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Modelling antisepsis using defined populations of facultative and anaerobic wound pathogens grown in a basally perfused biofilm model

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Running head: Modelling wound antisepsis

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Abstract

An *in vitro* model was developed to assess the effects of topical antimicrobials on taxonomically defined wound biofilms. Biofilms were exposed over 7d to povidone-iodine, silver acetate or polyhexamethylene biguanide (PHMB) at concentrations used in wound dressings. The rank order of susceptibility in multi-species biofilms, based on an analysis of the average bacterial counts over time (low to high) was *P. aeruginosa*>Methicillin-resistant *Staphylococcus aureus* (MRSA)>*B. fragilis*>*S. pyogenes*. The rank order of effectiveness for the antimicrobials in the biofilm model was povidone-iodine>PHMB>silver acetate. None of the test compounds eradicated *P. aeruginosa* or MRSA from the biofilms although all compounds except silver acetate eliminated *S. pyogenes*. Antimicrobial effectiveness against bacteria grown in multi-species biofilms did not correlate with planktonic susceptibility. Defined biofilm populations of mixed-species wound pathogens could be maintained in the basal perfusion model, facilitating the efficacy testing of treatments regimens and potential dressings against multi-species biofilms composed of wound isolates.

Introduction

Chronic wounds represent a considerable challenge to wound care professionals and healthcare resources, having a significant bearing upon patient morbidity and mortality (Scali and Kunimoto 2013). Whilst the processes influencing the transition to a chronic wound state are complex and depend on host factors such as wound aetiology, co-morbidities and anatomical location, infection and the presence of biofilms have been implicated as important contributors (Roy et al. 2014, Scali and Kunimoto 2013, Schierle et al. 2009). Biofilms have been described as structured microbial communities encased in an exopolymer matrix (Costerton 1999, Hall-Stoodley et al. 2004) that are normally taxonomically diverse and adapted to survive inimical conditions (Gilbert et al. 2002, Watanabe et al. 1998). Evidence suggests that through cellular proximity, biofilm formation can enhance horizontal gene transfer rates and may therefore drive bacterial the transfer of resistance determinants (Hausner and Wuertz 1999, Sorensen et al. 2005) and microbial evolution over longer timescales (as reviewed by Claessen *et al.,* 2014). Through genotypic and phenotypic heterogeneity, biofilms exhibit substantially increased tolerance to antimicrobial therapies and host immune responses (Gilbert et al. 2002, Pedersen 1992, Stewart and Costerton 2001).

The association of biofilms with chronic wounds (Bjarnsholt et al. 2008, Kanno et al. 2009, Neut et al. 2011, Roy et al. 2014, Wolcott and Rhoads 2008) has driven the development and application of antimicrobial dressings based on their effectiveness against the most recalcitrant forms of microbial growth present in this environment, which includes organisms displaying biochemical resistance, such as *Pseudomonas* spp. (Ramos et al. 2010, Rochex and Lebeault 2007, Walters et al. 2003), Methicillin resistant *Staphylococcus aureus* (MRSA) (McCarthy et al. 2015, Ohadian Moghadam et al. 2014) and microbial biofilms, that generally exhibit greater tolerance to antimicrobials than their planktonic counterparts regardless of taxonomic composition (Gilbert et al. 2002).

A variety of antimicrobial wound dressings are available incorporating active compounds such as iodine and silver that are designed with broad-spectrum antimicrobial activity in mind (Barnea et al. 2010, Bradley et al. 1999, Butcher 2012). *In vitro* models supporting the growth of biofilm communities (Oates and McBain 2016) can be used in the preclinical investigation of antimicrobial dressings for their potential effectiveness against wound bacteria (Humphreys et al. 2011, Werthén et al. 2010, Wilkinson et al. 2016, Woods et al. 2012). Several models have been developed specifically for investigating wound-associated biofilms, including the Lubbock Chronic Wound Biofilm, which is a closed, static media system (Sun et al. 2008), a flat-bed perfusion model that comprises a continuous media flow system with an inert substratum (Thorn and Greenman 2009), and the modified drip-slide reactor (Lipp et al. 2010). Various other continuous culture systems have also been developed for biofilm applications including the Constant Depth Film Fermenter (CDFF) and devices housing Sorbarods (cylindrical cellulose filters). These models have been used for a range of applications including wound biofilm investigations (Hill et al. 2010, Hodgson et al. 1995). Axenic or combined cultures of bacteria of relevance to wounds such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* have been utilised in *in vitro* models in several previous investigations (Oates and McBain 2016, Thorn and Greenman 2009, Werthén, Henriksson, Jensen, Sternberg, Givskov and Bjarnsholt 2010) and can generate robust and reproducible data, allowing for example, the examination of axenic biofilm growth dynamics and responses to antimicrobials. Defined community systems where a small number of culturable bacteria are grown together in a biofilm model are however, likely to be more representative of the communities which they aim to represent as opposed to single species systems (Thorn and Greenman 2009).

In the current investigation, a model in which the effectiveness of topical antimicrobials can be assessed against four functionally distinct wound pathogens grown together in biofilms was developed. The system has several features of use for preclinical evaluation of antimicrobials for

application to wounds since biofilms grown in the system are fed basally like the nutrient supply in chronic wounds, and it supports the growth of obligate aerobic, anaerobic and facultative bacterial organisms, enabling assessment of the differential effects of distinct classes of antimicrobial against broad physiological groups of wound pathogens.

Methods

Bacteria

Four bacteria of significance to wound infections and with variable growth requirements were selected for inclusion in the defined biofilm consortium. *Pseudomonas aeruginosa* and *Streptococcus pyogenes* were isolated from infected wounds. Methicillin resistant *Staphylococcus aureus* (MRSA) NCTC 11939 and *Bacteriodes fragilis* NCTC 9343 were obtained from Public health England Southampton, UK.

Chemicals and media

Vantocil (a 20% [vol/vol] aqueous solution of polyhexamethylene biguanide) was obtained from Arch Biocides, Inc. (Manchester, United Kingdom). Dehydrated bacteriological media were obtained from Oxoid (Basingstoke, Hampshire, U.K.) and reconstituted per instructions supplied by the manufacturer. Unless otherwise stated all other chemicals used were supplied by Sigma (Poole, Dorset, U.K.).

Model Development and Optimisation

Relative fitness assays

To assess whether the four bacterial strains selected for the mixed wound consortium could grow stably when combined, relative fitness was assessed in pair-wise combinations during growth on 5mm x 5mm mono-acetate filters (BullBrand Ltd. Keighthly, UK) using a modified competition

127 assay (Lenski et al. 1991, Rozen et al. 2007), as follows: stationary phase cultures were grown
128 overnight in a simulated wound fluid (SWF) medium consisting of foetal calf serum (49% v/v),
129 Mueller-Hinton broth (49% v/v) and laked horse blood (2% v/v). Axenic cultures were then adjusted
130 to achieve an initial inoculum density of *c.* 1 x 10^8 CFU/ml and combined in a 1:1 ratio with a
131 partner culture and then further diluted in fresh sterile SWF to give a final density of *c.* 1 x 10^6
132 CFU/ml. The final combined inoculum was dispensed (1 ml) onto 5mm x 5mm monoacetate filters
133 (n=6) housed within the wells of a microtitre plate. To determine the initial starting CFU/mm^3 of
134 each bacterial isolate, three filters were aseptically removed, aseptically sectioned, with sections
135 placed in a plastic Universal bottle (Scientific Laboratory Supplies, Nottingham, UK) containing 9
136 ml of half-strength Thiogylcolate Broth and 5 mm sterile glass beads (n = 5) (Merck, Darmstadt,
137 Germany). To ensure uniform distribution of cells throughout the diluent, Universals were vortexed
138 for 10 sec., serially diluted in half strength thioglycolate broth and plated onto suitable selective
139 agars, as follows: Mannitol Salt Agar, Pseudomonas CFC selective agar, G-N Anaerobe Selective
140 Agar and Streptococcus Selective Agar. The remaining filters were incubated for 48 h and then
141 viable counts were performed to determine the endpoint CFU/mm^3.
142
143 ***Basal perfusion wound biofilm models***
144 A diagram of the basal perfusion model is shown in Figure 1 and 2. Briefly, the system consists of a
145 hollow cylinder Pyrex glass outer casing (8 cm Ø x 4.5 cm depth) housing three machined
146 polytetrafluoroethylene (PTFE) components comprising a lid, a central receptacle and a waste
147 reservoir. The lid measures c. 8 cm Ø and contains a central inlet tube to deliver media to the central
148 receptacle. The central receptacle is machined from PTFE and contains a central fluid basin (2.2 cm
149 Ø x 3 cm), which is fed with media via the lid inlet tube (Figure 1). There are 8 channels that lead
150 away from the central fluid basin towards 8 individual filter receptacles (1 cm Ø) where each filter
151 resides. Each filter receptacle incorporates a lower fluid bulk section and an upper level receptacle

152 separated by a small PTFE rim (0.5 cm Ø) upon which 5mm x 5mm mono-acetate filters (Bullbrand
153 Ltd. Keighthly, UK) are placed. Medium fed into the central fluid basin is distributed into the lower
154 fluid bulk section of the filter receptacles via the channels shown in Figure 1. The fibres of the 5mm
155 x 5mm mono-acetate filter act as a wick, drawing media up from the bulk fluid, saturating the filter.
156 Drainage channels leading away from the filter receptacles allow the removal of excess media and
157 waste products into the waste reservoir, where it is drained from the whole system via the outlet tube
158 in the Pyrex glass casing. Temperature (37°C) and atmosphere (O_2) were maintained by placing the
159 device in an aerobic incubator. SWF was continually supplied to the device at a rate of *c.* 1.5 ± 0.1
160 ml h^{-1} by peristaltic pump (Minipulse 3, Gilson, Villiers-Le-Bel, France). Once inoculated with all
161 four bacteria, the model was run for up to 8 days.
162
163 ***Inoculation and population stability assays***
164 Initial trials into the inoculation and maintenance of the defined wound consortium (MRSA, *S.*
165 *pyogenes, P. aeruginosa* and *B. fragilis*) determined that a staggered inoculation process was
166 required (data not shown). Prior to inoculation, the system was preconditioned with SWF for 1 h.
167 Stationary phase axenic cultures of MRSA and *S. pyogenes* were each adjusted to give an inoculum
168 density of *c.* 1 x 10^8 CFU/ml, combined in a 1:1 ratio and a further diluted to give cellular density of
169 each organism of *c.* 1 x 10^7 CFU/ml. Filters were inoculated in situ with 1 ml of the dual species
170 inoculum. The basal perfusion model was continuously fed at a rate of 1.5 ± 0.1 ml h^{-1} by peristaltic
171 pump. After 24 h, stationary phase cultures of *P. aeruginosa* and *B. fragilis* were similarly adjusted
172 and combined to give a density of *c*. 1 x 10^7 CFU/ml of each organism and 1 ml of this mixed
173 inoculum was used to inoculate each filter. The model was incubated for a further 24 h under
174 continuous culture conditions before sampling. Filters were then removed every 24 h and placed in 9
175 ml of half strength thioglycolate broth containing 5 mm sterile glass beads (n=5) (Merck, Darmstadt,
176 Germany), vortexed for 10 s, serially diluted in half strength thioglycolate broth and plated onto

177 suitable selective agar as follows to allow for viable counts of each distinct organism, as outlined
178 above. All experiments were undertaken independently, in triplicate.
179
180 ***Homogeneity of population densities across filters***
181 The basal perfusion model was designed to grow wound associated bacterial communities and assess
182 the response to antimicrobial therapies over time. To ensure that the population densities in each
183 filter were homogenous with those in the other filters i.e. a population density of a randomly sampled
184 filter would be approximately the same in another filter within the same model, several independent
185 basal perfusion models were set up as previously described and run to a timed endpoint. Four filters
186 were randomly selected and removed at each end-point and viable counts preformed as previously
187 described. This was continued until viable count data for 4 filters for each individual day over 8 d
188 were completed. Statistical significance was determined using analysis of variance (ANOVA)
189 combined with post-hoc analysis of least significant difference (LSD) test to determine significant
190 difference.
191
192 ***Imaging of biofilms using environmental scanning electron microscopy***
193 Environmental scanning electron microscopy (ESEM) was used to image biofilm communities. A
194 FEI Quanta 200 ESEM was used under a low vacuum (<0.83 torr), permitting interrogation of
195 putative biofilm structures and microcolonies whilst conserving the hydrated state of the sample.
196 Filters were sectioned both transversally and horizontally to ensure that representative images were
197 obtained.
198
199 ***Determination of antimicrobial susceptibility in planktonic culture***
200 The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were
201 determined axenic planktonic populations. Briefly, stationary phase cultures of MRSA, *S. pyogenes,*

P. aeruginosa and *B. fragilis* grown in SWF, were adjusted in fresh sterile SWF media to give a cell density of *c*. 1 x 10^6 CFU/ml. Inocula were dispensed (100 µl) in wells of a flat bottom 96 well microtitre plate within 30 min. of preparation. Stock antimicrobial solutions were prepared in SWF and dispensed (100 µl) into the first column containing bacterial inocula in the microtitre plate. Doubling dilutions of solutions were then made. Plates were incubated in a wet box at 37°C for 24 h. The MIC endpoint was determined as the lowest concentration of antimicrobial at with no visible growth. The MBC was determined by transferring 10 µl of inocula (that showed no growth) and spot plating onto agar plates. Agar plates were incubated for 24 h and then examined for colony growth. The MBC endpoint was determined as the lowest concentration that resulted in no visual growth of colonies.

Assessment of differential antisepsis in the wound model system

To assess the effect of the selected antiseptic agents on biofilm communities a series of basal perfusion models were set up. Concentrations commonly utilized topically in wound care (Bolton 2006, Burks 1998, Butcher 2012, Fong and Wood 2006, Goldenheim 1993) were used that were in all cases greater than the corresponding MICs of the test bacteria. These were povidone-iodine (10% w/v); polyhexamethylene biguanide (PHMB) (0.5 % v/v) and silver acetate (0.05 % w/v). Once systems were established, one untreated filter was removed on day 1 (baseline) and viable counts performed as previously described. The perfused media were then supplemented with the antimicrobial of interest (added to media to produce the desired concentration) and systems were fed with the antimicrobial dosed SWF at rate of 1.5 ± 0.1 ml h^{-1} by peristaltic pump for the remainder of the model run. Filters were removed daily, and viable counts performed as previously described.

Statistical Analysis

Data analysis was conducted using a linear mixed-effects model to analyse the average bacterial count over time to answer two questions. The first pertained to the stability of the model and the second the effect of each treatment against the control. For both questions, a step-wise nested modelling approach was taken where after each step a p-value from the likelihood ratio-test was reported.

For model stability we first analysed the technical replicates and assumed they are independent from each other; that is, we ignored which biological replicate and which bacteria the technical replicate was from. We then assessed the importance of knowing which biological replicate the technical replicates belonged to. The final step assessed the importance of knowing which bacteria the biological replicate belonged to. For the assessment of treatment effect, the model stability data (control data) was pooled with the treated data for each treatment. This pooled data was first analysed by ignoring whether the data was from treated or control group. We then assessed the importance of knowing for which time-series belonged to the control versus treated group. For each treatment we reported the size of the effect compared to control as a % drop with 95% confidence intervals. All data was Log_{10} transformed for the analysis and was conducted in Rv3.1.1 using the *nlme* library.

Results

Developing, Characterising and Optimising the Basally Perfused Model

Relative fitness assay

The growth of wound bacteria in binary culture was assessed using monoacetate filters and SFW in a competition assay. A relative fitness close to 1.0 was achieved for all combinations (Table 1). Values

below 1.0 would indicate that the bacterium was less competitive than its congener. Values above 1.0 indicate the advantageous growth of the organism in co-culture, at the cost of growth of the other organism. A small reduction in the relative fitness was observed for *B. fragilis* when combined with all organisms. This reduction was however interpreted as a feature of the growth of this bacterium in relative fitness model due to the requirement for anaerobiosis. Overall data indicate that stable maintenance of the consortium was achievable.

Inoculation and population stability

Using a sequential inoculation approach, defined communities were established within 24 h after the final inoculation phase, with each individual species maintaining dynamic stability for a further 8 d (Figure 3). Bacterial populations obtained an overall mean density of *c.* 6 Log_{10} CFU/mm^3 with values of *c.* 6 Log_{10} CFU/mm^3 (0.87 sd) for MRSA, *c.* 5 Log_{10} CFU/mm^3 (0.68 sd) for *S. pyogenesis*, *c.* 8 Log_{10} CFU/mm^3 (1.07 sd) for *P. aeruginosa* and *c.* 6 Log_{10} CFU/mm^3 (0.74 sd) for *B. fragilis*.

Microbial population stability within biofilm models

The step-wise inclusion of each source of variability in the analysis of the average bacterial count over time for the Model Stability data set can be seen in Table 2. Data suggests that knowing which technical replicate is associated with which biological replicate is important and subsequently knowing which biological replicate belongs to which bacteria is also important. Overall this suggests that the within bacteria variability is lower than the between bacteria variability which implies the experimental system is stable in that it can distinguish between different bacteria.

Homogeneity of microbial populations across filters

To assess the homogeneity of population densities in each 5mm x 5mm monoacetate filters were relative to each other at the same time point, communities were grown to a specific timed endpoint

and viable counts performed. Table 3 shows the mean, maximum, minimum and standard deviations of viable counts from four filters at each time point. Statistical significance was determined using analysis of variance (ANOVA) combined with post-hoc analysis of least significant difference (LSD) test to determine significant difference. No significant difference was found between viable counts of MRSA, *S. pyogenes*, *P. aeruginosa* or *B. fragilis* between the filters on each day sampled. The basal perfusion model was validated longitudinally and cross-sectionally; a dynamic steady state was achieved up to 8 d in independent runs and comparable populations were obtained between filters harvested at the same time point.

Antimicrobial exposure

Minimum inhibitory and minimum bactericidal concentrations

Data in Table 4 show the minimum inhibitory and minimum bactericidal concentrations for povidone-iodine, PHMB and silver acetate determined for each organism. All MICs were lower than selected for assessment in the biofilm model, which represent concentrations typically used in topical wound treatments (Bolton 2006, Burks 1998, Butcher 2012, Goldenheim 1993).

Antimicrobial effects on four-species biofilm consortia

The impact of the exposure regimens on the individual populations in the bacterial consortia varied considerably (Figures 4-6). For both PHMB and povidone-iodine, populations of Group A *Streptococcus* became undetectable after dosing, with communities of MRSA and *B. fragilis* also significantly affected, although viable populations of these organisms were detected with increasing frequency (across replicate systems) towards the later phases of dosing in replicated experiments (day 4 onwards). Data for silver acetate dosing show that overall bacterial viability for *P. aeruginosa* remained largely unaffected, with cell counts remaining relatively stable for the remainder of the model runs. There was however, considerable reduction in the viable populations of MRSA, Group

A *Streptococcus* and *B. fragilis* 24 h after dosing began but the detectable populations stayed relatively stable for the remainder of the study albeit at a numerically lower density. The overall rank order of effectiveness for the antimicrobials was povidone-iodine > PHMB > silver acetate**.** The analysis of the effect of each treatment on the average bacterial count over time can be seen in Table 5. The magnitude of the treatment effect on the bacterial population varies across the treatments. The largest reduction in bacterial count was seen for povidone-iodine (57%) followed by PHMB (44%) and then silver acetate (27%).

Biofilm imaging

ESEM revealed afferent bacteria and microcolonies of proportions, along with and putative biofilm EPS throughout inoculated filters (Figure 7 b-d). Comparisons with the negative control filter (Figure 7a) indicate that the microcolonies and putative EPS observed were the result of inoculation and environmental conditions of the model and were not an artifact of the filter structure or media.

Discussion

There is considerable interest in the development of effective regiments for the management of biofilms within complex wounds. *In vivo* and *in vitro* biofilm models have previously been used as research tools in this field. An issue of concern with biofilm models however is the ability to maintain populations comprising bacteria with distinct oxygen requirements in a system that facilitates the effects of antimicrobial compounds to be determined. With the broad aim of providing a platform in which this could be explored, an *in vitro* wound biofilm model was developed that reproduces the basolateral nutrient delivery of wounds, and which supports the growth of a defined consortium comprising of organisms of interest in wounds with distinct antimicrobial susceptibilities, general physiology and growth requirements.

Before designing the wound basal perfusion model, currently available model systems were assessed for their potential use. These included the Multiple Sorbarod Device (Ledder et al. 2006) and a Flat-Bed Perfusion Biofilm model (Thorn and Greenman 2009). To develop a model suitable for the required application, selected characteristics of these systems were adopted, including the use of filters as substrata and a nutrient delivery system which supplied nutrient media to the basally. The developed system combines a continuous perfusion, similar to exudate flow in a wound, with eight sampling positions to support the continuous assessments of a treatment over time or a single treatment point with multiple replicates.

In preliminary investigations, when models were concomitantly inoculated with MRSA, S. *pyrogens, P. aeruginosa and B. fragilis,* all species were initially detectable. However, after 24 h, MRSA and S. *pyogenes* rapidly declined and became undetectable, whilst populations of *P. aeruginosa* and *B. fragilis* expanded. To determine if the test bacteria could in principle grow in consortia under optimised conditions, relative fitness assays were performed in which the test organisms were grown together in all binary combinations with no evidence of specific growth inhibition. These data supported the initial observations of 24 h of growth in a prototype system, where viable Gram-positive organisms were detectable within 24 h of inoculation. Based on these observations a sequential inoculation procedure was developed in which MRSA and S. *pyogenes* were established prior to the introduction of the Gram-negative organisms including the obligate anaerobe, *Bacteroides fragilis* after 24 h. Using this approach, all four organisms became established within defined species wound-type biofilms for up to 8 d.

The inclusion of eight sampling positions in the biofilm model facilitated the collection of multiple baseline and treatment samples to monitor stability and reproducibility. The similarity in population densities between replicated model runs indicates that baseline values and daily sampling would provide a suitable platform through which to test the efficacy of antimicrobial therapies such as wound dressings over a 7 d period.

Three topical wound treatments with distinct modes of action were investigated; silver acetate, povidone-iodine and polyhexamethylene biguanide (PHMB) against the four-species consortium. For povidone-iodine and PHMB, concentrations of 10% and 0.5% (respectively) were selected based upon concentrations typically used in topical wound treatments (Burks 1998, Butcher 2012, Goldenheim 1993). Defining a relevant concentration for silver was however, more complicated, with previous reports of various silver compounds such as silver acetate, silver nitrate and nanoparticles being used at various concentrations in a range of commercially available wound dressings (Bolton 2006, Fong and Wood 2006). Following a literature review, silver acetate was selected and used at the highest soluble concentration achievable in the growth medium, which was greater than the MIC for silver acetate for each test bacterium.

Based on replicated determinations, the rank order of effectiveness for the antimicrobials in planktonic susceptibility tests was (highest to lowest) silver acetate > povidone iodine > PHMB. For biofilm communities however, it was povidone-iodine > PHMB > silver acetate. Possible reasons for this disparity include the fact that i) the mechanisms that underlie antimicrobial tolerance with biofilms are partly distinct from those that are assessed in planktonic susceptibility tests and ii) that povidone iodine was applied to the biofilms at 10% (w/v), polyhexamethylene biguanide at 0.5% and silver acetate at 0.05% (w/v), therefore planktonic susceptibility data (MIC and MBC) cannot necessarily be used to predict to the outcome of biofilm exposure. These two considerations are of relevance in the assessment of the effectiveness of antimicrobials for wound care, reinforcing the need to consider the biofilm phenotype in antimicrobial tests and to consider that the concentrations of distinct antimicrobial compounds used in wound care vary for reasons that include formulation, toxicity and regulation.

For data generated using the wound model, both povidone-iodine and PHMB had the greatest impact on MRSA, *B. fragilis* and Group A *Streptococcus*, with the latter undetectable in both the povidone-iodine and PHMB dosed systems from second day of dosing. For povidone-iodine, PHMB

and silver acetate-dosed systems; *P. aeruginosa* was detected throughout, with silver acetate being the least effective of the antimicrobials tested against this bacterium. In some cases, following the initial inactivation of bacteria, partial recovery of cell counts was observed during continual exposure to antimicrobials. This was apparent following initial exposure and was notable for *P. aeruginosa* during exposure to povidone-iodine and PHMB and for MRSA during exposure to PHMB and silver acetate.

When a microbial community is exposed to an antimicrobial agent, organisms will generally be inactivated in order of their susceptibility. The well documented tolerance of biofilms towards antimicrobials may increase this effect, since it is less likely that a biofilm community will be completed inactivated than the same organisms growing in dispersed, planktonic form. An additional consideration is that the tolerance conferred by biofilm growth may be greater for some organisms than for others. In the current context, the recalcitrance of *P. aeruginosa* (Pedersen, 1992; Gilbert et al., 2002; Walters et al., 2003), its tendency to form biofilms, and the recalcitrance of those biofilms once developed, has been widely reported. Thus, the relative susceptibility of bacteria observed in the current study, and the survival of *P. aeruginosa* in exposed biofilms could be largely predicted based on known features of this bacterium. The observed inactivation kinetics could be explained by phenotypic adaptation within the biofilm, whilst the varied tolerance to antimicrobial exposure observed in the basal perfusion model against the three compounds resembles that previously reported by Forstner et al. (2013) using a porcine wound model. Since distinct bacteria often exhibited distinct antimicrobial susceptibly profiles to antiseptics as well as to antibiotics, such variable antibacterial effects may be an important but relatively little studied variable in wound antisepsis (Davis et al. 2006, James et al. 2008, Roy et al. 2014, Saye 2007).

In conclusion, the primary objective of the current investigation was to develop an *in vitro* biofilm model which would i) support the growth of a mixed species consortia; ii) could be used model antisepsis and iii) would facilitate the establishment of replicated biofilm consortia for

temporal studies. A defined species biofilm consortium containing aerobic, facultative and anaerobic species was grown reproducibly using the basally perfused model. The effect of topical wound antimicrobials was examined and variable susceptibility in the four test bacteria was evident in biofilm consortia despite concentrations used being considerably higher than planktonic susceptibility, a phenomenon often associated with biofilms.

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Transparency declaration

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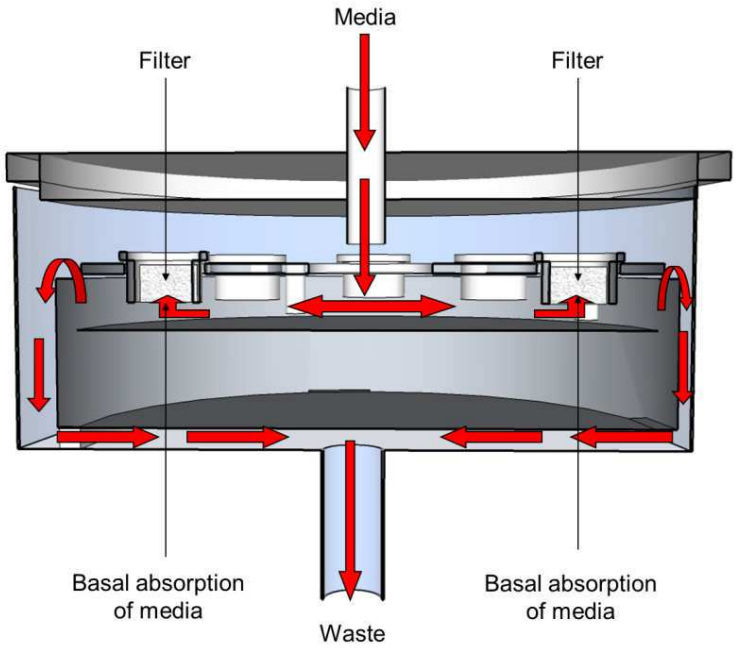


Figure 1. A diagrammatical cross-sectional view of the basal perfusion model illustrating the media delivery and waste outflow. The filters on which biofilms are grown are 5mm x 5mm in size and are placed within the individual holes (8 in total) of the central fluid basin. The upper section and lower section of these individual holes are separated by a small rim/ledge upon which the filter rest.

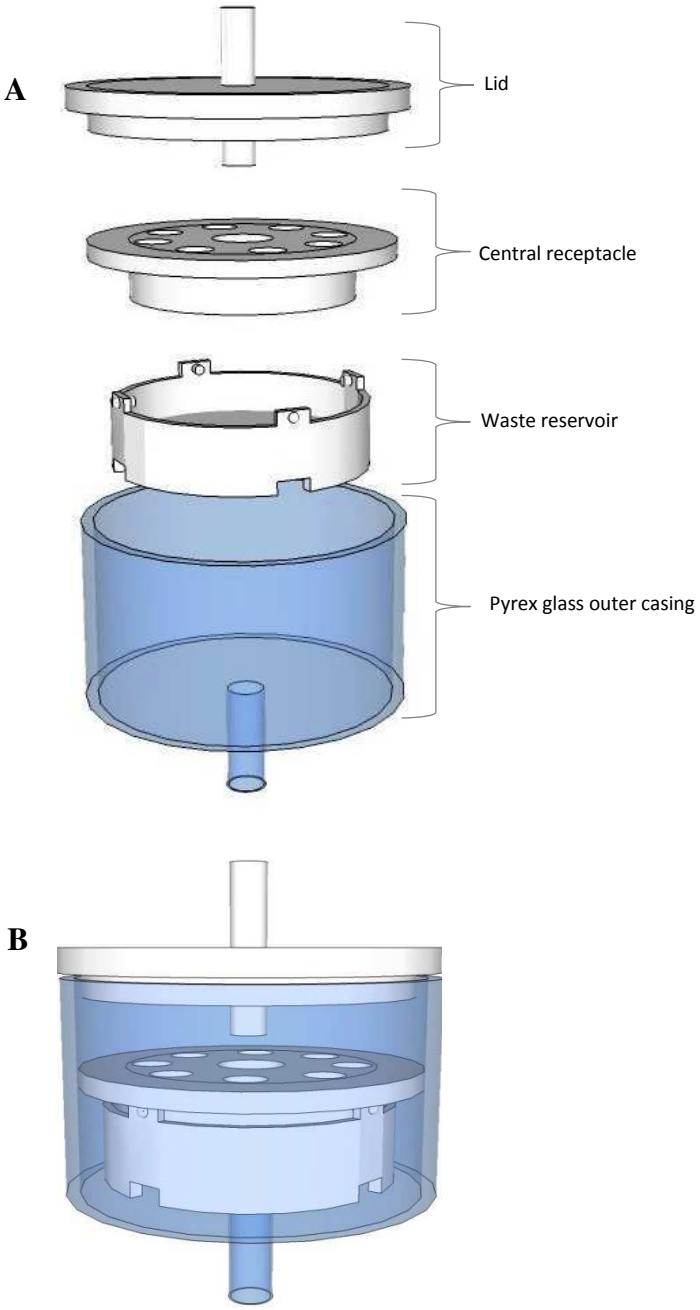


Figure 2. A diagrammatical view of the individual components (A) and combined (B) components of the basal perfusion wound model.

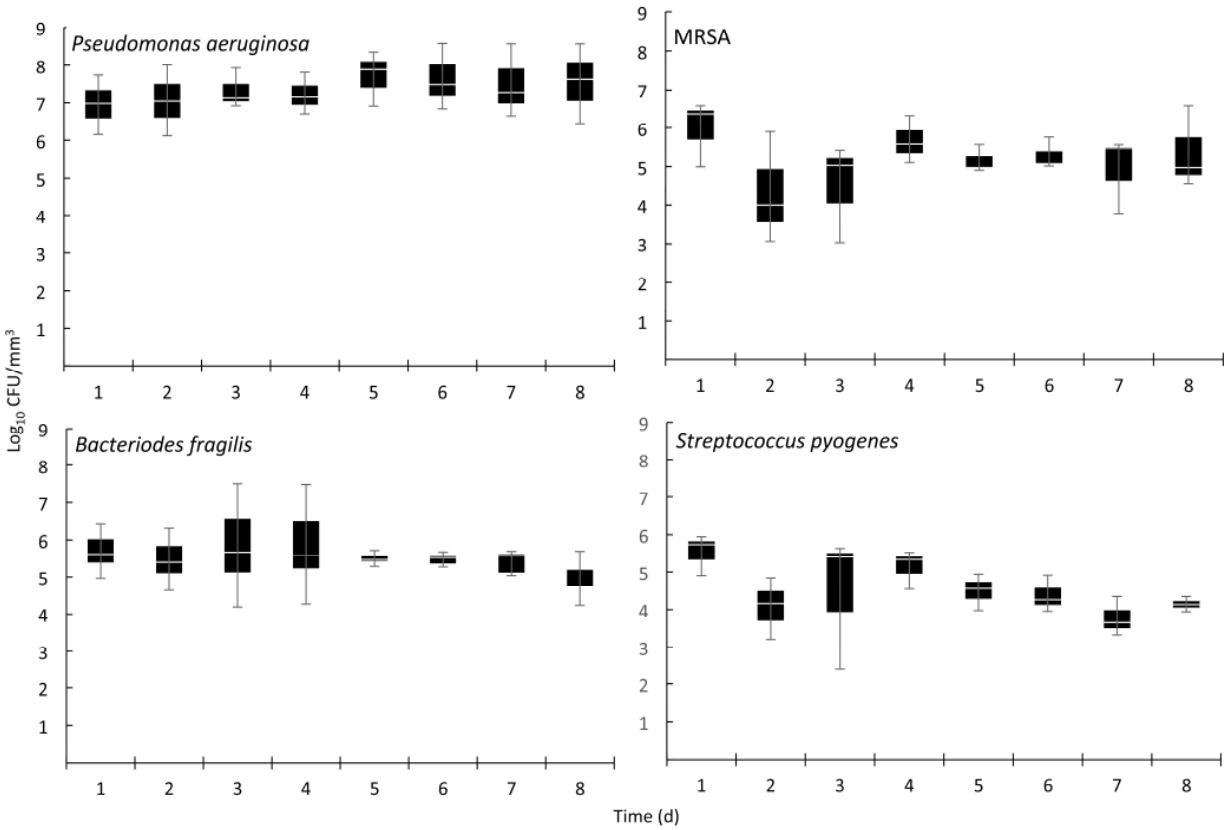
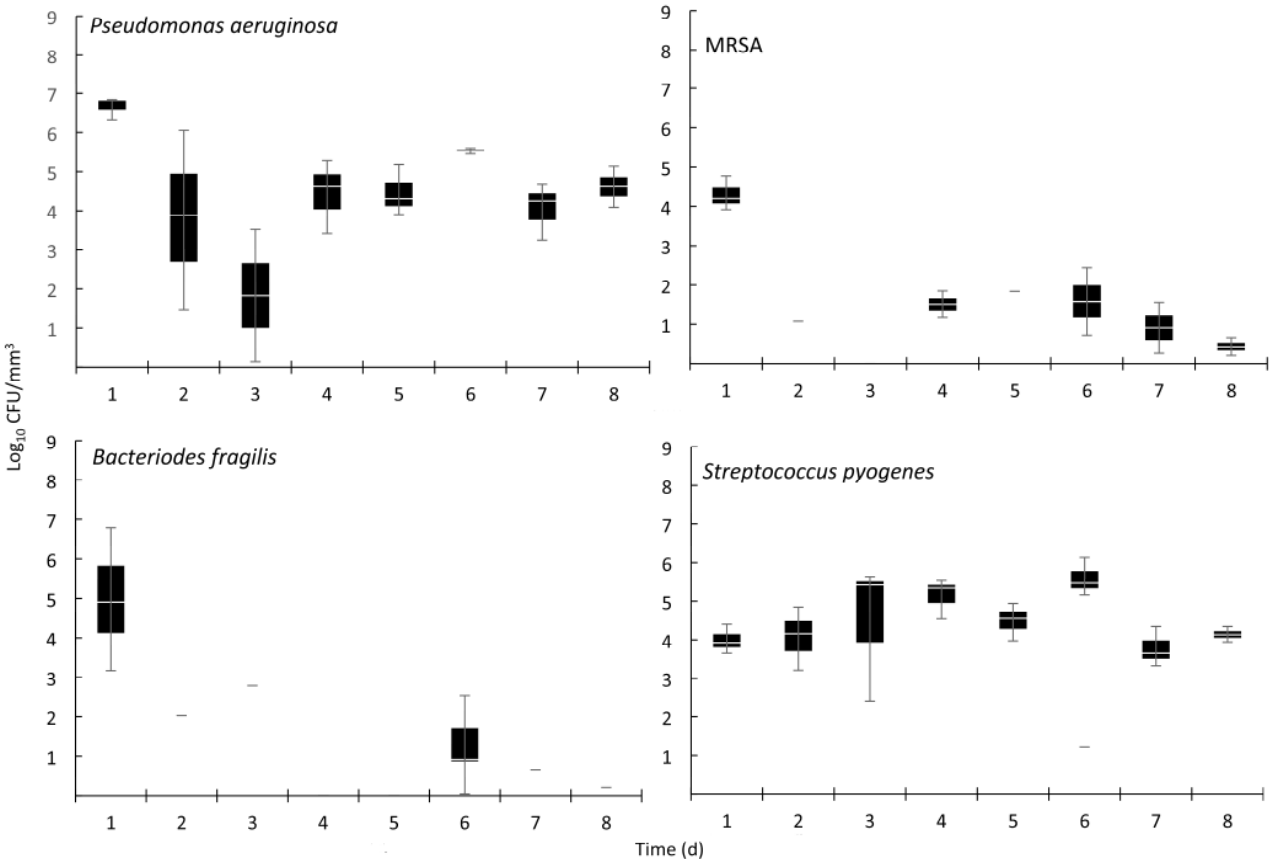


Figure 3. Population densities of the four species wound consortia grown and maintained for 8 d in the three independent basal perfusion models. The boxes represent the interquartile range with the lower and upper boundaries of the boxes representing quartiles 1 and 3; the central line is the median. The whiskers represent the maximum and minimum values. Data represent mean and ranges for three independent experiments. Detection threshold, <1.0 Log_{10} CFU/mm^3



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580
581 **Figure 4.** Population densities the four species wound consortia grown in the basal perfusion models
582 and exposed to 10% povidone-iodine. Single horizontal lines indicate viable count data for one
583 replicate above the detection threshold. See legend to Figure 3.
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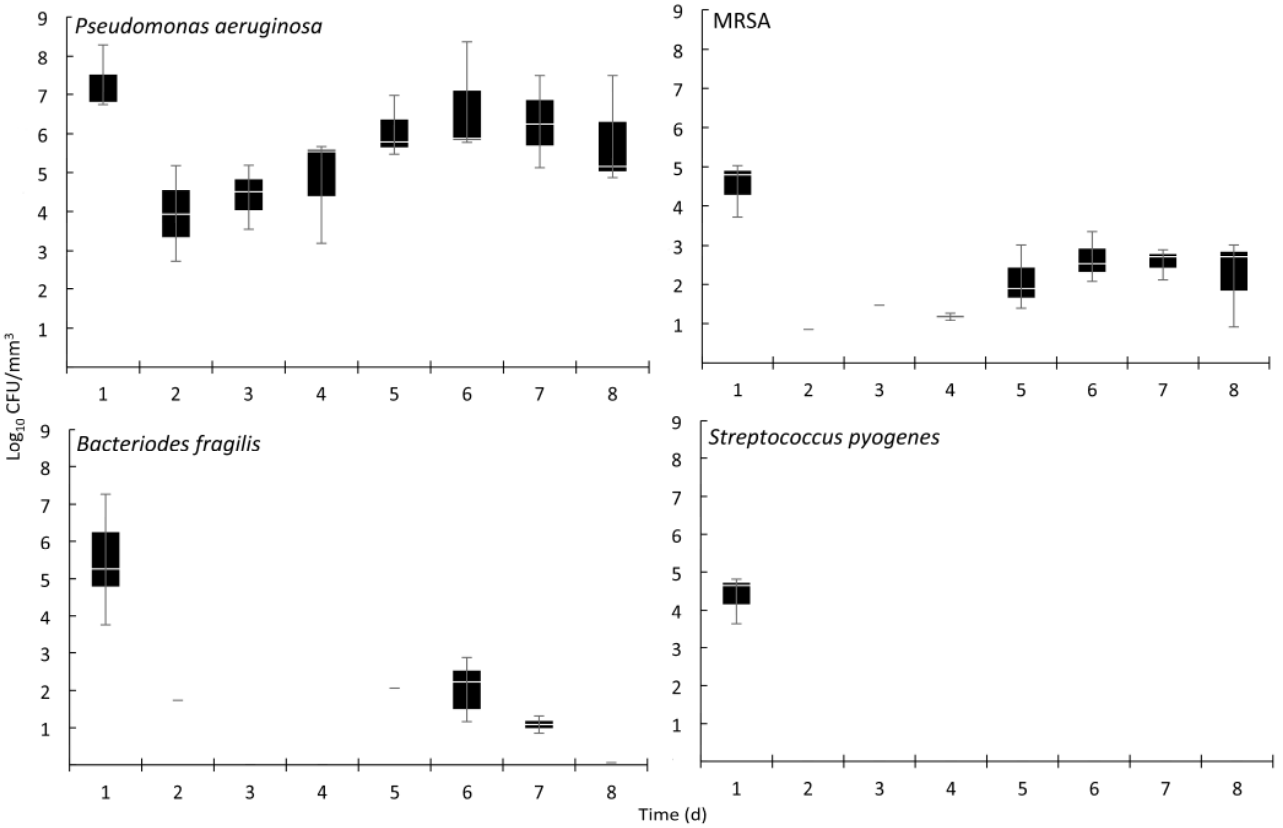

Figure 5. Population densities the four species wound consortia grown in the basal perfusion models and exposed to 0.5% PHMB. See legend to Figure 3.

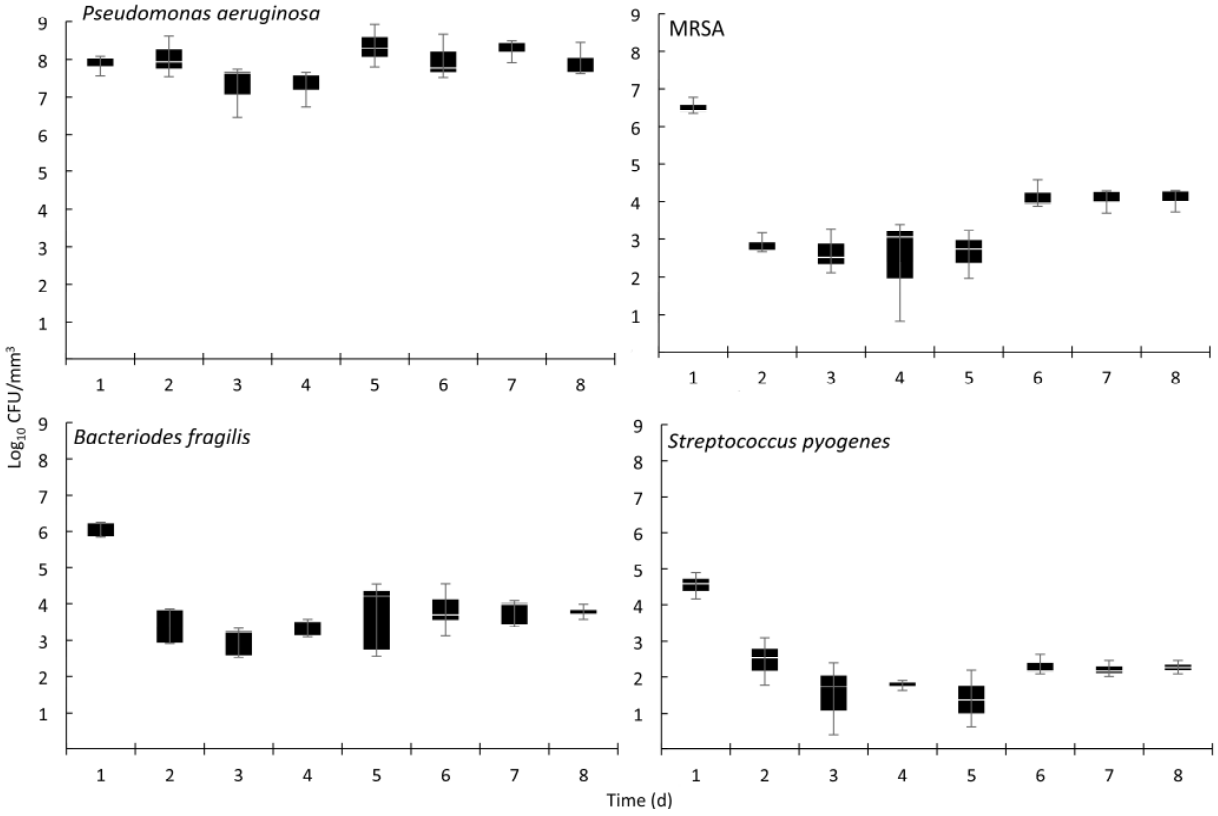

Figure 6. Population densities the four species wound consortia grown in the basal perfusion models and exposed to 0.05 % silver acetate. See legend to Figure 3.

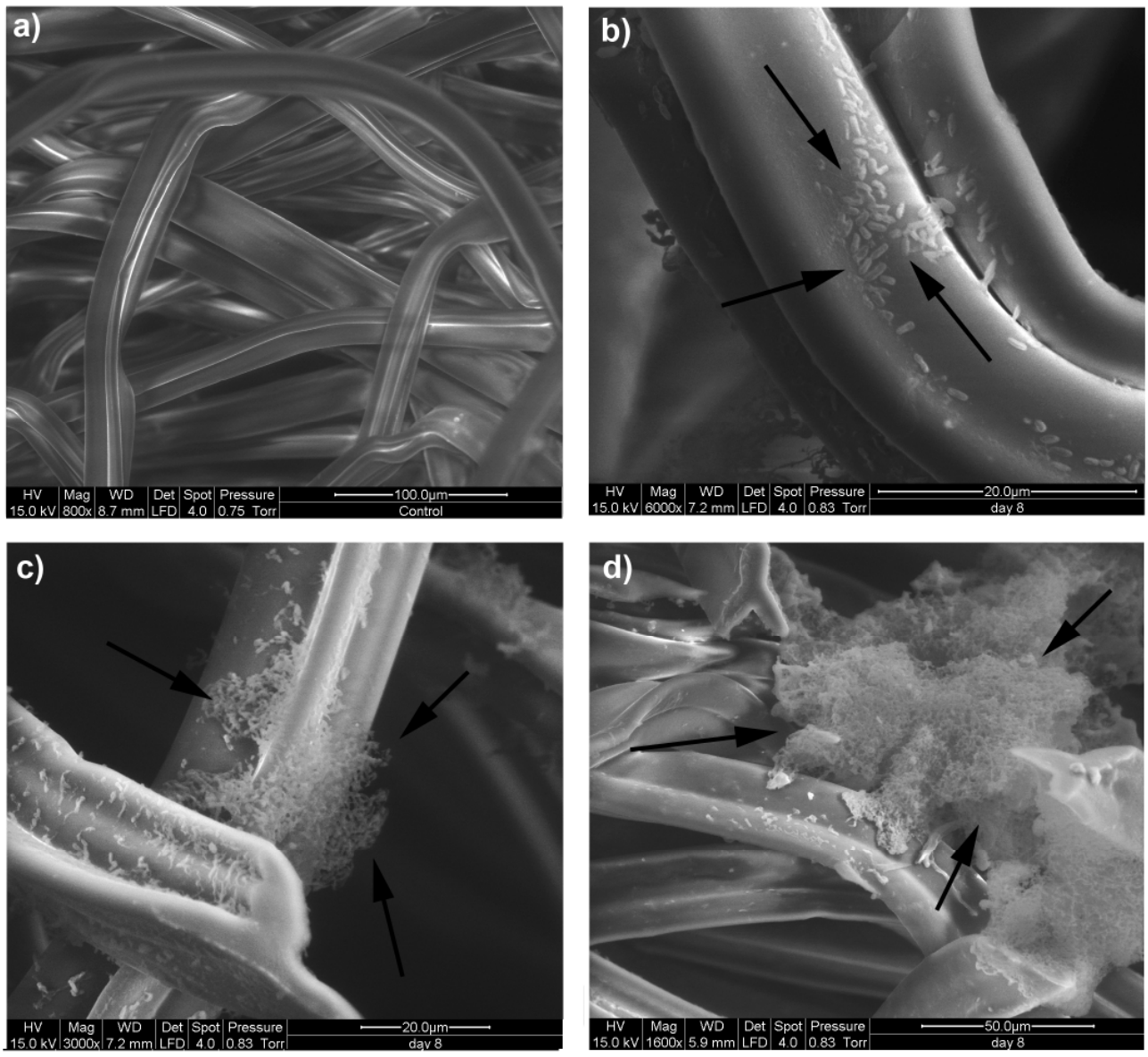

Figure 7. ESEM images showing (a) an uninoculated filter; (b) adherent bacteria (mainly rod morphology); (c) a microcolony and (d) a larger colony/biofilm of coccoid bacteria on filter samples taken from inoculated models.

635 **Table 1.** The relative fitness of organisms in the modified competition assay

Relative fitness of → versus ↓	Bacterium			
	MRSA	*S. pyogenes*	*P. aeruginosa*	*B. fragilis*
MRSA	na	1.05 (0.02)	0.96 (0.09)	0.83 (0.03)
S. pyogenes	0.95 (0.04)	na	1.13 (0.04)	0.84 (0.03)
P. aeruginosa	1.04 (0.14)	0.89 (0.07)	na	0.78 (0.15)
B. fragilis	1.21 (0.05)	1.19 (0.10)	1.28 (0.15)	na

636 na, not applicable. Data are means (SEM) of three separate experiments.
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Table 2. The goodness of fit value, -2LL, for each iteration of the model, together with p-value from the likelihood ratio test for model stability assays

Replicate type	-2LL (p-value)
Technical Replicates (1)	567
Technical Replicates in Biological replicates (2)	542 (2 v 1: $p < 0.001$)
Technical Replicates in Biological replicates in type of Bacteria (3)	533 (3 v 2: $p = 0.004$)

Table 3. The mean, maximum, minimum and standard deviation of population densities from four separate mono-acetate filters sampled at the same time point

Day	Bacterium															
	MRSA				*S. pyogenes*				*P. aeruginosa*				*B. fragilis*			
	Mean	Max	Min	Stdev	Mean	Max	Min	Stdev	Mean	Max	Min	Stdev	Mean	Max	Min	Stdev
1	7.72	7.93	7.52	0.17	8.58	9.10	8.25	0.38	9.52	9.60	9.43	0.08	9.32	9.35	9.26	0.04
2	7.73	7.84	7.68	0.07	7.62	7.73	7.49	0.11	9.53	9.62	9.43	0.10	9.28	9.32	9.23	0.04
3	7.31	7.59	7.11	0.22	7.42	7.60	7.24	0.17	9.45	9.47	9.43	0.02	9.12	9.17	9.04	0.06
4	7.28	7.38	7.19	0.09	7.62	7.76	7.50	0.11	9.31	9.35	9.25	0.04	9.24	9.39	9.07	0.13
5	7.34	7.52	7.24	0.13	7.08	7.22	6.96	0.11	9.26	9.42	9.05	0.16	8.92	9.09	8.65	0.19
6	7.50	7.68	7.26	0.20	7.00	7.20	6.81	0.16	9.40	9.48	9.36	0.05	9.13	9.57	8.94	0.30
7	7.06	7.68	6.46	0.66	6.99	7.24	6.77	0.20	9.37	9.39	9.35	0.02	8.07	8.22	7.93	0.12
8	6.45	6.55	6.23	0.15	7.62	8.08	6.96	0.53	9.46	9.51	9.40	0.06	7.98	8.04	7.92	0.06

Data are Log_{10} CFU per mm^3.

Table 4. Planktonic susceptibility determined in simulated wound fluid

Bacterium	Planktonic susceptibility					
	MIC			MBC		
	PHMB	Silver acetate	Povidone-iodine	PHMB	Silver acetate	Povidone-iodine
MRSA	7.94 (2.75)	0.08	5210 (1800)	101 (44.01)	1.25	25000
S. pyogenes	4.76	0.02	490 (4100)	22.23 (14.55)	0.26 (0.09)	20839 (7220)
P. aeruginosa	152	0.04	8330 (3610)	2032 (704)	1.25	25000
B. fragilis	6.35 (2.75)	0.07 (0.02)	3130	25.00	nd	25000

All concentrations are mg/L. Standard deviations are given in parenthesis, nd, not determined (>1.25mg/l).
Data are means from three separate determinations.

Table 5. The difference in -2LL between each treatment, control and model stability showing p-values derived from the likelihood ratio test for the treatment assays

Treatment	-2LL Drop (p-value)	% Relative Drop (95% CI)
Povidone-iodine	46 (p<0.001)	57 (51, 63)
PHMB	27 (p<0.001)	44 (35, 53)
Silver acetate	12 (p<0.001)	27 (15, 39)

CI, confidence interval.