of infection in this NICE report. Further investigation with silver wound dressings with clinical isolates of *P. aeruginosa* and *S. aureus* would help to further assess the utility of silver

wound dressings in microbial resolution alongside incorporation into simple wound healing models (Jordana-Lluch et al. 2020).

Limitations of this study include the *in vitro* nature of the model, which lacks host-derived macromolecules, immune system components, and some wound fluid components. The model is grown on solid agar with a limited amount of moisture and lack of dynamic flow conditions, which could be observed in highly exuding wounds. The model is grown in an oxygenated environment and may not replicate microaerophilic and anaerobic conditions, which exist in *in vivo* wound conditions. Anaerobic bacteria have often been observed in metagenomic studies of wound biofilms and this model does not include strict anaerobes, but the model is amenable to use in microaerobic conditions. The model is stable for 72 h but was unstable at 96 h, this demonstrates that the model has temporary stability a common limitation observed in, *in vitro* models, e.g. cystic fibrosis (O'Brien et al. 2021); however, our model generally has greater throughput and a lower cost scale.

Human serum albumin composes up to 60% of the whole blood protein and therefore be an important molecule in wounds. Silver nanoparticles in the selected wounds dressings are used to generate silver ions; however, albumin both human (Gnanadhas et al. 2013) and bovine (Grade et al. 2012) has been demonstrated to bind silver and silver ions reducing their efficacy. The model could be improved by the addition of human serum albumin, which has also been demonstrated to attenuate killing of *S. aureus* by *P. aeruginosa* (Smith et al. 2017), which could lead to an increase in model stability. Furthermore, nutrient conditions could be modified further to influence antagonistic interactions between *S. aureus* and *P. aeruginosa*. The model could be modified to include dynamic flow of microbial growth medium or simulated wound fluid to further challenge wound dressings, or to include host cell macromolecules such as collagen.

## Conclusions

The polymicrobial colony biofilm model presented shows promise as a medium throughput, low-cost model to study the effectiveness of antimicrobials and novel compounds, which could be standardized. Treatment with silver-containing antimicrobial wound dressings in this model did not result in significant log reduction of microorganisms when tested against whole biofilms with minimal log reductions versus the disrupted colony biofilm. Cadexomer iodine wound dressing eradicated both monospecies and polymicrobial biofilms; however, iodine-containing wound dressings have contraindications in some patients (Formulary 2023). This result suggests differences in the mechanism of action in biofilms between released ingredients in antimicrobial wound care products and those retained within the wound dressing. Differences in antimicrobial activity between products may also result from differences in neutralization kinetics by biofilm macromolecules or kinetics in release of antimicrobial molecules. These results suggest there may be differences in clinical effectiveness between products as tested in the whole and disrupted biofilm models.

The polymicrobial colony biofilm model represents also a useful tool to study interactions between clinically relevant microbes, investigating phenotypic interactions and the regulation of cross-signalling processes. Media choice can be

adapted to those that mimics *in vivo* conditions, e.g. SCFM2 for Cystic Fibrosis (Turner et al. 2015) and wound like media (Sun et al. 2008, Pouget et al. 2022). The easy handleability of the polycarbonate membrane discs lends itself to good throughput for multiple end point assays, from basic microbial enumeration, microscopic examination, viability PCR through to mass spectrometry analysis of key microbial metabolites (Robertson et al. 2023).

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## Author contributions

Shaun N. Robertson (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing), Manuel Romero (Investigation, Methodology, Resources, Visualization, Writing – original draft, Writing – review & editing), Samuel Fenn (Methodology, Resources, Writing – original draft, Writing – review & editing), Petra L. Kohler Riedi (Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing) and Miguel Cámara (Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing).

## Data availability

The datasets generated are available from the National Biofilms Innovation Centre at the University of Nottingham. DOI: 10.17639/nott.7324.

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