An *in vitro* model of chronic wound biofilms to test wound dressings and assess antimicrobial susceptibilities

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Objectives: The targeted disruption of biofilms in chronic wounds is an important treatment strategy and the subject of intense research. In the present study, an *in vitro* model of chronic wound biofilms was developed to assess the efficacy of antimicrobial treatments for use in the wound environment.

Methods: Using chronic wound isolates, assays of bacterial coaggregation established that aerobic and anaerobic wound bacteria were able to coaggregate and form biofilms. A constant depth film fermenter (CDFF) was used to develop wound biofilms *in vitro*, which were analysed using light microscopy and scanning electron microscopy. The susceptibility of bacteria within these biofilms was examined in response to the most frequently prescribed 'chronic wound' antibiotics and a series of iodine- and silver-containing commercial antimicrobial products and lactoferrin.

Results: Defined biofilms were rapidly established within 1–2 days. Antibiotic treatment demonstrated that mixed *Pseudomonas* and *Staphylococcus* biofilms were not affected by ciprofloxacin (5 mg/L) or flucloxacillin (15 mg/L), even at concentrations equivalent to twice the observed peak serum levels. The results contrasted with the ability of povidone–iodine (1%) to disrupt the wound biofilm; an effect that was particularly pronounced in the dressing testing where iodine-based dressings completely disrupted established 7 day biofilms. In contrast, only two of six silver-containing dressings exhibited any effect on 3 day biofilms, with no effect on 7 day biofilms.

Conclusions: This wound model emphasizes the potential role of the biofilm phenotype in the observed resistance to antibiotic therapies that may occur in chronic wounds *in vivo*.

Keywords: chronic venous leg ulcer, constant depth film fermenter, antibiotic resistance, biocide resistance, coaggregation, lactoferrin

Introduction

Chronic wounds harbour a diverse microflora and are a repository of complex polymicrobial communities (which include both aerobic and anaerobic species).[1] The precise role of these organisms in mediating the observed impairment of wound healing is complex and may include both direct and indirect mechanisms.[2,3] The importance of individual species, multiple species or microbial density in relation to healing, however, remains unclear.[4–6] Anaerobic species constitute ~45% of the total microbial population in non-infected venous leg ulcers,[7–9] which increases to 49% in clinically infected chronic venous leg ulcers (CVLUs).[7] Whilst all wounds are colonized by bacteria, not all wounds are clinically infected;[4] the definition of inflammation and infection requiring clinical experience to avoid the

unnecessary prescription of antibiotics in this 'at-risk' population.[10,11]

Considerable attention has recently been focused on the ability of bacteria within chronic wounds to form and exist in biofilms.[12–14] Bacterial biofilms consist of a complex microenvironment of single or mixed bacterial species encased within an extracellular polymeric substance (EPS) or glycocalyx which the bacteria themselves produce. The moist wound surface, with its adhesive, proteinaceous substrate and a ready supply of nutrients, represents (conceptually at least) the ideal environment for biofilm development.[15] Researchers have demonstrated that bacteria within the wound environment possess the ability to form biofilms.[12,13,16,17] Moreover, it has recently been suggested that acute partial thickness wounds[13,18] may harbour bacterial

biofilms growing on the wound surface. In addition, EPS has been visualized by epifluorescence and light microscopy on chronic wound smears.[16,19] Individual bacteria and bacterial microcolonies have also been observed using fluorescence *in situ* hybridization (FISH) on chronic wound biopsy sections.[12,20] Such biofilms may play an important role in the ability of wounds to resist antimicrobial and antibiotic treatments.

In the formation of biofilms, coaggregation is a specific mechanism of bacterial cell-to-cell adhesion that plays a key role in biofilm formation. Coaggregation is mediated by specific growth-phase-dependent adhesin–receptor interactions[21,22] with bacteria from biofilm communities showing an increased tendency to coaggregate compared with planktonic bacteria.[23] This coaggregation has also been shown to contribute a metabolic advantage by facilitating the survival of obligate anaerobic species in aerated environments.[24] Apart from oral plaque bacteria, coaggregation has also been shown to occur between bacteria isolated from other ecosystems such as the gastrointestinal and urogenital tracts[25–28] as well as wastewater and food processing environments.[29–31] Despite the importance of coaggregation in biofilm establishment, the coaggregation phenotype of chronic wound bacteria remains to be studied.

In attempts to model dental plaque biofilm formation *in vitro*, the constant depth film fermenter (CDFF) was developed.[32–34] The CDFF allows the generation of identical, multiple biofilms of uniform depth for sequential analysis (including gene, protein and cellular/structural analysis). Importantly, the flexibility of the system allows key parameters, including nutrient source, temperature, oxygen availability and substrata to be varied. Schematic representation of the CDFF has already been published elsewhere.[35,36] The CDFF model has consequently been extensively used to study various aspects of biofilm physiology as well as for testing antimicrobial therapies e.g. chlorhexidine, sodium hypochlorite, tetracycline and silver.[31,37–39] In addition to the study of human disease causing biofilms, it has also been utilized to model bacteria in other ecosystems, such as wastewater.[33]

In this study, we sought to develop a reliable *in vitro* model of chronic wound biofilms, initially testing the coaggregating ability of bacteria derived directly from chronic wounds, before establishing biofilms in the CDFF system. The model was then used to test and compare the efficacy of conventional antibacterial wound therapies on biofilms.

Materials and methods

Bacterial strains and media

Bacterial species were selected from a previous prospective study of 70 patients with newly diagnosed CVLUs at the Wound Healing Research Unit in Cardiff, with informed consent.[5,40,41] These included the most frequently encountered species, namely those of the *Pseudomonas*, *Staphylococcus*, *Micrococcus* and *Streptococcus* genera, as well as a range of strictly anaerobic bacteria (Table 1).

For the CDFF, seven bacterial species with good coaggregating ability were selected to represent the polymicrobial nature of chronic wound beds. Davies *et al.*[5] found that the mean number of organisms per wound (for both deep tissue or wound surface) was fewer than three, but had a range of one to six. Hence, up to six organisms were used at any one time for the model wound biofilm, using both aerobic and anaerobic species. In later experiments this number was reduced to four aerobic species. From our previous work,[5,40] two of the most frequently isolated

Table 1. Bacterial isolates from CVLUs used in coaggregation assays

Wound isolate ID No.	Bacterial isolate
G68	*Staphylococcus aureus*
D58	*Staphylococcus aureus* (methicillin resistant)
D76	*Staphylococcus aureus*
C49	*Staphylococcus aureus*
C72	*Staphylococcus aureus*
D21	*Staphylococcus aureus*
B60	*Pseudomonas aeruginosa*
C21	*Pseudomonas aeruginosa*
D40	*Pseudomonas aeruginosa*
D49	*Pseudomonas aeruginosa*
B43	*Pseudomonas aeruginosa*
C31	*Pseudomonas aeruginosa*
B64	coagulase-negative *Staphylococcus*
C33	coagulase-negative *Staphylococcus*
D56	coagulase-negative *Staphylococcus*
C45	*Micrococcus* sp.
C7	*Micrococcus* sp.
B81	*Micrococcus luteus*
D74	*Streptococcus agalactiae*
B52	*Streptococcus oralis*
H56	*Streptococcus adjacens*
B12	*Peptostreptococcus anaerobius*
E67	*Propionibacterium acnes*
B14	*Finegoldia magna*
B48	*Peptoniphilus asaccharolyticus/indoliticus*
D72	*Peptoniphilus asaccharolyticus*
F21	*Micromonas micros*
G34	*Finegoldia magna*
G60	*Finegoldia magna*
F69	*Bacteroides levii*
B11	*Bacteroides fragilis*
B76	*Eubacterium lentum*

wound bacteria were *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Strains of *S. aureus* (D76, methicillin susceptible) and *P. aeruginosa* (D40), were therefore selected from wound isolates to be components of the biofilm consortium. In addition, *Micrococcus luteus* (B81) and *Streptococcus oralis* (B52) were selected, not only on the basis of their relative ability to coaggregate, but also on their relatively fast growth rates. This was done with the caveat that both pseudomonads and staphylococci could potentially out-compete other slower-growing organisms in mixed culture. The anaerobic bacteria selected included *Propionibacterium acnes* (E67), *Bacteroides fragilis* (B11) and *Peptostreptococcus anaerobius* (B12), although only two of these species were used at any one time in the mixed bacterial biofilm in the CDFF.

Aerobic isolates were routinely grown on blood agar No. 2 (BA; Lab M) and anaerobes on fastidious anaerobe agar (FAA; Lab M), both supplemented with 5% (v/v) defibrinated sheep blood. BA plates were incubated aerobically at 37°C for 2–3 days. FAA plates were incubated in an anaerobic environment (10% CO_2, 10% H_2, 80% N_2) also at 37°C for up to 7 days. Fastidious anaerobe broth (FAB; Oxoid) was used for liquid culture.

Coaggregation testing

The ability of bacteria from wounds to initiate biofilm formation was investigated by testing chronic wound isolates for their ability to

coaggregate (both intra- and inter-generically). Isolates with the highest coaggregating ability were subsequently used to produce a mixed species biofilm in the CDFF (see below).

Thirty-one bacterial wound isolates from CVLUs were selected for testing in the coaggregation assay (Table 1). These included *S. aureus* (*n*=6), *P. aeruginosa* (*n*=6), coagulase-negative *Staphylococcus* spp. (*n*=3), *Micrococcus* spp. (*n*=3), *Streptococcus* spp. (*n*=3) and anaerobic isolates (comprising seven peptostreptococci, two *Bacteroides* spp. and one *Eubacterium lentum*). A standard coaggregation assay was used to test the ability of the wound bacteria to coaggregate in suspension.[24,42] A Gram-negative dental plaque anaerobe, *Fusobacterium nucleatum*, was also tested against wound isolates, due to its ability to coaggregate with all species of oral bacteria tested.[43,44]

Shaking overnight cultures (or 3–5 day cultures for slower-growing organisms) of aerobic isolates (300 mL) were grown at 37°C in BM medium[45] (proteose peptone, 10 g/L; trypticase peptone, 5 g/L; yeast extract, 5 g/L and KCl, 2.5 g/L) with addition of haemin, 0.005 g/L; vitamin K_1, 0.001 g/L; L-cysteine HCL, 0.5 g/L and glucose, 10 g/L. The bacterial growth was then harvested by centrifugation (8000 **g**; 10 min). The anaerobic isolates were grown in BM medium (600 mL) as static cultures in an anaerobic atmosphere (80% N_2, 10% CO_2, 10% H_2) for 5–7 days at 37°C. All cultures were grown to stationary phase and the cells were then harvested by centrifugation at 8000 **g** for 10 min. Harvested cells were washed twice in 100 mL coaggregation buffer (0.1 mM $CaCl_2$, 0.1 M $MgCl_2$, 0.15 M NaCl, 1 mM Tris–HCl, pH 8).[46] Anaerobes that showed poor growth in BM medium (i.e. little or no biomass even after several days growth) were subsequently grown in FAB.

Washed cells were re-suspended in coaggregation buffer to give a final optical density of 2.0–2.5 at 660 nm. Coaggregation tests were initially carried out for pairs of species, although these were subsequently also performed in triplicate (or combinations of four species). For each test, 1 mL of bacterial cell suspension was mixed by vortexing for 2 min and left to stand for 90 min at room temperature prior to scoring. The coaggregation score was recorded using the visual scale described by Cisar *et al*.[46]; see footnote of Table 2. Control bacterial suspensions were also dispensed singly to assess autoaggregation (i.e. self-coaggregation). The effect on coaggregation of components in the growth medium was also investigated through the use of media supplemented with 10% or 50% (v/v) fetal calf serum.

Biofilm formation using the CDFF

Simple beaker microcosms, containing 100 mL brain heart infusion (BHI) broth, established that coaggregating wound bacteria were able to form biofilms. Initial experiments in the CDFF determined whether a biofilm could be produced using bacteria isolated from chronic wound environments.

Production of biofilms

Biofilms were cultured in a CDFF maintained at 37°C with plug inserts recessed to a depth of 400 μm. To prepare the biofilms, the bacteria were initially cultured overnight at 37°C in FAB. As described previously, aerobes were cultured in a shaking incubator whilst anaerobes were grown statically in an anaerobic environment. Prior to inoculation, culture medium was recirculated through the CDFF for 30 min to simulate a conditioning film with a turntable speed of 20 r.p.m.

Five microlitres of each wound isolate was added separately to BM medium (1000 mL) and recirculated through the CDFF for 24 h to 'seed' the system. After this time, the inoculum was disconnected and fresh uninoculated medium was fed into the CDFF; the CDFF waste being collected in a separate effluent bottle. The growth medium was delivered at a rate of 30 mL/h using a peristaltic pump (Watson–Marlow).

Quantification of bacteria in the chronic wound biofilm

A single pan holding five plug inserts was removed aseptically at each sample timepoint from the CDFF for viable cell count estimation. Individual plugs were removed aseptically from the pan and the biofilms from two plugs (×2 for duplicate samples) were pooled and resuspended in 5 mL PBS by vortexing for 2 min. Serial dilutions of the resuspensions were prepared and samples plated on to BA and FAA, and incubated as previously described to give duplicate bacterial counts.

EPS staining of wound biofilms with ethidium bromide and calcofluor white

Mixed chronic wound biofilms were stained with ethidium bromide and calcofluor white as described by Davis *et al*.[13] Briefly, biofilm samples

Table 2. Coaggregation scores[a] of pairs of chronic wound bacterial species

	Species	1 D40	2 D49	3 D76	4 C49	5 B12	6 B14	7 B48	8 D72	9 F21[b]	10 G34	11 G60
1	*P. aeruginosa* D40											
2	*P. aeruginosa* D49	1+										
3	*S. aureus* D76	0	1+									
4	*S. aureus* C49	0	1+	0								
5	*P. anaerobius* B12	2+	3+	0	0							
6	*F. magna* B14	0	0	0	0	0						
7	*P. asaccharolyticus/indoliticus* B48	2+	2+	1+	2+	2+	0					
8	*P. asaccharolyticus* D72	0	1+	0	0	0	0	1+				
9	*M. micros* F21[b]	3–4+	2–3+	2–3+	3+	4+	2+	3+	3+			
10	*F. magna* G34	0	1+	0	0	4+	0	ND	ND	ND		
11	*F. magna* G60	0	1+	0	0	4+	0	ND	ND	ND	ND	

ND, not determined.
[a]The coaggregation score was recorded using the visual scale described by Cisar *et al*.[46] and reported as: 0, no flocs in suspension; 1+, very small uniform flocs in a turbid suspension; 3+, clearly visible flocs that settle leaving a clear supernatant; 4+, very large flocs of coaggregates that settle almost immediately, leaving a clear supernatant. Scores of 3+ and 4+ indicate high coaggregation scores.
[b]*M. micros* F21 showed high levels of autoaggregation.

were smeared on to glass slides, fixed with 2.5% formalin and stained with ethidium bromide (500 mg/mL) for 15 min. Samples were then washed in distilled water and stained with calcofluor white (0.1%) for 15 min before epifluorescence microscopy. Calcofluor white stains carbohydrates (EPS) blue, with DNA (in bacterial cells) stained red by ethidium bromide.

EPS staining of wound biofilms with Congo Red and Ziehl carbol fuchsin

Mixed chronic wound biofilms were also stained with Congo Red and Ziehl carbol fuchsin as described by Serralta *et al.*[18] Briefly, biofilm samples smeared on to glass slides were covered with 10 mM cetylpyridinium chloride. Slides were allowed to air dry for 20–30 min, fixed by gentle heating by transient passage over a Bunsen burner flame and allowed to cool. Slides were then stained for 15 min with a 2:1 mixture of saturated Congo Red solution and 10% Tween 80, and rinsed in distilled H_2O. Slides were then counter-stained with 10% Ziehl carbol fuchsin for 6 min, rinsed in distilled H_2O and dried at 37°C prior to visualization by epifluorescence microscopy. Biofilm EPS stains orange/pink with Congo Red whilst Ziehl carbol fuchsin stains bacterial cells purple/red.

Structural characterization of the chronic wound biofilm

The remaining fifth plug from the CDFF pan was fixed in 2.5% (v/v) paraformaldehyde for subsequent scanning electron microscopy (SEM) analysis. The samples were freeze-dried, mounted on aluminium stubs, sputter coated with gold in a sputter-coater (EM Scope; model Sc500) and then viewed using an EBT1 Scanning Electron Microscope (SEM Tech Ltd).

Antibiotic/biocide therapy of CDFF-generated biofilms

Antibiotic and biocide susceptibility testing of the established *in vitro* chronic wound biofilms in the CDFF was undertaken with individual antibiotics (flucloxacillin 15 mg/L or ciprofloxacin 5 mg/L; these concentrations being equivalent to twice the peak serum doses normally attained, with both antibiotics routinely prescribed at the equivalent of half these peak serum doses in chronic wound patients) and the biocide povidone–iodine (PVP1; 1% w/v). Each antimicrobial was continuously pumped into the CDFF with the growth medium. Bacterial counts from the CDFF-generated biofilms were performed at ∼48 h intervals over the length of the experiment (5–8 days). Efficacy of the individual antimicrobials in the growth media mix was confirmed for up to 48 h at room temperature by determining the MIC of the antibiotic/biocide for control strains (results not shown).

Lactoferrin, a component of the innate immune system found in human mucosal secretions and known to have anti-biofilm properties, was also tested in the CDFF. Conditioning the CDFF with lactoferrin (20 mg/L) for 1 h prior to inoculation was undertaken to see whether this agent would affect formation of the biofilm.

Antimicrobial susceptibility testing of planktonic cultures

In addition to assessing the effect of antimicrobial agents on established biofilms (see below), the MIC of the antimicrobials (flucloxacillin, ciprofloxacin and PVP1) for planktonically cultured aerobic species was determined using a broth macrodilution method.[47]

Antimicrobial effects of wound dressings on CDFF-generated biofilms

The dressings examined, any incorporated antimicrobials and their respective manufacturers were as follows: Aquacel® Ag, ionic silver (Convatec); Contreet® silver, ionic silver (Coloplast); Acticoat® N (nano-crystalline/elemental silver), Acticoat® A (absorbent, silcryst nanocrystals, elemental silver), Iodoflex® (cadexomer iodine paste) (Smith & Nephew); Silvercel® (hydroalginate with elemental silver), Actisorb® silver 220 (silver-impregnated activated charcoal), Nuderm® alginate (non-silver-impregnated alginate control), Inadine® (povidone–iodine) (Johnson & Johnson); and Topper Gauze with 1 g of added Betadine® cream (povidone–iodine) smeared aseptically as a thin film on the gauze (Seton Healthcare Group plc).

Dressing testing was performed using CDFF-generated biofilms grown using the six-member wound consortium described above. Pans were removed aseptically from the CDFF after 3 or 7 days and inverted on a previously moistened dressing in a sterile 140 mm Petri dish. The lid was secured on top of the inverted pans and the whole Petri dish sealed in a plastic bag to prevent dehydration. Experiments were performed using dressings soaked in a 20% excess of broth [tryptone soya broth (TSB) or BM]; the percentage of excess fluid being calculated from the differing fluid absorbance of each dressing (values not shown). After incubation at 37°C for 24 h, the inverted pan was placed on a fresh moistened dressing, again for 24 h incubation, with daily changes continuing in this manner for 7 days. On day 8, the plugs were sampled as previously described for total bacterial counts. Counts for the biofilm at the beginning of each experiment were performed on untreated plugs from the CDFF; the biofilm from two plugs (×2 for duplicate samples) being pooled as previously described to give an initial control biofilm count. Dressing tests were performed 'blindly', having been supplied in unmarked packaging and identified only by an alphabetical code.

To check that the results obtained were not pH related, the dressings were also tested with phosphate-buffered TSB broth (Na_2HPO_4, 10 g/L; NaH_2PO_4, 4 g/L; pH 7.0). Tests were also repeated in the absence and presence of 2% BSA.

Results

Coaggregation studies

Coaggregation tests were repeated in triplicate for the 31 chronic wound isolates. Table 2 shows typical data from the coaggregation testing. All wound isolates exhibited coaggregation with at least one other wound isolate, with both intra- and inter-generic interactions evident. Typically, scores between 0 and 2+ were recorded, although two strains, *Micromonas micros* F21 and *P. anaerobius* B12 gave higher scores. Interestingly, *M. micros* F21 exhibited high coaggregation scores with all organisms tested, although this could be attributed to autoaggregation as evident in the control. In contrast, *P. anaerobius* B12 showed very high, but selective coaggregation. The positive scores for *P. anaerobius* B12 were either between 2+ and 4+ (with pseudomonads and particular anaerobes), or there was a complete absence of coaggregation, e.g. with staphylococci.

Although coaggregation scores for wound bacteria in this study were typically lower than those reported in the literature for dental plaque isolates, increased coaggregation scores were recorded for each of the 15 combinations tested after the addition of the dental plaque isolate *F. nucleatum* (Table 3), with all showing increased coaggregation scores of between one and four orders of magnitude.

Table 3. Coaggregation scores[a] of pairs of chronic wound bacterial species with and without the addition of *F. nucleatum*

Species	1 D74[b]	2 C7	3 D40	4 B60	5 D58
2 *Micrococcus* sp. C7	0 (3+)				
3 *P. aeruginosa* D40	0 (3+)	0 (1+)			
4 *P. aeruginosa* B60	1 (3+)	0 (1+)	0 (1+)		
5 *S. aureus* D58	0 (4+)	1+ (3+)	1+ (3+)	1+ (3+)	
6 *S. aureus* D76	0 (4+)	0 (3+)	1+ (3+)	1+ (3+)	0 (4+)

The coaggregation score as a result of the addition of the dental isolate *F. nucleatum* is shown in parentheses.
[a]Key to the coaggregation scores is in the footnote of Table 2.
[b]*S. agalactiae* D74.

The coaggregation phenotype was in some cases shown to be variously affected by components in the growth media. The addition of fetal calf serum to the growth media had no affect on the ability to coaggregate in almost half the combinations tested (results not shown). An increase in the coaggregation scores was seen in 35% (8/23) of combinations tested with the addition of 10% fetal calf serum.

The CDFF wound model

Using the CDFF system it was evident that a wound biofilm could successfully be established *in vitro* and be maintained in a steady state over 7 days (Figure 1). In the quantitative analysis of the *in vitro* biofilms, *S. aureus* and *P. aeruginosa* predominated in all of the various wound consortia analysed; this situation being directly analogous to that found in the quantitative analysis of the microflora of chronic skin wounds *in vivo*.[40] Bacterial counts of *S. oralis* and *B. fragilis* were close to the minimum detection levels, whilst *P. anaerobius* and *M. luteus* were consistently not detected. Establishment of the chronic wound biofilm was confirmed by specific staining of the biofilm with calcofluor white (Figure 2a) and Congo Red (Figure 2b) with EPS strongly evident, and by scanning electron microscopy (Figure 2c).

The growth medium employed in the CDFF system had an effect on the ability to isolate organisms from the system. In BM medium (Figure 1a), only *P. aeruginosa* and *S. aureus* grew well, whilst *S. oralis* was close to the limits of detection and the other three strains were not detected at all. In contrast, all four aerobic species were more readily isolated when grown in BHI medium (Figure 1b). Here again though, *M. luteus* was at or close to the limits of detection.

### Antimicrobial efficacy against biofilm-grown wound bacteria

Peak serum concentrations achieved for a typical single oral dose[48] of 250 mg (flucloxacillin) and 500 mg (ciprofloxacin) range between 6.0 and 9.0, and 2.0 and 2.9 mg/L, respectively.[49] The mid-point in this range was taken as the peak serum concentration, and twice this dose was used to test the antimicrobial efficacy of these antibiotics against the chronic wound biofilms. No change in *S. aureus* counts was observed in response to flucloxacillin dosing at 15 mg/L (equivalent to twice the
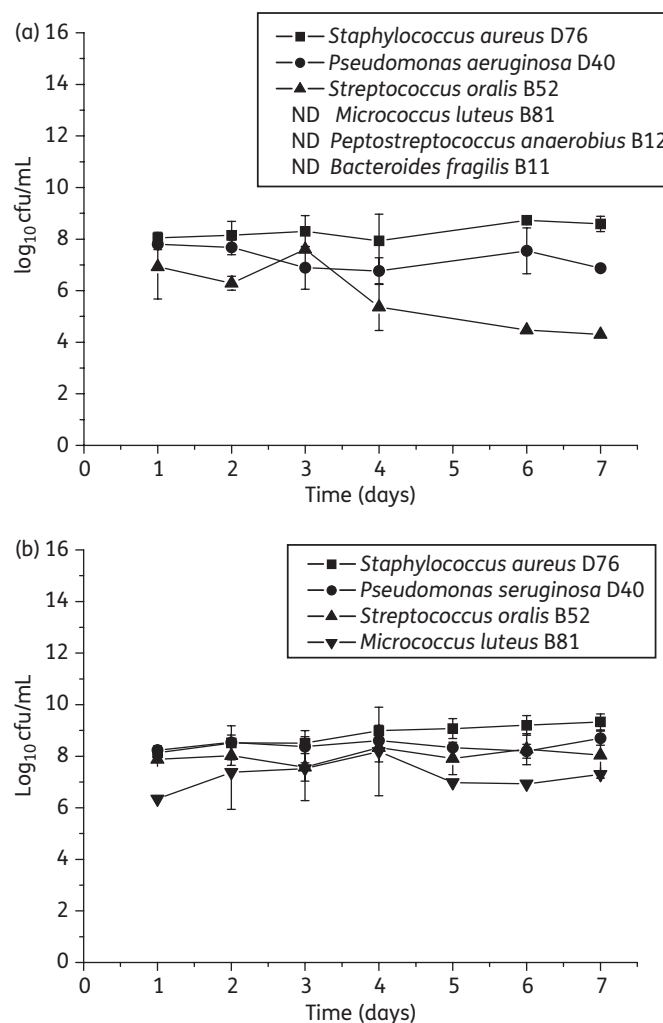
Figure 1. CDFF chronic wound model biofilm grown over 7 days: detection of (a) a six-member wound bacteria consortium grown in BM and (b) a four-member wound bacteria consortium grown in BHI. (Error bars represent standard deviations from two experiments.)

recommended therapeutic dose) with counts remaining ~8.0 log$_{10}$ cfu/mL for the 5 day duration of the antimicrobial treatment (Figure 3a). In contrast, a small 1.5-fold log decrease in counts for *P. aeruginosa* in response to treatment with ciprofloxacin at 5 mg/L (equivalent to twice the therapeutic dose) was observed (Figure 3b). The MICs for the planktonically grown wound isolates (*S. aureus* D46 and *P. aeruginosa* D40) were below the levels of antimicrobial used in the CDFF for these experiments (both at 1 mg/L of flucloxacillin and ciprofloxacin). The biofilm however, failed to respond (in the case of *S. aureus*) or had little response (for *P. aeruginosa*) to these antimicrobials at concentrations 5 or 15 × the MIC.

PVP1, used at a concentration of 1% (w/v) as a biocide in solution, showed minimal efficacy against wound biofilms (Figure 4). Prolonged PVP1 treatment (Figure 4) again showed a slight drop in *P. aeruginosa* and *S. aureus* counts by nearly 2-log fold. Importantly however, the effect was rapidly lost when PVP1 therapy was ceased; numbers of both organisms
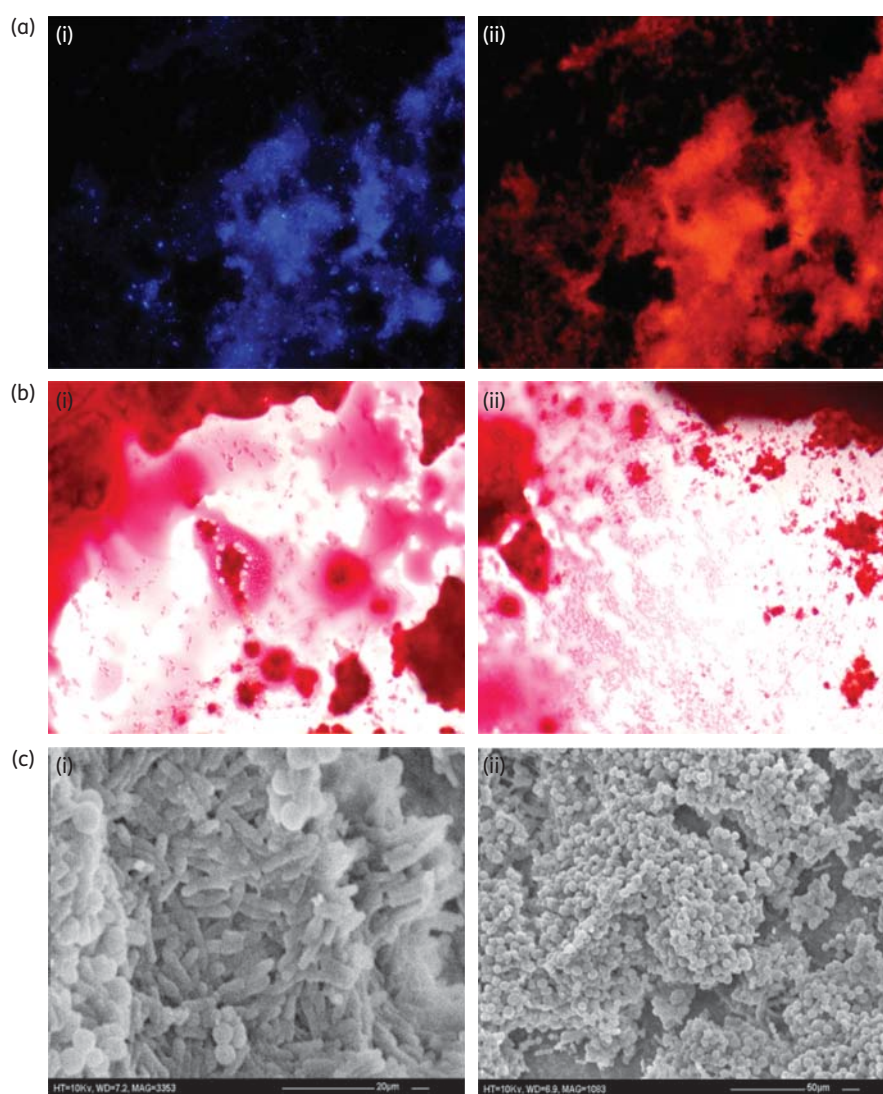
Figure 2. Visualization of the biofilm by: (a) (i) calcofluor white (stains EPS blue) and (ii) ethidium bromide (stains nucleic acids red) staining of CDFF-generated biofilms (×20); (b) (i) and (ii) Congo Red (stains polysaccharide orange/pink) and Ziehl carbol fuchsin (stains bacterial cells purple/red) staining of CDFF-generated biofilms (×100); (c) SEM pictures showing a build-up of bacterial cells with time in the CDFF with formation of distinct areas of predominantly (i) bacilli and (ii) cocci within the biofilm.

rapidly returning to their pre-treatment levels. The MIC of PVP1 for both planktonically grown *P. aeruginosa* and *S. aureus* was 1% (w/v).

Conditioning of the CDFF with lactoferrin (20 mg/L) for 1 h prior to inoculation resulted in no difference in bacterial biofilm counts or levels of bacteria attained for any species (results not shown).

Dressing testing of CDFF-generated biofilms

A schematic representation of the dressing test using biofilms generated in the CDFF is shown in Figure 5. The PVP1-containing Inadine® dressing and cadexomer iodine paste-containing Iodo-flex® dressing showed complete and efficient killing of all the bacteria in the biofilm (Figure 6). No bacteria were recovered

for either 3 or 7 day CDFF-generated biofilms (Figure 6d or b, respectively) after completion of the 7 day test for these two dressings. The therapeutic dose of PVP1 used in dressings such as Inadine® and Iodoflex® is 10% (equivalent to 1% available iodine and 0.9% w/w, respectively). Dressing testing with the other iodine-impregnated dressing, Betadine® cream/gauze showed a slight reduction in *S. aureus* bacterial counts to 10^6 cfu/mL for the 7 day biofilms (Figure 6b) and a slightly greater reduction to 10^4 cfu/mL for the 3 day biofilms (Figure 6d). However, no significant reduction in *P. aeruginosa* count was observed for the Betadine® cream/gauze.

From experiments using phosphate-buffered TSB pH 7.0, it was apparent that the Iodoflex® dressing was very acidic in both unbuffered (pH 3.59–3.18) and buffered TSB (pH 5.24 or 3.83) when tested by the addition of 10 or 5 mL TSB, respectively.
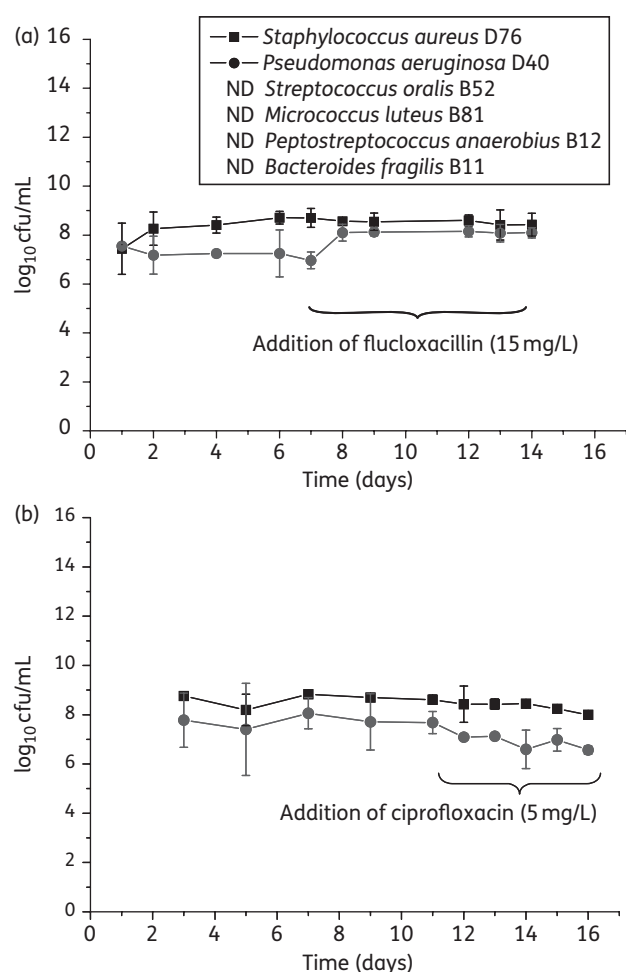
Figure 3. Effect of antibiotic treatment on a six-member wound bacteria biofilm, grown in BM in the CDFF (a) flucloxacillin 15 mg/L; (b) ciprofloxacin 5 mg/L. (Error bars represent standard deviations from at least two experiments.)

In contrast, buffering of TSB for all the other dressings (with pH values ranging from pH 6.04 to 8.26) brought most of the pH values close to pH 7.0 (with values then ranging from pH 6.43 to 7.22). Dressing tests using buffered TSB with 3 day biofilms produced similar results to those obtained previously with unbuffered TSB, showing that the antimicrobial effect of the dressings was unaffected by the pH of the dressing used. Importantly however, pH may play a role in the antimicrobial activity of Iodoflex®, as buffering appeared to have a minimal effect on neutralizing the pH of this dressing, it being still very acidic (pH < 5.25).

In contrast to the iodine dressings, silver-containing dressings tested on 7 day biofilms showed no difference between bacterial counts at the end of the test, and control plugs sampled at the beginning of the test. *S. aureus* and *P. aeruginosa* counts remained at ~10^7–10^8 cfu/mL for all silver dressings tested (Figure 6a). There was also no significant difference between the silver dressings and the unimpregnated control dressing, Nuderm®. This was the case with either BM or TSB (results not shown) showing that it was not a medium-related result. It was also not related to the presence or absence of 2% BSA (results not shown).
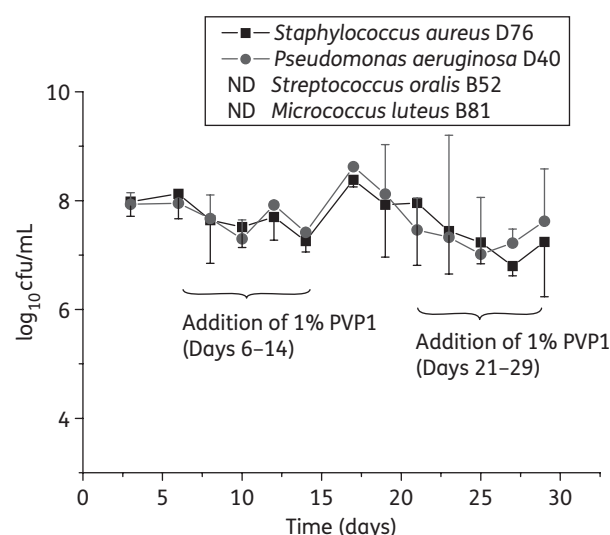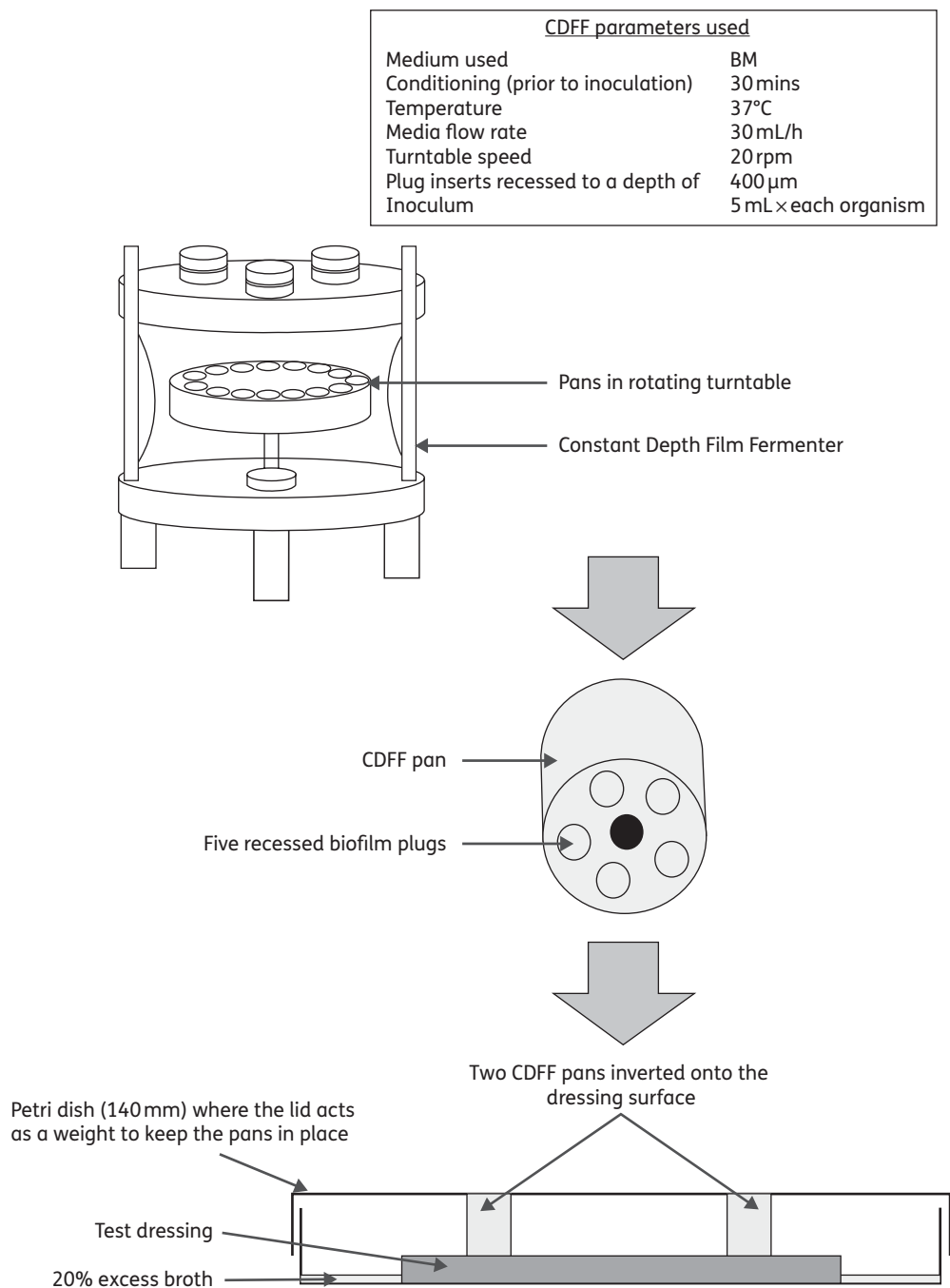

Figure 4. Effect of PVP1 on a mixed four-member wound bacteria consortium grown in BM. (Error bars represent standard deviations from two experiments.)

When the dressing tests were repeated with less mature 3 day CDFF-generated biofilms (Figure 6c) this resulted in a more noticeable decrease in numbers of both *S. aureus* and *P. aeruginosa* for Acticoat N® (1-log reduction) and for staphylococcal counts for the Actisorb silver 220® dressing (2- to 3-fold log reduction). No differences in bacterial counts were seen for any of the other silver dressings. Instead, counts for the other silver-containing dressings tested were comparable to both the non-silver-impregnated Nuderm® control and to the initial control biofilm counts.

Discussion

We have described the development of a reliable *in vitro* model of a chronic wound biofilm. In coaggregation studies, it was evident that the chronic wound bacteria (particularly the pseudomonads and staphylococci) had a high propensity to coaggregate and thus potentially form biofilms *in vitro*. Most wound isolates displayed the coaggregation phenotype, enabling them to come into close contact with one another, one of the first steps necessary for biofilm formation to occur *in vivo*.[21] These data are in keeping with histological examination of chronic and acute wound bacterial biofilms[12,13] which demonstrate the ability of these bacteria to produce large amounts of EPS.

Specificity was evident in the ability of chronic wound bacteria to coaggregate; the ability to coaggregate not being demonstrable in all combinations tested. This observation is consistent with the concept of specific adhesin–receptor interactions in coaggregation.[43] The low coaggregation scores seen with the chronic wound bacteria is not surprising given their natural habitat; a 'strong' coaggregation phenotype being more likely to occur in organisms exposed to high shear forces.[23] In these other non-wound environments, e.g. the oral cavity or fast-flowing streams, bacteria are more likely to exhibit strong coaggregation phenotypes.

| CDFF parameters used	
Medium used	BM
Conditioning (prior to inoculation)	30 mins
Temperature	37°C
Media flow rate	30 mL/h
Turntable speed	20 rpm
Plug inserts recessed to a depth of	400 μm
Inoculum	5 mL × each organism

Figure 5. Schematic representation of dressing test using biofilms generated in the CDFF and CDFF parameters used.

Wound bacteria were readily grown in the CDFF environment, being able to form biofilms within 24 h, in keeping with previous *in vitro* findings.[19] Reproducible, multi-species, CDFF wound biofilms could be maintained over a period of up to 4 weeks *in vitro*. In addition, the ability of the CDFF model system to allow the generation of multiple identical biofilms (simultaneously) is clearly beneficial in the comparative testing of antimicrobial therapies and dressings. With the exception of *P. acnes*, anaerobes were not detected in the consortium. In practice, in this system, the aerobic species tended to out-compete the anaerobes. This finding is not likely to be simply related to the presence of an aerobic environment within the fermenter; anaerobic species having been shown to survive oxygen stress when interacting with facultative and aerobic species.[24,50,51] This observation is more likely to be related to the inherent slower growth rates of the anaerobes compared with the pseudomonads and staphylococci. This is supported by PCR analysis of *in vitro* biofilms showing that, with the exception of *Finegoldia*
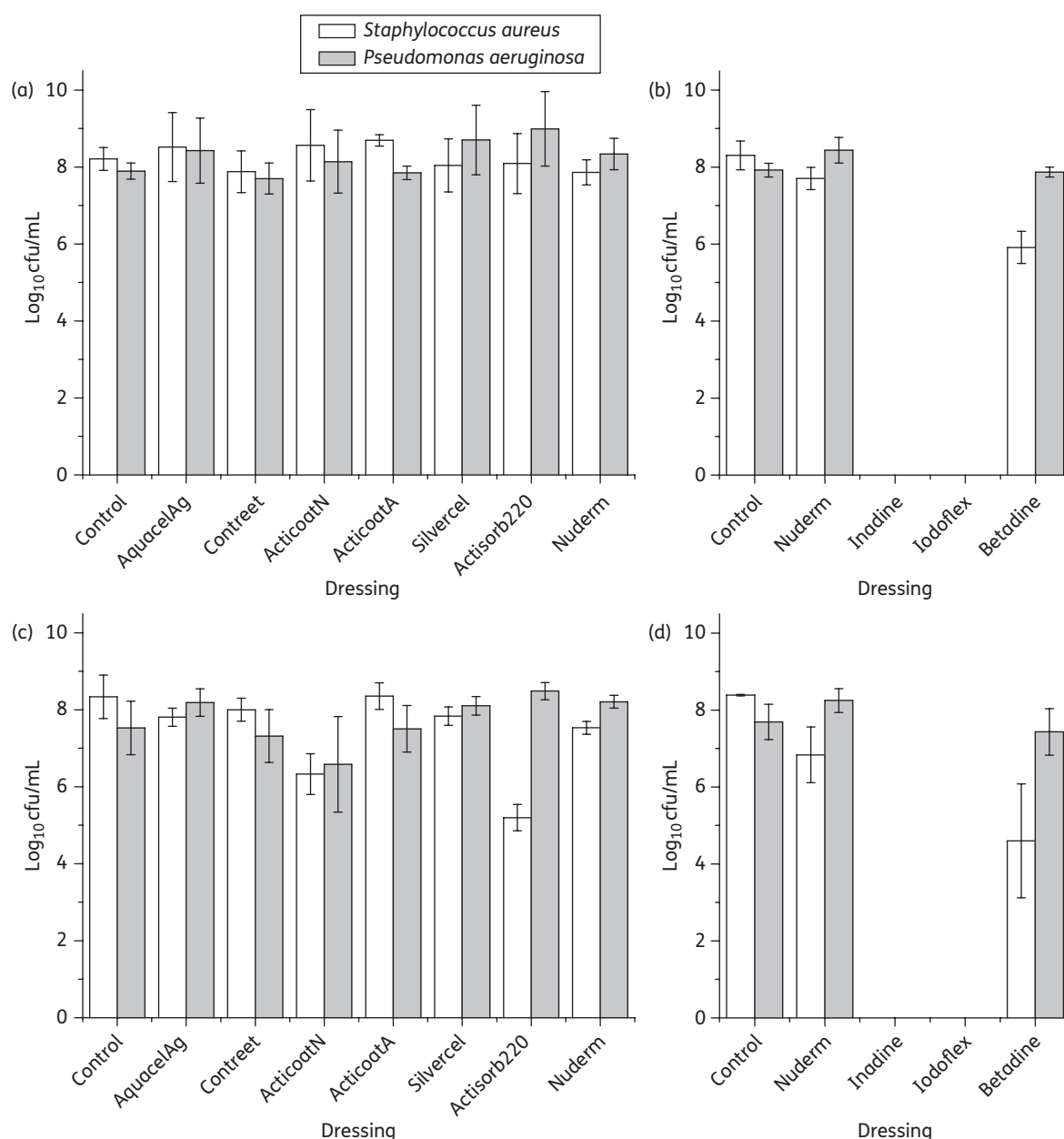
Figure 6. Dressing testing with silver (a, c) and iodine (b, d) dressings in TSB of (a, b) 7 day CDFF-generated biofilms and (c, d) 3 day CDFF-generated biofilms. (Error bars represent standard deviations from $n \geq 4$.) Only *S. aureus* and *P. aeruginosa* were detected.

magna (35%), anaerobes made up <5% of mixed chronic wound populations.[51] This could in the future be partly overcome by sequential inoculation of the CDFF model, which could potentially facilitate improved colonization by anaerobic species after aerobic species are established.

The finding that chronic wound bacteria in biofilms exhibit altered phenotypes compared with their planktonic (free-living) equivalents (in resistance to antibiotic therapy) is unsurprising. Biofilms also exhibit intercellular communication via quorum-sensing pathways and are protected from host defences and conventional antimicrobial therapies. Previous studies have demonstrated that biofilm-grown bacteria can be up to 1000

times more resistant to antibiotics than planktonically grown cells;[52] bacterial EPS also plays an important role in protecting a biofilm from external attack. In addition to this, bacteria within biofilms themselves are known to employ distinct mechanisms to resist the action of antimicrobial agents:[53] bacterial periplasmic glucans are able to bind to and physically sequester antibiotics.

The ineffectiveness of ciprofloxacin and flucloxacillin against wound biofilms, even when used at twice peak serum levels and at 5 and 15 times their MICs, respectively, was evident. The mechanism for this resistance is probably related to oxygen limitation and low metabolic activity beneath the biofilm surface, rather

than poor antibiotic penetration.[54] Borriello *et al.*[55] demonstrated that only organisms at the biofilm/air interface are metabolically active. In consideration of the antibiotic treatment of chronic wounds, the penetration of antibiotics into healthy subcutaneous tissue is relatively poor.[56] The penetration of antibiotics into the chronic wound bed in patients with peripheral vascular disease and circulatory impairment is, moreover, likely to be further impaired *in vivo*. These findings of increased biofilm resistance to antibiotics are therefore likely to contribute to treatment failure *in vivo*, especially if delivered topically.

Iodine has a long history as an antimicrobial therapy (both for treatment and prophylaxis of infection) and for many years has been incorporated into a range of wound dressings used routinely in the treatment of chronic wounds. The contrast between the effectiveness of iodine used in a topical dressing and when used in solution was striking. Almost 2-log reductions in bacterial numbers of both pseudomonads and staphylococci were observed when PVP1 was applied to the CDFF as a biocide (in solution). However, the 8 day PVP1 treatment failed to have a significant impact on the microbial flora; its effect only being sustained whilst the biocide was applied. The bacterial population rapidly regained their pre-treatment levels as soon as PVP1 treatment was stopped. To mimic the concentrations used in wound dressings, PVP1 was used in this study at the actual MIC for both species. Increasing this 5- or 10-fold may have more impact on wound biofilms.

The effect of iodine on wound biofilms was significantly different when iodine was presented in a wound dressing format. The iodine-impregnated dressings, Inadine® and Iodoflex®, proved to be extremely efficient antibiofilm agents. Interestingly, Betadine® cream, containing povidone–iodine was less effective, presumably due to the fact that iodine in the Inadine® and Iodoflex® dressings is delivered from a gel excipient (a polyethylene glycol or cadexomer base, respectively) giving them a more prolonged efficacy. This gel base may further aid breakdown of the EPS surrounding the biofilm, thereby more readily exposing the bacteria directly to the antimicrobial. Interestingly, whilst the Iodoflex® dressing was extremely effective against biofilms, it appeared to be more acidic compared with the other dressings tested (pH < 5.25), a property perhaps contributing to its significant wound de-sloughing properties.

Dressing testing showed that iodine was much more effective against wound biofilms than silver. Of the silver dressings, only the silver-impregnated activated charcoal, Actisorb silver 220® and the nanocrystalline silver Acticoat N® had any effect on wound biofilms, but then only in a limited manner and only against 3 day biofilms. These results contrast directly with previous studies which suggest that silver is an effective antimicrobial against wound biofilms,[14,57,58] possibly as a result of the sequestration of matrix metalloproteinases by silver-containing wound care products.[59] Bjarnsholt *et al.*[60] have suggested that the concentrations of silver currently available in wound dressings are too low (<11 mg/cm^2) to be effective against chronic wound biofilms. Silver concentrations for Acticoat N® and Actisorb Silver 220® have previously been determined,[61] and both values are considerably lower than that reported by Bjarnsholt *et al.*[60] However, the actual level of silver released into the wound is dependent upon the wound environment, not the level of silver in the dressing. Once the wound environment is saturated, no additional silver will be solubilized. The remaining silver in the dressing acts as a reservoir, replenishing the supply to the wound and maintaining this level of saturation. Testing on many silver-releasing dressings has shown that this level of silver can be maintained in the wound for many days, with ionic silver dressings typically lasting a shorter time than the elemental silver-containing dressings. While the level of silver in the wound may provide an insight into the relative ineffectiveness of the silver-containing dressings tested in this *in vitro* model, it does not explain the positive effects observed with Actisorb silver 220®. This dressing is a silver-impregnated charcoal dressing and as such does not release significant levels of silver; its antimicrobial efficacy relates to its ability to bind bacteria and bacterial endotoxins.

Lactoferrin is a component of the innate immune system that is found in many human external secretions, and has been postulated to play a potential therapeutic role in preventing biofilm development.[62] By chelating iron, lactoferrin stimulates a form of cellular motility that encourages bacterial cells to be motile rather than adhering and forming biofilms. This effect has been demonstrated at lactoferrin concentrations below those that kill or prevent growth.[62] Lactoferrin has also been shown to increase antimicrobial susceptibility to particular antibiotics.[63] Moreover, deficiencies in synthesis of innate lactoferrin appear to predispose certain individuals to increased risk of infection e.g. biofilm-associated chronic rhinosinusitis.[64] In this model, pre-treatment with lactoferrin did not affect biofilm formation or bacterial numbers in the CDFF. Hence, a single-dose treatment in this way appears to be ineffective as an anti-biofilm therapy, with continuous exposure of biofilms to lactoferrin apparently necessary to have an effect.[62,63]

In the present study, the benefits of using the CDFF chronic wound biofilm model in testing the effectiveness of currently employed antimicrobial agents, has clearly been demonstrated. The reproducibility of identical biofilms makes the CDFF an ideal model to test the efficacy of therapeutic interventions. Moreover, the ability to image the bacterial communities within biofilm systems in three dimensions and real-time,[20] is a distinct advantage. Further refinement of the model (e.g. the addition of collagen or fibronectin substrates) may be considered in the future to more closely mimic the *in vivo* situation. If the bacteria in chronic wounds exhibit a 'biofilm-like' phenotype, then the importance of physical disruption of this biofilm and, moreover, alternative, i.e. non-antibiotic, antimicrobial strategies is likely to be increasingly important in the future management of patients.

Transparency declarations

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