Controlled-release iodine foam dressings demonstrate broad-spectrum biofilm management in several in vitro models

Anne-Marie Salisbury | Marc Mullin | Lauren Foulkes | Rui Chen | Steven L. Percival

Abstract
Multiple in vitro models were utilised to evaluate the biofilm management capabilities of seven commercially-available wound dressings, varying in composition and antibacterial ingredients, to reduce common aerobic, anaerobic, and multispecies biofilms. The Center for Disease Control bioreactor was used to evaluate single species Pseudomonas aeruginosa (P. aeruginosa) and Staphylococcus aureus (S. aureus) 24 and 48 hours biofilms, as well as a multispecies biofilm consisting of these two organisms in addition to Enterococcus faecalis (E. faecalis). As wound biofilms often exist in hypoxic wound environments, a direct contact anaerobic model system was used to evaluate efficacy on Bacteroides fragilis (B. fragilis). Biofilm control was evaluated against P. aeruginosa in the drip flow bioreactor model, where a constant flow of proteinaceous media is used to create a more challenging and wound-like model. The results demonstrated that biofilm management capabilities varied amongst wound dressings. Two dressings, a controlled-release iodine foam dressing and a silver nanocrystalline dressing, showed potent >4 log reductions in recovered organisms compared with untreated controls in all biofilm models evaluated. The effectiveness of other dressings to manage bioburden varied between dressing, test organism, and model system. A silver foam dressing showed moderate biofilm control in some models. However, biofilm exposure to methylene blue and gentian violet-containing foam dressings showed negligible log reductions in all in vitro biofilm methods examined. The data outlined in this in vitro study support the use of the iodine foam dressing for wounds with infection and biofilm.

Abbreviations: B. fragilis, Bacteroides fragilis; BSN, Broad Spectrum Neutralizer (including 30 g/L polysorbate 80; 3 g/L lecithin; 2 g/L cysteine; and 1 g/L histidine); CDC, center for disease control; CFU, colony forming units; CMC, carboxymethyl cellulose; E. faecalis, Enterococcus faecalis; EPS, extracellular polymeric substance; LD, log density; LR, log reduction; MB/GV, methylene blue and gentian violet; P. aeruginosa, Pseudomonas aeruginosa; PU, polyurethane; PVA, polyvinyl alcohol; S. aureus, Staphylococcus aureus; TSA, Tryptone soy agar; TSB, Tryptone soy broth.

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**KEYWORDS**
biofilm, in vitro biofilm models, iodine foam dressing, wound healing

**Key Messages**
- in combination with debridement and systemic antibiotics, antibacterial dressings are utilised to manage infected wounds. Due to the broad mechanism of action of antibacterial dressings, bacterial resistance to them is often low or non-existent, making them an attractive alternative to antibiotic treatment
- in this study, the efficacy of biofilm management was evaluated against a multispecies biofilm of three of the most common bacterial species isolated from chronic wounds, an anaerobic strain, *B. fragilis*, as well as evaluation against mono species *P. aeruginosa* and *S. aureus* biofilms in several in vitro biofilm models: CDC bioreactor, multispecies biofilm model, drip flow bioreactor, direct contact anaerobic model
- the data outlined in this in vitro study demonstrated that iodine foam dressing has broad-spectrum biofilm management ability, which supports the use of the iodine foam dressing for wounds with infection and biofilm

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**1 | INTRODUCTION**

Wound healing occurs through a process of several steps including inflammation, cell proliferation, and cellular remodelling. Disruption in these steps can prevent wound healing and lead to the formation of chronic wounds. Bacterial colonisation of wounds and the formation of biofilms have been shown to have a significant role in delayed wound healing. When wounds become colonised at levels of $10^5$ viable organisms per gram of tissue or greater, they are said to be critically colonised (the authors of this publication prefer the use of the terms subclinical infection), which significantly increases the risk of infection and subsequent biofilm formation in the wound and surrounding tissues.

Biofilm formation can be initiated by various environmental cues and starts when bacterial cells attach to a surface and each other and form aggregates. Biofilms consist of microorganisms encased in a matrix of extracellular polymeric substance (EPS) which is composed of water, polysaccharides, nucleic acids (extracellular DNA) and proteins. Biofilms have been shown to be up to 1000× more recalcitrant to antibiotics than their planktonic counterparts making them difficult to treat. Multiple mechanisms are thought to contribute to biofilm recalcitrance to antibiotics including slow growth of the bacterial cells and also the EPS, which diffuses and lowers the concentration of antibiotic reaching the bacterial cells.

In a recent systemic review and meta-analysis of published data, it was estimated that the prevalence of biofilms in chronic wounds was 78.2%. Treatment options for chronic wounds that have become infected include cleansing and debridement of the wound, use of topical antiseptics, and use of antimicrobial dressings. Silver dressings are the most widely used antimicrobial dressings and have been shown to be effective against biofilms; however, cytotoxicity and bacterial resistance to silver have also been found.

Iodine is a broad-spectrum antiseptic that has demonstrated antimicrobial activity against Gram-negative bacteria, Gram-positive bacteria, fungi, protozoa and viruses. Iodine-based dressings have been shown to be effective at debriding wounds and killing bacteria, which may lead to the promotion of wound healing. In addition, in contrast to clinically used antibiotics and some antimicrobials, such as silver, no bacterial resistance has been found against iodine to date. This study aimed to evaluate the ability of an iodine-based dressing to reduce biofilm in comparison to other commercially available dressings. The dressings were evaluated against immature and mature biofilms of representative common wound pathogens including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, a multispecies biofilm (*S. aureus, P. aeruginosa, and Enterococcus faecalis*), and the anaerobic strain, *Bacteroides fragilis*.

**2 | MATERIALS AND METHODS**

2.1 | Test articles

In this study, the ability to control biofilm within an iodine foam dressing was evaluated against six...
comparator dressings (Table 1). An untreated biofilm growth control was included in each experiment to compare the biofilms treated with each dressing to biofilms grown under the same conditions with no dressing added.

### 2.2 Methods

Biofilms were created using the Center for Disease Control (CDC) Bioreactor (single and mixed species), the drip flow bioreactor, and a direct contact anaerobic biofilm model. Biofilm growth conditions and microbes evaluated are described in Table 2.

#### 2.3 CDC bioreactor

The ability of each dressing to reduce biofilm was determined against a 24 and 48 hours biofilm of *P. aeruginosa* ATCC 15442 and *S. aureus* ATCC 6538. The CDC bioreactor assay was performed following an adapted version of ASTM 2871 - 19. Overnight cultures of *P. aeruginosa* ATCC 15442 and *S. aureus* ATCC 6538 were set up by inoculating 10 mL of TSB with a single colony of the strains. Cultures were incubated for 24 hours at 37°C and 125 rpm in an orbital shaking incubator. The overnight culture of *P. aeruginosa* was adjusted to 0.5 McFarland (1 × 10⁸ CFU/mL) and used to inoculate the CDC bioreactor by adding 1 to 300 mL TSB. The CDC bioreactor was incubated in batch phase at room temperature and 125 rpm on a magnetic stir plate for 24 hours. The overnight culture of *S. aureus* was centrifuged at 4800 rpm for 3 minutes. The cell pellet was resuspended in 1 mL TSB and used to inoculate the CDC bioreactor. The CDC bioreactor was made up to a final volume of 300 mL TSB and incubated in batch phase at 37°C and 80 rpm for 24 hours. The 24 hours biofilms were grown in batch phase for the duration of the incubation. Forty-eight hour biofilms were grown in batch phase for 24 hours followed by continuous phase for an additional 24 hours, with temperature and stirring conditions kept the same as batch phase. Reactors were connected to a nutrient carboy containing 100 mg/L TSB (*P. aeruginosa* and *S. aureus*) and incubated at a flow rate of 11.67 ± 0.2 mL/min.

After 24 or 48 hours biofilm growth, rods were washed twice in sterile distilled water (SDW) and

### Table 1: Dressings tested

| Dressing number | Dressing                          | Dressing type                                      |
|-----------------|-----------------------------------|---------------------------------------------------|
| 1               | Optifoam, Medline Industries Inc  | Foam                                              |
| 2               | Hydrofera BLUE CLASSIC, Hydrofera LLC | Methylene blue and gentian violet polyvinyl alcohol foam (MB/GV PVA foam) |
| 3               | Hydrofera BLUE READY, Hydrofera LLC | Methylene blue and gentian violet polyurethane foam (MB/GV PU foam) |
| 4               | AQUACEL Ag Advantage, ConvaTec Inc | Silver carboxymethyl cellulose (CMC)              |
| 5               | Mepilex Ag, Mölnlycke Health Care Ltd | Silver foam                                        |
| 6               | IoPlex, Medline Industries Inc    | Controlled-release iodine foam                     |
| 7               | ACTICOAT 7, Smith & Nephew Inc    | Silver nanocrystalline                             |

### Table 2: Methods summary

| Method            | Single or multispecies biofilm | Organism(s) tested    | Aerobic or anaerobic | Biofilm growth time (h) | Dressing challenge time (h) |
|-------------------|--------------------------------|-----------------------|----------------------|-------------------------|---------------------------|
| CDC bioreactor    | Single                         | *P. aeruginosa* ATCC 15442 | Aerobic             | 24                      | 24                        |
| CDC bioreactor    | Single                         | *S. aureus* ATCC 6538  | Aerobic             | 24                      | 24                        |
| CDC bioreactor    | Single                         | *P. aeruginosa* ATCC 15442 | Aerobic             | 48                      | 24                        |
| CDC bioreactor    | Single                         | *S. aureus* ATCC 6538  | Aerobic             | 48                      | 24                        |
| CDC bioreactor    | Multispecies                   | *P. aeruginosa* ATCC 15442 | Aerobic             | 24                      | 24                        |
|                   |                                 | *S. aureus* ATCC 29213 |                     |                         |                           |
|                   |                                 | *E. faecalis* ATCC 29212 |                     |                         |                           |
| Direct contact    | Single                         | *B. fragilis* ATCC 25285 | Anaerobic           | 48                      | 24                        |
| Drip flow bioreactor | Single                        | *P. aeruginosa* ATCC 70088 | Aerobic             | 24                      | 24                        |

Abbreviations: *B. fragilis*, *Bacteroides fragilis*; *E. faecalis*, *Enterococcus faecalis*; *P. aeruginosa*, *Pseudomonas aeruginosa*; *S. aureus*, *Staphylococcus aureus*.
individual coupon discs were placed into 12-well plates. A 2.5 × 5 cm² section (this size of dressing was necessary to ensure complete encasing of the coupons - if smaller dressing sizes were used it was found that those dressings that shrink significantly once hydrated that is, CMC dressing regions of the coupon were not covered leading to significant biofilm growth on the coupon) of each dressing was then added to each coupon, in triplicate, by encasing the coupon discs in the dressing and ensuring the coupons were in complete contact with the dressings. Each dressing was hydrated to full saturation point with SDW and incubated for 24 hours at room temperature. The following day, coupons were removed from the wells and added to 10 mL Dey Engley neutralising broth (silver-based dressings) or BSN 1 (all other dressings), before being sonicated at full power for 30 minutes. All tubes were vortexed briefly, samples were serially diluted 1:10 in phosphate buffered saline (PBS), and dilutions were plated onto TSA in duplicate by pipetting 50 μL onto each half and spreading. Plates were incubated at 37°C overnight and the following day colonies were enumerated.

### 2.4 Multispecies biofilm model

The ability of the dressings to reduce biofilm was determined against a 24 hours multispecies biofilm of *P. aeruginosa* ATCC 15442, *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212. The CDC bioreactor assay was performed as described above, except for the inoculation step. For inoculation of the multispecies biofilm model, the overnight cultures of *P. aeruginosa* and *E. faecalis* were adjusted to $1 \times 10^8$ CFU/mL and used to inoculate the CDC bioreactor by adding 1 mL of each strain to 300 mL TSB. The overnight culture of *S. aureus* was centrifuged at 4800 rpm for 3 minutes, before discarding the supernatant and resuspending the cell pellet in 1 mL of TSB. The resuspended cell pellet was then used to inoculate the CDC bioreactor by adding it to 300 mL TSB. The CDC bioreactor was incubated at 37°C and 125 rpm for 24 hours.

### 2.5 Drip flow bioreactor

The drip flow bioreactor was prepared following the method described by Bourdillon et al to represent an exudative wound environment. A 2.5 cm² absorbent pad was attached to the centre of a clean borosilicate microscope slide using non-toxic glue and placed in each channel of the drip flow bioreactor. The device was then autoclaved at 121°C for 15 minutes.

Next, an overnight inoculum was set up by inoculating 10 mL TSB with a single colony of *P. aeruginosa* ATCC 70088 and incubating at 37°C and 125 rpm. The following day, the absorbent pads were moistened with TSB and 2 cm² Durapore membrane filter discs (0.1 μM) were added to each pad. The overnight culture was adjusted to $1 \times 10^8$ CFU/mL and 10 μL of adjusted culture was added to each filter membrane. The drip flow bioreactor was then placed level and the adjusted culture allowed to air dry for 30 minutes. The drip flow was then secured and connected to a nutrient flow of 270 mg/L TSB at 5 mL/h/channel. After 24 hours, 2.5 cm² samples of each test dressing were added onto each filter membrane and the drip flow was run for a further 24 hours. Each dressing was tested in triplicate. After the challenge period, each filter membrane was sonicated in 10 mL Dey Engley neutralising broth (Ag-based dressings) or BSN 1 (all other dressings), serial diluted 1:10 in PBS and plated in duplicate onto TSA by pipetting 50 μL onto each side and spreading. The plates were incubated overnight at 37°C and the following day colonies were enumerated.

### 2.6 Direct contact anaerobic model

The efficacy of the dressings to manage biofilm was determined against a 48 hours anaerobic biofilm of *B. fragilis* ATCC 25285 by growing the biofilm in 12 well plates. A single colony of *B. fragilis* ATCC 25285 was inoculated into TSB + 5% laked horse blood and incubated under anaerobic conditions (9%-13% CO2) at 37°C and 125 rpm for 24 hours. The overnight culture was added to each well of a 12-well plate by adding 1 mL per well. The plate was incubated statically for 48 hours under anaerobic conditions at 37°C. After incubation, the liquid was removed ensuring not to disturb the biofilm. Test dressings were hydrated with SDW and added to wells in triplicate ensuring the entire surface of the biofilm was covered. The plates were then incubated statically for 24 hours at 37°C under anaerobic conditions. After the challenge period, dressings were removed and 1 mL Dey Engley neutralising broth (Ag-based dressings) or BSN 1 (all other dressings) was added to each well and the biofilm was scrapped off the surface using sterile pipette tips. The contents of each well were transferred to Falcon tubes containing 9 mL neutraliser and sonicated on full power for 30 minutes. Samples were then vortexed briefly, serial diluted 1:10 in PBS and plated onto TSA + 5% sheep blood using the Miles Misra method, by plating 10 μL of each dilution onto agar and allowing to dry. The plates were then incubated for 48 hours at
37°C under anaerobic conditions and colonies were enumerated.

2.7 | Statistical analysis

The number of colony forming units (CFU) per plate was enumerated and raw data were input into Microsoft Excel. The log density (LD) of each sample was determined using the following formula:

\[
\text{Log density (LD)} = \log_{10}(\text{CFU/cm}^2) = \log_{10}(X/B)(V/A)(D),
\]

where

- \( X \) = mean CFU
- \( B \) = volume plated
- \( V \) = final volume
- \( A \) = surface area scraped
- \( D \) = dilution.

The overall log10 density per group was calculated by taking the mean of the log10 densities, \( n = 3 \) per group, unless otherwise noted. CDC bioreactor and drip flow bioreactor dilutions were plated in duplicate and the average was used in calculations; direct contact anaerobic dilutions were plated once. The mean log10 density (CFU/cm²) of each test dressing was subtracted from the mean LD of the untreated controls to determine log reduction (LR) in recovered organisms. For experiments where no colonies were observed at any dilution plated, a value of 0.5 CFU was assigned to the lowest dilution counted. To determine if there was a statistical difference in the LD (log10[CFU/cm²]) of recovered organism between the untreated control and the treated biofilms, a one-way ANOVA with Dunnett’s multiple comparisons test was conducted using GraphPad Prism 7, version 7.0c software (* \( p < .05 \)). Normal distribution of the residuals from all one-way ANOVAs was evaluated using the D’Agostino’s and Pearson normality test.

3 | RESULTS

3.1 | CDC bioreactor

To measure the effectiveness of dressings to reduce biofilm bioburden in the CDC bioreactor model system, single species biofilms (\( P. \) aeruginosa [ATCC 15442] and \( S. \) aureus [ATCC 6538]) were independently grown on coupons for either 24 and 48 hours. Biofilm-covered coupons were then left untreated or exposed to test dressings for 24 hours, neutralised, sonicated, and enumerated.

3.1.1 | \( P. \) aeruginosa

The 24 hours \( P. \) aeruginosa untreated biofilm growth control had a mean log10 density (±SD) of 7.18 (±0.12) CFU/cm². In contrast, no colonies were observed following biofilm exposure to the iodine foam, silver nanocrystalline, and silver foam dressings, corresponding with 6.1 LR in recovered organisms compared with the untreated control (* \( p < .05 \)). The mean log10 density following biofilm exposure to the MB/GV PU foam and a silver CMC dressing was 4.92 (±0.46) and 4.31 (±0.95) CFU/cm², corresponding with 2.3 and 2.9 LRs compared with the untreated control, respectively (* \( p < .05 \)). Biofilm exposure to the MB/GV PVA foam and an inactive control foam dressing, resulted in mean log10 densities of 5.40 (±1.06) and 5.87 (±0.34) CFU/cm², or 1.8 and 1.3 LRs compared with the untreated control, respectively (* \( p < .05 \) (Figure 1A). To determine if the amount of recovered organisms from coupons exposed to the controlled-release iodine foam was statistically significantly different from the amount of recovered organisms from biofilm-covered coupons exposed to other dressings, separate statistical analyses were performed. The amount of recovered organisms in coupons exposed to the iodine foam dressing was significantly lower than the inactive control foam, MB/GV PVA foam, MB/GV PU foam, and silver CMC dressings (* \( p < .05 \) (Figure 1A).

The 48 hours \( P. \) aeruginosa untreated biofilm growth control had a mean log10 density of 8.93 (±0.35) CFU/cm². In similar to the 24 hours \( P. \) aeruginosa CDC bioreactor experiment, no colonies were observed following biofilm exposure to the iodine foam or the silver nanocrystalline dressing, corresponding with 7.8 LRs in recovered organisms compared with the untreated control (* \( p < .05 \). In contrast to the 24 hours biofilm where no colonies were observed following exposure to the silver foam dressing, the 48 hours biofilm was more recalcitrant. The mean log10 density recovered following exposure to the silver foam dressing was 3.82 (±0.31), corresponding with a 5.1 LR compared with the untreated control (* \( p < .05 \). Exposure to the MB/GV PU foam, MB/GV PVA foam, and the silver CMC dressings (* \( p < .05 \) (Figure 1B). To determine if the amount of recovered organisms from biofilms exposed to the controlled-release
iodine foam was statistically different from the amount of recovered organisms from biofilm-covered coupons exposed to other dressings, separate statistical analyses were performed. The amount of recovered organisms in coupons exposed to the iodine foam dressing was statistically significantly lower than the inactive control foam, MB/GV PVA foam, MB/GV PU foam, silver CMC, and silver foam dressings ($p < .05$) (Figure 1B).

### 3.1.2 Staphylococcus aureus

The 24 hours S. aureus untreated biofilm growth control had a mean log$_{10}$ density (±SD) of 7.12 (±0.15) CFU/cm$^2$. Conversely, no colonies were observed following biofilm exposure to the iodine foam, silver foam, and silver nanocrystalline dressings, corresponding with 6.0 LRs in recovered organisms, compared with the untreated control ($p < .05$). No other dressings achieved the desired >4.0 LR threshold. The mean log$_{10}$ densities following biofilm exposure to the inactive control, MB/GV PU foam, silver CMC, and MB/GV PVA foam dressings were 6.30 (±0.52), 5.98 (±0.28), 4.85 (±1.02), and 3.66 (±0.03) CFU/cm$^2$, or 0.8, 1.1, 2.3, 3.3, and 3.5 LR in recovered organisms, respectively. There was no statistically significant difference between LD of recovered organisms between the untreated control and the inactive control foam dressing ($p < .05$). The amount of recovered organisms from biofilm exposed to the iodine-impregnated dressing was significantly lower than all other dressings tested, with the exception of the silver foam and silver nanocrystalline dressings, which had similar recovery ($p < .05$) (Figure 1C).

The 48 hours S. aureus untreated biofilm growth control had a mean log$_{10}$ density of 5.8 (±0.23) CFU/cm$^2$. No colonies were observed following biofilm exposure to the iodine foam, silver foam, and the silver nanocrystalline dressings, corresponding with 4.7 LRs in recovered organisms compared with the untreated control ($p < .05$). Minimal LRs were observed with other
dressings. The mean log_{10} densities following biofilm exposure to the inactive control, MB/GV PU foam, silver CMC, and MB/GV PVA foam dressings were 4.71 (±0.67), 4.48 (±0.42), 3.87 (±0.92), and 4.13 (±0.77) CFU/cm², or 1.1, 1.3, 1.9, and 1.7 LR in recovered organisms, respectively (*p < .05). The log_{10} density of recovered organisms from biofilm exposed to the iodine foam dressing was statistically significantly lower than all other dressings tested, with the exception of the silver foam and silver nanocrystalline dressings, which had similar recoveries (*p < .05) (Figure 1D).

3.1.3 | Multispecies biofilm

The CDC bioreactor was also used to evaluate 24 hours multispecies (*P. aeruginosa* ATCC 15442, *S. aureus* ATCC 29213, and *E. faecalis* ATCC 29212) biofilms. In this model, three dressings were able to reduce bioburden >4 logs, when compared with untreated control, including the controlled-release iodine foam, silver foam, and silver nanocrystalline dressings. No colonies were observed following biofilm exposure these dressings, corresponding with mean log_{10} densities of 1.12 (±0.00) CFU/cm² and 5.04 LR. The mean log_{10} density of recovered organisms was 2.96 (±1.60) CFU/cm² and 3.2 LR compared with untreated control for the silver CMC dressing. Negligible LRs were observed with other dressings. The mean log_{10} densities following biofilm exposure to the inactive control, MB/GV PU foam, and MB/GV PVA foam dressings were 5.44 (±0.54), 4.59 (±0.06), and 4.11 (±0.42) CFU/cm², or 0.7, 1.6, and 2.0 LR in recovered organisms, respectively. The mean log_{10} density of recovered organisms from the inactive control dressing was not statistically significantly different from the untreated control (*p < .05). The log_{10} density of recovered organisms from biofilm exposed to the iodine foam dressing was statistically significantly lower than all other dressings tested, with the exception of the silver foam and silver nanocrystalline dressings, which had similar recoveries (*p < .05) (Figure 2).

3.2 | Drip flow bioreactor

The drip flow bioreactor is advantageous for characterising wound dressing effectiveness at reducing bioburden because it creates biofilms at an air-liquid interface, and is thus the *in vitro* model most similar to a wound environment. Using the drip flow, *P. aeruginosa* ATCC 70088 biofilm was grown on membranes for 24 hours and then membranes were either left untreated or exposed to wound dressings for 24 hours. In this model, three dressings were able to reduce bioburden >4 logs, when compared with untreated control, including the controlled-release iodine foam, silver foam, and silver nanocrystalline dressings. No colonies were observed following biofilm exposure to the iodine foam and silver nanocrystalline dressings, corresponding with mean log_{10} densities of 0.90 (±0.00) CFU/cm² and 6.0 LR. The mean log_{10} density of recovered organisms was 2.02 (±0.99) CFU/cm² and 4.9 LR compared with untreated control for the silver CMC dressing. Minimal LRs were observed with other dressings. The mean log_{10} densities following biofilm exposure to the inactive control, silver foam, MB/GV PU foam, and MB/GV PVA foam dressings were 5.76 (±0.43), 2.99 (±1.26), 6.70 (±0.08), and 6.40 (±0.04) CFU/cm², or 1.1, 3.9, 0.2, and 0.5 LR in recovered organisms, respectively. The mean log_{10} density of recovered organisms from the MB/GV PU, MB/GV PVA, and inactive control foam dressings were not statistically different from the untreated control (*p < .05). The amount of recovered organisms from

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**FIGURE 2** Multispecies CDC bioreactor model system. Mean log_{10} densities of recovered organisms following multi-species (*Pseudomonas aeruginosa* ATCC 15442, *S. aureus* ATCC 29213, and *E. faecalis* ATCC 29212) 24 hours CDC bioreactor-generated biofilm exposure to various wound dressings. Data are presented as mean log_{10} density (CFU/cm²) ± SD. n = 3 per group. *p < .05 one-way ANOVA with Dunnett’s post-test, where the log_{10} densities of recovered organisms from the untreated control biofilm were compared with the log_{10} densities of test dressings. #p < .05 one-way ANOVA with Sidak post-test, where all dressings (but not the untreated control) were compared with the iodine foam dressings.
biofilm exposed to the iodine-impregnated dressing was significantly lower than all other dressings tested, with the exception of the silver CMC and silver nanocrystalline dressings, which had similar recoveries (*p < .05) (Figure 3).

### 3.3 Anaerobic model

To test the effectiveness of wound dressings at managing biofilm created under anaerobic conditions, *B. fragilis* ATCC 25285 biofilms were grown for 48 hours and then either left untreated or exposed to wound dressings for 24 hours. In this model, only two dressings, the controlled-release iodine foam and the silver nanocrystalline, were able to reduce bioburden ≥4 logs, when compared with untreated control. No colonies were observed following biofilm exposure to the iodine foam and silver nanocrystalline dressings, corresponding with mean log_{10} densities of 1.20 (±0.00) CFU/cm² and 4.0 log_{10} reductions. Exposure to the inactive control, MB/GV PU, and MB/GV PVA dressings had little effect and were not statistically significantly different from the untreated control. The mean log_{10} density of recovered organisms was 5.55 (±0.11), 4.89 (±0.22), and 5.03 (±0.39) CFU/cm², corresponding with 0.4, 0.2, and 0.2 LR compared with untreated control (*p < .05). The amount of recovered organisms from biofilm exposed to the iodine-impregnated dressing was significantly lower than all other dressings tested, with the exception of the silver nanocrystalline dressing, which had similar recovery (*p < .05) (Figure 4).

### 4 DISCUSSION

In combination with debridement and systemic antibiotics, antibacterial dressings are utilised to manage infected wounds. Due to the broad mechanism of action of antibacterial dressings, bacterial resistance to them is often low or non-existent, making them an attractive alternative to antibiotic treatment.26
*P. aeruginosa* and *S. aureus* are the most common microorganisms isolated from chronic wounds, causing infection and delayed wound healing through biofilm formation. A large study analysing the chronic wound microbiota of 2963 patients found 63% and 25% of wounds contained *Staphylococcus* and *Pseudomonas* species, respectively. In addition, *P. aeruginosa* and *S. aureus* are also both frequently associated with multidrug resistant infections that are resilient to clinically used antibiotics. Though *S. aureus* and *P. aeruginosa* are often the predominant aerobic bacterial strains in chronic wounds, wounds are often colonised by several microbial species that can exist as mono aggregates or mixed species aggregates, with mixed species biofilms often working together synergistically to survive. For example, one study demonstrated 4-hydroxy-2-heptylquinoline-N-oxide (HQNO), a by-product of *P. aeruginosa*, protects *S. aureus* from killing by Tobramycin, an aminoglycoside antibiotic, when the two organisms are cocultured together. Mixed species aggregates can also act as a reservoir for antimicrobial resistant genes, promoting genetic exchange and antimicrobial resistant infections. Therefore, in this study the efficacy of biofilm management was evaluated against a multispecies biofilm of three of the most common bacterial species isolated from chronic wounds, an anaerobic strain, *B. fragilis*, as well as evaluation against mono species *P. aeruginosa* and *S. aureus* biofilms.

Silver is the most widely used antimicrobial in wound care, incorporated into many different platforms; therefore, several silver dressings were included in this study as a benchmark comparison for the iodine-based dressing. In the CDC bioreactor model, the iodine-based dressing demonstrated comparable ability to reduce biofilm to the silver nanocrystalline dressing and was superior in efficacy in comparison to the silver CMC and the silver foam dressings. Following a 24 hours contact time of the iodine-based dressing with *P. aeruginosa* and *S. aureus* immature (24 hours) and mature (48 hours) CDC bioreactor biofilms and also with a 24 hours multispecies biofilm of *P. aeruginosa*, *S. aureus*, and *E. faecalis*, no detectable level of biofilm was found. Two of the silver dressings, the nanocrystalline dressing and the foam dressing also demonstrated no detectable level of biofilm, except for the silver foam dressing against the 48 hours *P. aeruginosa*. The silver CMC dressing showed some activity with a <3 LR in the *P. aeruginosa* and *S. aureus* biofilms, as compared with untreated control, as well as a 3.2 LR the multispecies biofilm.

Methylene Blue and Gentian Violet are known inorganic antimicrobials, widely accepted to kill Gram-negative and Gram-positive bacteria, respectively. The MB/GV dressings contain the two antimicrobials bound to the foam inside the dressing and while the mechanism of action is not fully understood, it has been suggested that they inhibit bacterial metabolism by altering the oxidation/reduction potential inside the dressing, creating an environment not suitable for bacterial growth or attachment. The MB/GV dressings in this study have been previously shown to be non-cytotoxic and to exert antimicrobial activity against *S. aureus* and *P. aeruginosa*. In one study, MB/GV PVA foam was used to treat 29 patients with chronic wounds over a 4 week period and demonstrated a significant reduction in wound surface area and significant improvement in pressure ulcer scale for healing scores, as well as a significant reduction in coverage of devitalised tissue and infection. MB/GV PVA foam is an antibacterial foam dressing made of PVA sponge and containing ≤0.00025 g/g of both methylene blue and gentian violet. MB/GV PU foam is an antibacterial foam dressing made of PU foam and containing ≤0.00035 g/g of both methylene blue and gentian violet. In this study, the different dressing material and slight difference in maximum concentration of methylene blue and gentian violet did not show any major differences in ability to manage the 24 and 48 hours biofilms, as well as the multispecies CDC bioreactor biofilms, and neither were able to achieve a >4 LR when compared with the untreated control. Moreover, the iodine-based dressing in this *in vitro* model demonstrated clear, superior reduction of biofilm when compared with the MB/GV dressings in the CDC bioreactor model.

The biofilm management efficacy was also evaluated in the drip flow bioreactor model to represent an *in vitro* exuding wound environment. In this model, the biofilms were grown under a constant supply of fresh media and were also challenged with the test dressings under the same conditions. The drip flow bioreactor model provides a more challenging ‘dynamic’ environment for the test dressings in comparison to the ‘static’ CDC bioreactor model, as there is a constant presence of proteinaceous media, which actsives eluted from the dressings can bind to and which can also wash away actives; therefore, lowering the availability of active to exert antimicrobial efficacy. Following 24 hours treatment of a *P. aeruginosa* biofilm in this model, the iodine-based dressing demonstrated no detectable level of biofilm, showing further potent reduction of biofilm in this more challenging model. The silver nanocrystalline dressing also demonstrated potent reduction of biofilm of the *P. aeruginosa* to below detectable levels. In comparison, reduced efficacy was observed with the silver foam dressing in comparison to that in the CDC bioreactor model with a 3.9 LR in comparison to the untreated control. The silver CMC dressing showed a 4.9 LR, demonstrating slightly greater efficacy in this model compared with the CDC bioreactor model. This was an interesting result considering the more challenging nature of the drip flow bioreactor
model and could indicate that the constant hydrated environment of the drip flow bioreactor was more favourable for silver, ethylenediaminetetraacetic acid and benzalkonium chloride release from this type of dressing or form of silver. Silver dressings have been shown to often dose dump silver when in a liquid environment and an initial large release of silver may have contributed to this slightly greater efficacy in this model. The MB/GV foams both showed <1 LR of the \textit{P. aeruginosa} biofilm in the drip flow bioreactor model, showing potential inferior management of biofilm than the iodine and silver-based dressings. These data suggest that the MB/GV foams may be less suitable for treating exuding wounds. Interestingly, the three foam dressings including the two MB/GV dressings and the silver foam dressing were the least efficacious in this model, which could perhaps be due to the type of dressing and/or antimicrobial release from it.

Lastly, the dressings were tested against a representative anaerobic wound pathogen, \textit{B. fragilis}, by utilising a Direct Contact anaerobic model system. Multiple clinical studies have shown the presence of anaerobic species in chronic wounds, with \textit{Bacteroides} species being frequently present, as well as other anaerobic species such as \textit{Finegoldia} species and \textit{Anaerococcus} species.\textsuperscript{2,32,33,45} Wolcott et al\textsuperscript{13} showed of 2963 wounds analysed, 25% and 24% had \textit{Finegoldia} species and \textit{Anaerococcus} species present, respectively. While there is currently a lack of evidence supporting a pathogenic role of anaerobic bacteria in chronic wounds some studies have shown synergism between aerobic and anaerobic species \textit{in vitro} with an increase in pathogenicity observed when grown together. Some evidence suggests that presence of \textit{Staphylococcus} species may encourage anaerobic species to grow, while presence of \textit{Prevotella} species has been shown to increase the pathogenicity of \textit{S. aureus} in a rat infection model.\textsuperscript{36,37} Following 24 hours exposure of the 48 hours \textit{B. fragilis} biofilm in this model with the iodine-based dressing, the biofilm was reduced to below detectable levels, showing potent efficacy against this anaerobic strain as well as the aerobic species tested in this study. Treatment with the silver nanocrystalline dressing also showed strong management of the anaerobic biofilm showing comparable efficacy to that seen against the aerobic species. In comparison to the iodine-based dressing and silver crystalline dressing, the other dressings showed less efficacy against \textit{B. fragilis}. The silver CMC dressing and the silver foam dressing showed a 2.3 and 1.5 LR in the \textit{B. fragilis} biofilm. In comparison to efficacy against the aerobic bacterial biofilms tested in the CDC bioreactor model, the silver CMC dressing showed comparable biofilm management against this anaerobic biofilm and the silver foam dressing showed less biofilm management. The MB/GV dressings showed no biofilm efficacy against the \textit{B. fragilis} biofilm in this model and were comparable to the non-antimicrobial foam dressing.

Although there are inherent study limitations due to the small sample size, and in some cases, possible non-normal data distribution and the use of more static biofilm models, the iodine dressing in this study showed significant reduction of aerobic immature and mature \textit{P. aeruginosa} and \textit{S. aureus} biofilms, as well as an anaerobic strain \textit{B. fragilis}, eliminating all the biofilms following 24 hours treatment. The iodine dressing also demonstrated the same level of potency against tougher biofilm models utilised in this study, showing no detectable level of the \textit{P. aeruginosa} biofilm in the drip flow bioreactor model or the multispecies biofilm in the CDC bioreactor model. The silver nanocrystalline dressing showed comparable management of biofilm to the iodine dressing, also lowering biofilm below detectable levels in all the models. The controlled-release iodine dressing is a highly absorbent foam dressing, effectively removing exudate and debris and allowing slow release of iodine for sustained infection management. The silver nanocrystalline dressing consists of three layers of silver-coated polyethylene net with two layers of absorbent rayon/polyester between them, helping to maintain a moist environment and sustaining an antimicrobial barrier for at least 7 days. Both dressings demonstrated higher potency than the other dressings in the biofilm models in this study and were comparable to each other in antibiofilm efficacy. The silver foam dressing showed biofilm management against \textit{P. aeruginosa}, \textit{S. aureus} and the multispecies biofilm; however, reduced efficacy was observed against \textit{P. aeruginosa} in the drip flow bioreactor model and against the anaerobic strain \textit{B. fragilis}. The silver CMC dressing showed some control over biofilm growth across all of the models; however, was overall less potent than the iodine-based dressing and the other silver dressings. Interestingly, the silver CMC dressing was most efficacious in the drip flow bioreactor model, which may be explained by the constant flow of media encouraging the silver to elute out with this particular dressing. Overall, the MB/GV dressings were less efficacious than the other dressings, showing some biofilm management activity against \textit{S. aureus}, \textit{P. aeruginosa} and the multispecies biofilms in the CDC bioreactor; however, no management of biofilm was observed in the more challenging drip flow bioreactor model or against the anaerobic strain \textit{B. fragilis}.

An ex vivo porcine skin explant biofilm model was used to compare antibiofilm efficacy of wound dressings with different topical agents and various formulations, moisture retention by Phillips et al.\textsuperscript{48} In total 15 dressings were tested, only a cadexomer iodine dressing effectively eliminated 24 and 72 hours \textit{P. aeruginosa} biofilms grown on dermis of pig skin explants, while 14 other wound
dressings containing various microbicidal agents only partially reduced CFUs. A further in vivo study by Roche et al. demonstrated cadexomer iodine significant reduced biofilm growth in a pig wound model, while a silver CMC dressings had minimal impact on biofilm in the model. All these studies and our results presented herein strongly supports the use of the iodine-based dressing for wounds with infection and biofilm.

In this study, we applied multiple in vitro models to evaluate the biofilm management capability of wound dressings in different biofilm growth conditions, such as aerobic/anaerobic, static/dynamic, to mimic as best as possible the wound environment. Though the study provided a more comprehensive picture than single model, the results obtained from in vitro study may not be representative of the actual clinical situation where the mature bacterial biofilms on/in the dermal tissue or in the eschar of chronic skin wounds will form. In more clinical biofilms, the microbial load may actually be at lower levels than biofilms levels used in this study. The biofilm model used in this study, such as, CDC and drip flow in vitro biofilm model, in which biofilms were cultured on synthesis polymer materials, do not reproduce the exact growth of microbial biofilms found on and in the extracellular matrix (ECM) of the dermal tissue of chronic skin wounds. The microbicidal agents in the tested wound dressings will interact with the components in the ECM of chronic wound beds, which can lead to both a biological demand and inactivation of many microbicidal agents. For examples, silver ions can be inactivated by the biologically relevant compounds that contain reduced thiol groups such as glutathione and thiol and cysteines, also, the antibacterial activity of Iodine potassium iodide is reported to be inactivated by type-I collagen and other compounds find in the ECM. Consequently, based on the limitations of all in vitro models further more complex in vitro models and in vivo studies are needed to determine the antibiofilm efficacy of the tested wound dressing to provide more clinically relevant data.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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