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# Comparison of *in vitro* antibacterial activity of streptomycin-diclofenac loaded composite biomaterial dressings with commercial silver based antimicrobial wound dressings

Harshavardhan V. Pawar [a], John Tetteh [a], Philip Debrah [b], Joshua S. Boateng [a,*]

[a] Department of Pharmaceutical, Chemical & Environmental Sciences, School of Science, University of Greenwich at Medway, Central Avenue, Chatham Maritime, ME4 4TB Kent, UK
[b] Department of Pharmaceutics and Microbiology, School of Pharmacy, College of Health Sciences, University of Ghana, Legon, P.O. Box LG43, Legon, Accra, Ghana

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ABSTRACT

Infected chronic wounds heal slowly, exhibiting prolonged inflammation, biofilm formation, bacterial resistance, high exudate and ineffectiveness of systemic antimicrobials. Composite dressings (films and wafers) comprising polyox/carrageenan (POL-CAR) and polyox/sodium alginate (POL-SA), loaded with diclofenac (DLF) and streptomycin (STP) were formulated and tested for antibacterial activity against $2 \times 10^5$ CFU/mL of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* representing infected chronic wounds and compared with marketed silver dressings. Minimum inhibitory concentration (MIC) showed higher values for DLF than STP due to non-conventional antibacterial activity of DLF. The DLF and STP loaded dressings were highly effective against *E. coli*, *P. aeruginosa* and *S. aureus*. POL-SA dressings were more effective against the three types of bacteria compared to POL-CAR formulations, while the DLF and STP loaded dressings showed greater antibacterial activity than the silver-based dressings. The films, showed greater antibacterial efficacy than both wafers and silver dressings. STP and DLF can act synergistically not only to kill the bacteria but also prevent their resistance and biofilm formation compared to silver dressings, while reducing chronic inflammation associated with infection.


## 1. Introduction

A wound is an interruption in the defensive role of the skin in protecting against harmful environmental agents [1]. Injury evokes wound healing comprising distinct phases (haemostasis, inflammation, proliferation, migration and maturation) involving biochemical, and molecular events that work sequentially towards tissue regeneration [2]. However, wounds can get contaminated by microorganisms, especially during the proliferation stage leading to infection. Persistent infection impairs wound healing causing repeating inflammatory cycle, resulting in chronic wounds [3,4]. Prevention and control of infection have been identified as essential aspects of wound management [5]. Effective management requires reducing exogenous microbial contamination, debridement, using appropriate dressing(s) and administration of topical and systemic broad-spectrum antimicrobial agents [6]. Topical agents such as povidone iodine and chlorhexidine acid are commonly employed, though their use is currently restricted to wound cleansing and skin swabs before surgical incisions [1]. However, antibiotics have high specificity against infection and ultimately improve wound healing at low concentrations [1,7]. Various commercial dressings have been developed that release silver to prevent wound infections both *in vitro* [8] and *in vivo* [9]. The emergence of microbial resistance has resulted in the need for more effective treatments for wound infections [1]. Further, systemic antibiotic treatment is difficult in chronic wounds such as diabetic foot ulcers due to poor blood circulation at the extremities of diabetics [6].

Chronic wound infection also causes pain, excessive exudation and patient discomfort and is a major source of cross-infection particularly antibiotic-resistant species. Burns for example provide a protein-rich environment, favourable for microbial colonization [10]. Most infected wounds involve *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococci* and *Escherichia coli*. *S. aureus* is considered a challenging microorganism in wound infections [6] due to its ability to develop resistance against first line antibiotics.

Streptomycin (STP) has been used to treat wound infections [11] and for reducing infection before skin grafting [12]. It's reported that diclofenac (DLF) has antibacterial activity and acts synergistically with STP against Mycobacterium tuberculosis after systemic administration [13]. Systemic STP in combination with DLF demonstrated synergistic activity against 45 different strains of mycobacteria [14,15].

This paper reports on the evaluation of antibacterial activity of STP and DLF loaded film and wafer dressings against *S. aureus*, *E. coli* and *P. aeruginosa*. Minimum inhibitory concentration (MIC) of STP and DLF in the dressings and *in vitro* antibacterial efficacy (zone of inhibition) against the three microorganisms were evaluated using disk diffusion

* Corresponding author.
*E-mail address:* J.S.Boateng@gre.ac.uk (J.S. Boateng).

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assay and compared with three commercial silver containing dressings. To the best of our knowledge, this is the first study comparing the antibacterial performance of STP-DLF loaded medicated POL-CAR and POL-SA dressings with commercial silver loaded antimicrobial dressings for their antibacterial performance.

## 2. Methods

### 2.1. Materials

(Polyox™ WSR 301 ≈ 4000 kDa) was a gift from Colorcon Ltd. (Dartford, UK), κ-carrageenan (Gelcarin GP 812) was from IMCD Ltd. (Sutton, UK), Aquacel® Ag (ConvaTech, Ltd.), Melgisorb® Ag (Mölnlycke Health Care, Ltd.) were gifted by the manufacturers and Allevyn® Ag (Smith and Nephew, Ltd) obtained from a local pharmacy. Nutrient agar and nutrient broth were purchased from Oxoid, UK. Diclofenac sodium, streptomycin sulphate, glycerol, phosphate buffered saline (PBS) tablets, were purchased from Sigma-Aldrich, (Gillingham, UK). Sodium alginate was purchased from Fisher Scientific (Loughborough, UK). National Collection of Type Culture (NCTC) strains of *S. aureus* (A 29213), *E. coli* (DTCC 25922) and *P. aeruginosa* (A 10145), were used for microbiological assays.

### 2.2. Preparation of composite polymer based dressings

Composite films and wafers (Table 1) were prepared as previously reported [16,17]. In brief, blends of POL with CAR and POL with SA (weight ratio of 75/25 and 50/50 respectively) yielding 1% w/w of total polymer weight, were prepared by stirring on a magnetic stirrer at 70 °C to form a uniform gel (POL-CAR-BLK and POL-SA-BLK). The composition of the polymers and drugs used for the preparation of gels are summarised in Tables 2 and 3. DL gels of POL-SA and POL-CAR were prepared by adding 4 mL ethanolic solution of DLF containing 100 mg and 250 mg respectively of the drug to POL-SA gel to achieve 10% w/w and to achieve 25% w/w of DLF for POL-CAR in the polymeric gel. These gels were subsequently cooled to 40 °C with constant stirring. Similarly, a 4 mL aqueous solution containing 250 mg and 300 mg of STP was subsequently added to achieve a final STP concentration of 25% w/w (POL-SA) and 30% w/w (POL-CAR) respectively in the DL gels.

To obtain films, the solutions (25 g) were poured into Petri dishes (diameter 90 mm) and dried in an oven at 40 °C for 18 h, while unplasticised polymeric solutions (10 g) were freeze-dried to obtain wafers. To obtain the wafers, 10 g of each homogeneous gel was transferred into 6 well moulds (diameter 35 mm) (Thermo-Fisher Scientific Nunc, Leicestershire UK), placed in a Virtis Advantage XL 70 freeze dryer (Biopharma Process Systems, Winchester, UK) and lyophilised using the automated lyophilisation cycle. This involved initially cooling samples from room temperature to −5 °C and then −50 °C over a period of 10 h (at 200 mTorr). An annealing step at −25 °C for 2 h was applied based on the preliminary DSC studies and its effect on the different formulations was investigated. The frozen samples were then heated in a series of thermal steps to −25 °C under vacuum (20–50 mTorr) over a 24 h period. Secondary drying of the wafers was carried out at 20 °C (10 mTorr) for 7 h.

**Table 1**
Formulations used to evaluate antimicrobial efficacy against *S. aureus*, *P. aeruginosa* and *E. coli*.

| Formulation | Code |
|---|---|
| POL-CAR-BLK | A |
| POL-CAR-DL | B |
| POL-CAR-DL-20%GLY | C |
| POL-SA-BLK | D |
| POL-SA-DL | E |
| POL-SA-DL-9%GLY | F |
| POL-CAR-BLK-An | G |
| POL-CAR-DL-An | H |
| POL-SA-BLK-An | I |
| POL-SA-DL-An | J |
| Aquacel® Ag | K |
| Melgisorb® Ag | L |
| Allevyn® Ag | M |
| STP | N |
| DLF | O |

**Table 2**
Quantities of the polymers, drugs and GLY (varying amounts based on total solid weight) within composite polymer gels used for formulation of POL-CAR and POL-SA (BLK and DL) films.

| Formulation | POL (g) | CAR (g) | SA (g) | GLY (g) | DLF (g) | STP (g) | Total weight (g) | % GLY content |
|---|---|---|---|---|---|---|---|---|
| POL-CAR-BLK | 0.75 | 0.25 | – | 0.00 | – | – | 1.00 | 0.00 |
| POL-CAR-BLK | 0.75 | 0.25 | – | 0.10 | – | – | 1.10 | 9.09 |
| POL-CAR-BLK | 0.75 | 0.25 | – | 0.25 | – | – | 1.25 | 20.00 |
| POL-CAR-BLK | 0.75 | 0.25 | – | 0.50 | – | – | 1.50 | 33.33 |
| POL-CAR-BLK | 0.75 | 0.25 | – | 0.75 | – | – | 1.75 | 42.86 |
| POL-CAR-BLK | 0.75 | 0.25 | – | 1.00 | – | – | 2.00 | 50.00 |
| POL-CAR-DL | 0.75 | 0.25 | – | 0.00 | 0.10 | 0.30 | 1.40 | 0.00 |
| POL-CAR-DL | 0.75 | 0.25 | – | 0.10 | 0.10 | 0.30 | 1.50 | 6.67 |
| POL-CAR-DL | 0.75 | 0.25 | – | 0.25 | 0.10 | 0.30 | 1.65 | 15.15 |
| POL-CAR-DL | 0.75 | 0.25 | – | 0.50 | 0.10 | 0.30 | 1.90 | 26.32 |
| POL-CAR-DL | 0.75 | 0.25 | – | 0.75 | 0.10 | 0.30 | 2.15 | 34.88 |
| POL-CAR-DL | 0.75 | 0.25 | – | 1.00 | 0.10 | 0.30 | 2.40 | 41.67 |
| POL-SA-BLK | 0.50 | – | 0.50 | 0.00 | – | – | 1.00 | 0.00 |
| POL-SA-BLK | 0.50 | – | 0.50 | 0.10 | – | – | 1.10 | 9.09 |
| POL-SA-BLK | 0.50 | – | 0.50 | 0.25 | – | – | 1.25 | 20.00 |
| POL-SA-BLK | 0.50 | – | 0.50 | 0.50 | – | – | 1.50 | 33.33 |
| POL-SA-DL | 0.50 | – | 0.50 | 0.00 | 0.05 | 0.15 | 1.20 | 0.00 |
| POL-SA-DL | 0.50 | – | 0.50 | 0.10 | 0.05 | 0.15 | 1.30 | 7.69 |
| POL-SA-DL | 0.50 | – | 0.50 | 0.25 | 0.05 | 0.15 | 1.45 | 17.24 |
| POL-SA-DL | 0.50 | – | 0.50 | 0.50 | 0.05 | 0.15 | 1.70 | 34.48 |

### 2.3. Bacterial sample preparation

Fresh broth cultures were prepared as reported by Labovitiadi et al., [18] by transferring a single bead unit into 10 mL of nutrient broth and incubating for 24 h. A loop full of bacterial culture was streaked onto nutrient agar plate and incubated at 37 °C for 24 h to yield separate colonies. Overnight bacterial cultures were centrifuged at 4000 rpm for 10 min in an Accuspin 1 centrifuge (Fisher Scientific, UK), supernatant discarded and pellets suspended in 20 mL of simulated wound fluid (SWF) [16]. This process was repeated twice and final pellets resuspended in 5 mL SWF, followed by two fold dilutions in SWF. Bacterial density was determined by measuring the dilute suspension at 500 nm to yield the required density of $2 \times 10^5$ CFU/mL [18].

### 2.4. Minimum inhibitory concentration (MIC) of STP and DLF

The MIC for STP and DLF was evaluated as previously reported [19]. Briefly, three different stock solutions for each drug were prepared (Table 4) and STP required to obtain 10,000 mg/L was calculated using Eq. (1). Antimicrobial susceptibilities of *S. aureus*, *E. coli* and *P.*

**Table 3**
Composition of polymers and drugs (varying quantity) present in composite polymer gels used to produce composite freeze dried POL-CAR and POL-SA (BLK and DL) wafers.

| Pure material | POL-CAR-BLK (g) | POL-CAR-DL (g) | POL-SA-BLK (g) | POL-SA-DL (g) |
|---|---|---|---|---|
| POL | 0.75 | 0.75 | 0.50 | 0.50 |
| CAR | 0.25 | 0.25 | – | – |
| SA | – | – | 0.50 | 0.50 |
| STP | – | 0.30 | – | 0.25 |
| DLF | – | 0.25 | – | 0.10 |
| Total weight (g) | 1.00 | 1.55 | 1.00 | 1.35 |

**Table 4**
Stock solutions of STP and DLF used to evaluate MIC of *S. aureus*, *E. coli* and *P. aeruginosa* (mean ± SD, $n = 3$).

| | Stock solution 1 | Stock solution 2 | Stock solution 3 |
|---|---|---|---|
| STP | 10,000 mg/L (254 mg of STP + 20 mL of distilled water) | 1000 mg/L (1 mL of stock solution 1 + 9 mL of distilled water) | 100 mg/L (1 mL of stock solution 2 + 9 mL of distilled water) |
| DLF | 10,000 mg/L (200 mg of DLF + 20 mL of distilled water) | 1000 mg/L (1 mL of stock solution 1 + 9 mL of distilled water) | 100 mg/L (1 mL of stock solution 2 + 9 mL of distilled water) |

*aeruginosa* were determined by establishing the MIC using a standard agar dilution method and 0.25–512 mg/L calibration solutions of DLF and STP dilutions also prepared. 200 μL of stock and diluted solutions (10,000 mg/L, 1000 mg/L and 100 mg/L respectively) were transferred into a Petri plate and 20 mL of nutrient agar (stabilized at 45 °C) added and mixed. The agar was allowed to set at room temperature and 0.1 mL of $1 \times 10^5$ CFU/mL of *S. aureus*, *E. coli* and *P. aeruginosa* were spread on separate Petri plates. These plates were incubated at 37 °C for 24 h and ensuring that all microorganisms had grown on drug free control plate. MIC is the lowest concentration of antimicrobial at which there was no visible growth of organisms. Growth of one or two colonies or a fine film of growth was disregarded.

$$W = \frac{1000}{P} \times V \times C \quad (1)$$

$W$ is the weight of actives (mg) dissolved in volume $V$ (mL), $C$ is final concentration of solution (multiples of 1000 mg/L), $P$ (785 μg/mg) is the potency provided by the manufacturer.

### 2.5. In vitro antibacterial activity of antimicrobial films, wafers and marketed silver dressings

The disk diffusion method was used for the assessment of the antibacterial activity of the DL films, wafers and commercial silver dressings. Solutions ($2 \times 10^5$ CFU/mL) of each bacterial strain (*S. aureus*, *E. coli* and *P. aeruginosa*) were prepared as specified above (Section 2.3) and 0.1 mL of each strain spread separately on set nutrient agar media. The inoculated microorganisms were incubated at 37 ± 1 °C for 4 h to initiate growth of microorganisms on the inoculated culture medium before placing the films, wafers and marketed dressings. The films and marketed silver dressings were cut into 2 cm diameter disc shapes. However, due to difficulty of cutting thicker wafers into smaller discs, DL gels (2 g) were free-dried in 2 cm diameter containers to obtain the same diameter as the cut film discs. Further, circular Whatmann® paper discs (2 cm diameter), each wetted with reference solutions (80 μL) of STP and DLF at concentrations of 6 mg/mL and 5 mg/mL respectively were used as positive controls. Negative controls were BLK films and wafers (2 cm diameter) without any STP or DLF. The plates were then incubated at 37 ± 1 °C for 24 h after which the end zones of inhibition (ZOI) in millimetres, formed on the medium ($n = 3$), were measured.

### 2.6. Statistical analysis

Statistical data evaluation was performed using two tailed student *t*-test at 95% confidence interval (Graph Pad Prism 4 software) with $p$ value < 0.05 as the minimal level of significance.

## 3. Results

### 3.1. MIC of STP and DLF

The MIC of STP and DLF was determined for known densities ($2 \times 10^5$ CFU/mL) of *S. aureus P. aeruginosa E. coli* commonly associated with infected chronic wounds. The MICs of STP for *S. aureus* and *E. coli* ranged from 4 to 8 mg/L but ranged from 8 to 16 mg/L for *P. aeruginosa*. MIC for DLF against *P. aeruginosa* was >512 mg/L and 256–512 mg/L for *E. coli* and *S. aureus* respectively.

### 3.2. Antimicrobial activity of pure STP and DLF controls

The ZOI of the STP and DLF positive controls for *S. aureus*, *P. aeruginosa* and *E. coli* are shown in Fig. 1(N and O). STP showed significantly ($p < 0.05$) lower ZOI (3.2 ± 0.1 mm) for *S. aureus* compared to *P. aeruginosa* and *E. coli*. The maximum ZOI of *P. aeruginosa* was 4.1 ± 0.1 mm which was lower compared to *E. coli* (4.6 ± 0.1 mm) and was statistically significant ($p < 0.05$). DLF did not show ZOI for *S. aureus*, *P. aeruginosa* and *E. coli* though there was no bacteria growing directly under the DLF disc (Fig. 1, *E. coli* plate O) implying that their effectiveness alone as antibacterial may be limited in application to infected wounds.

### 3.3. Antibacterial activity of POL-CAR films ($2 \times 10^5$ CFU/mL)

Figs. 2(A) and 3(A, B and C) show ZOI of POL-CAR-DL and POL-CAR-DL-20% GLY films against *S. aureus*, *P. aeruginosa* and *E. coli*. There was a significant difference observed for all POL-CAR-DL films against strains of bacteria (compared to wafers and marketed dressing and DLF, STP discs. POL-CAR-DL and POL-CAR-DL-20% GLY films showed a smaller ZOI for *S. aureus* but increased for *P. aeruginosa* and *E. coli*. For *S. aureus* the ZOI for POL-CAR-DL and POL-CAR-DL-20%GLY films was 3.6 ± 0.1 mm and 3.5 ± 0.1 mm respectively which was significantly ($p < 0.05$) higher than pure STP (3.2 ± 0.1 mm). For *P. aeruginosa*, the observed ZOI was higher than *S. aureus* but less than *E. coli*. POL-CAR-DL and POL-CAR-DL-20%GLY films showed similar ZOI (4.3 ± 0.1 mm) for *P. aeruginosa* which was higher than the control STP (4.1 ± 0.1 mm), however, the difference was not statistically significant ($p > 0.05$). The maximum ZOI of POL-CAR-DL and POL-CAR-DL-20%GLY films was 4.8 ± 0.2 mm and 4.7 ± 0.1 mm respectively, for *E. coli* which though higher than 4.6 ± 0.2 mm for the control STP were not statistically significant ($p > 0.05$).

### 3.4. Antibacterial activity of POL-SA films ($2 \times 10^5$ CFU/mL)

Figs. 2(B) and 4(D, E and F) show the ZOI of POL-SA-BLK, POL-SA-DL and POL-SA-DL-9% GLY) films for *S. aureus*, *P. aeruginosa* and *E. coli*. For *S. aureus*, the observed ZOI for POL-SA-DL and POL-SA-DL-9%GLY films was 4.6 ± 0.2 mm and 4.1 ± 0.2 mm respectively which was significantly ($p < 0.05$) higher compared to the STP (3.2 ± 0.1 mm) control. The ZOI increased from 4.6 ± 0.2 mm (*S. aureus*) to 4.8 ± 0.2 mm (*P. aeruginosa*) and 5.0 ± 0.2 mm (*E. coli*) for POL-SA-DL films while for POL-SA-9%GLY films it increased from 4.1 ± 0.2 mm (*S. aureus*) to 5.1 ± 0.2 mm (*P. aeruginosa*) and 5.5 ± 0.2 mm (*E. coli*) respectively.

### 3.5. Antibacterial activity of POL-CAR and POL-SA wafers ($2 \times 10^5$ CFU/mL)

Figs. 2(C) and 5(G, H, I and J) show the ZOI of POL-CAR and POL-SA (BLK and DL) wafers for *S. aureus*, *P. aeruginosa* and *E. coli* bacterial strains. As was observed for the films, the BLK (no drug) wafers did not show any ZOIs against all three microorganisms (Fig. 5G and I). The ZOI of POL-CAR for *S. aureus* was 3.1 ± 0.1 mm which increased to 3.3 ± 0.1 mm for POL-SA whereas STP had a value of 3.2 ± 0.1 mm which was not statistically significant ($p > 0.05$). For *P. aeruginosa*, the

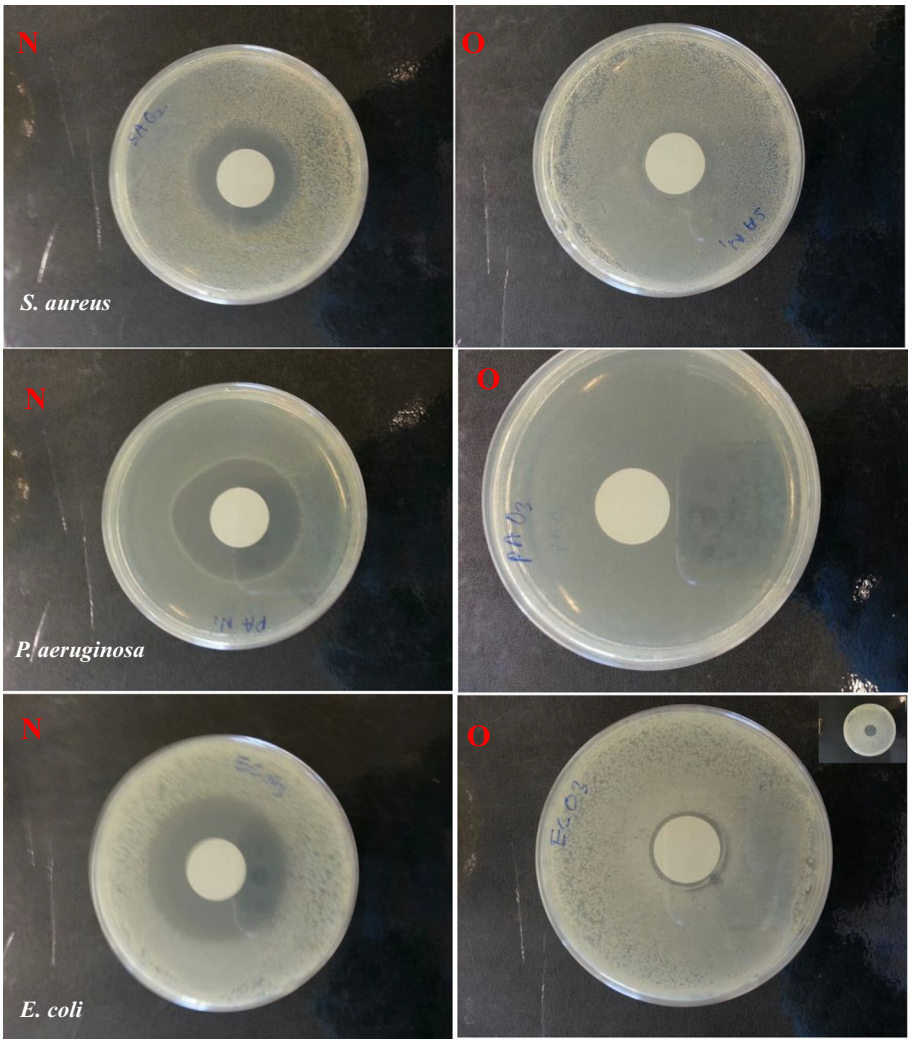


**Fig. 1.** ZOI of control STP (N) and control DLF (O) for *S. aureus*, *P. aeruginosa* and *E. coli*. The inset of control DLF shows the absence of bacteria around the applied area of the disk (mean ± SD, $n = 3$).

ZOI was higher than *S. aureus* but less than *E. coli*. POL-SA-DL and STP showed similar ZOI of 4.1 ± 0.2 mm which subsequently decreased for POL-CAR-DL (3.9 ± 0.1 mm). The maximum ZOI of POL-CAR-DL and POL-SA-DL wafers was respectively 4.5 ± 0.1 mm and 4.6 ± 0.3 mm for *E. coli*.

### 3.6. Antimicrobial efficacy of marketed wound dressings ($2 \times 10^5$ CFU/mL)

Figs. 2(D) and 6(K, L and M) show the ZOI of silver loaded marketed dressings (Table 5) (Aquacel® Ag, Melgisorb® Ag and Allevyn® Ag) for *S. aureus*, *P. aeruginosa* and *E. coli*. There were very small ZOIs observed for all three different strains of microorganisms in the presence of these marketed silver based dressings, though these bacteria were completely absent in the area directly underneath the dressing as shown in Fig. 6 inset (M, *S. aureus*). The ZOI for *S. aureus* was increased for Allevyn® Ag foam dressing (2.3 ± 0.1 mm) while all three marketed dressings showed a ZOI of 2.0 ± 0.1 mm for *P. aeruginosa*. The ZOI for *E. coli* was higher for Allevyn® Ag foam dressing (2.9 ± 0.0 mm) compared to Aquacel® Ag and Melgisorb® Ag (2.0 ± 0.0 mm).

## 4. Discussion

One of the overall objectives of the broader study was to compare the properties of dense dressings such as films to corresponding porous formulations such as freeze-dried wafers relative to commercial silver based dressings. Drying in an oven only yields non porous films and therefore it was important to freeze-dry other gels in a freeze-dryer. The reason for plasticising the films, was purely to improve the flexibility and ease of handling, to fulfil one of the key functional performance requirements for film dressings. The hypothesis for the comparison, was that the differences in physical properties (porosity), which are known to significantly affect rate of hydration and swelling, will also significantly affect the rate of drug diffusion out of the swollen gels and subsequently affect the degree of antibacterial efficacy.

Ineffective control of wound infections caused by antibiotic resistant strains of pathogens has intensified the need to consider modifying current approaches including use of medicated dressings which can overcome resistance and reduce bacterial biofilm formation. This study assessed the *in vitro* antibacterial activity of composite films and wafers combining antibacterial (STP) and anti-inflammatory (DLF) drugs for targeting two phases of wound healing. The two drugs were also selected based on their reported synergistic antibacterial effect when administered systemically [14]. Many texts refer to bacterial bio-burden $>10^5$ CFU/mL organisms per gram of tissue as a criterion for infection [3,6]. In this study we used $2 \times 10^5$ CFU/mL of *S. aureus*, *P. aeruginosa* and *E. coli* to evaluate antimicrobial efficacy of DL film and wafer dressings and compared their performance against marketed silver dressings.

POL-CAR-BLK films did not show any inhibition zone against all three different microorganisms (Fig. 3A) implying that the observed antibacterial effect was solely due to the presence of STP and DLF. The

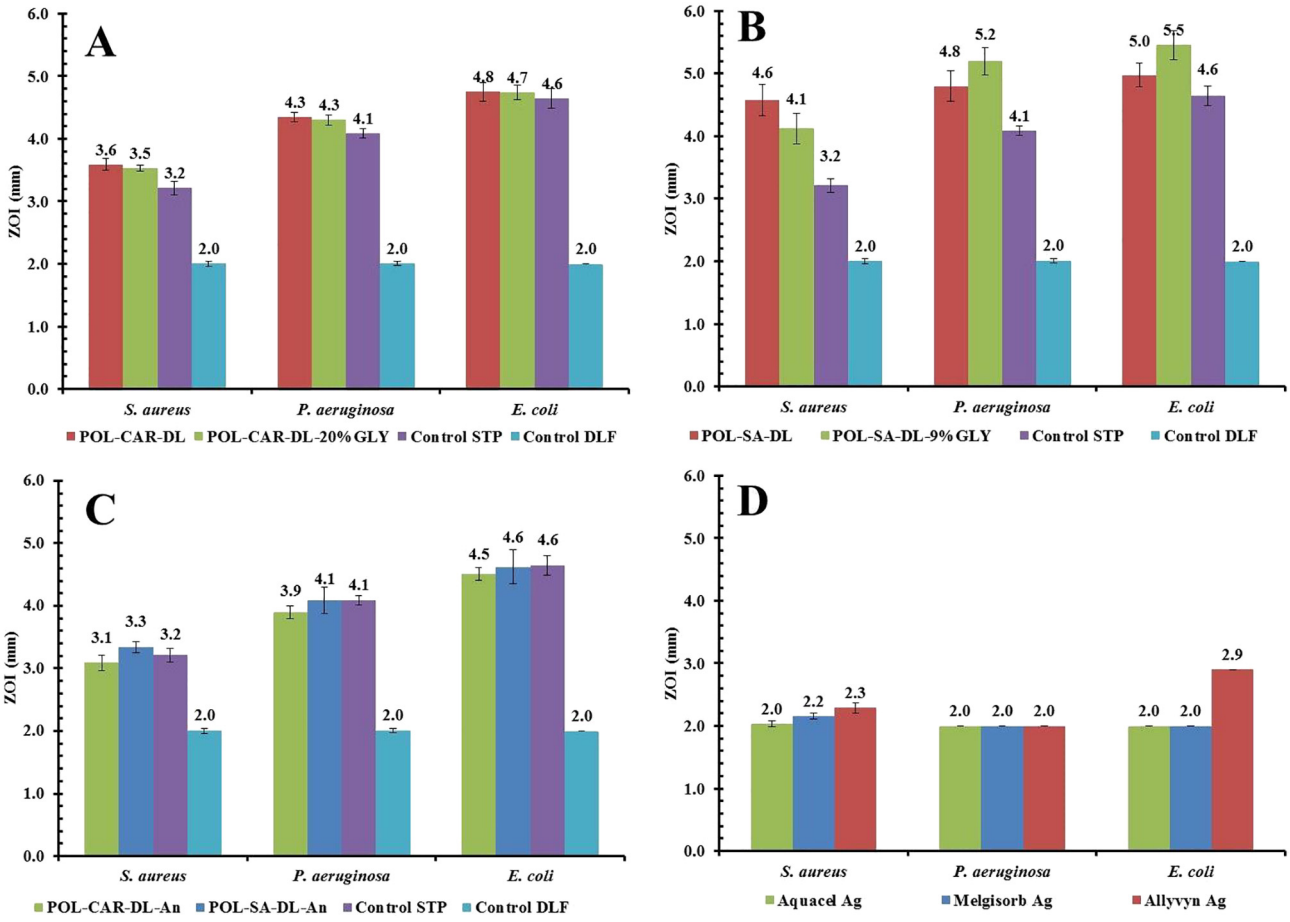


**Fig. 2.** Extracted data comparing the measured ZOI data (mm) of *S. aureus*, *P. aeruginosa* and *E. coli* for the various formulations and marketed dressings tested. (A) POL-CAR (DL and DL-20% GLY) films and STP and DLF (mean ± SD, $n = 3$). (B) POL-SA-DL and POL-SA-DL-9%GLY films and control STP and DLF (mean ± SD, $n = 3$). (C) POL-CAR-DL-An and POL-SA-DL-An wafers and control STP and DLF (mean ± SD, $n = 3$). (D) The marketed dressings (Aquacel® Ag, Melgisorb® Ag, Allevyn® Ag (mean ± SD, $n = 3$).

formulated films, wafers and marketed dressings showed antibacterial efficacy against bacterial bio-burden $2 \times 10^5$ CFU/mL of *S. aureus*, *P. aeruginosa* and *E. coli*. Both *P. aeruginosa* and *E. coli* are Gram-negative microorganisms and required a higher MIC of STP compared to *S. aureus*. This means STP is more effective against the Gram-positive microorganism *S. aureus* than the Gram-negative *E. coli* and *P. aeruginosa* which is interesting, given the fact the *S. aureus* and related species are a major cause of antibiotic resistance [14].

During the antibacterial study, the films and wafers swelled when placed on the highly water saturated agar gel under incubation, simulating a broken skin (wound) surface and this is to be expected. The swelling of the drug loaded polymeric dressings is an important characteristic as that is important to ensure ease of drug dissolution, diffusion out of the swollen gel and eventually release to reach the target bacterial organisms.

To kill the bacteria, STP and DLF must interact with the binding site, occupy a critical number of sites of the bacteria and remain there long enough to inhibit normal biochemical reactions [20]. It's been reported that antimicrobial activity is either concentration or time dependent [21,22]. Concentration dependent drugs include aminoglycosides (*e.g.* STP), whose ability to kill bacteria is dependent on the presence of high concentrations at the site of infection. At least a ratio of 10:1 is required for such concentration dependent antibiotics to effectively kill bacteria and prevent development of resistance [21,23–25]. On the other hand, drug concentrations above the MIC should remain for long periods of time at the site of infection in order to achieve antibacterial action [21,22].

In previous studies [13–15], it has been demonstrated that the concentrations required to kill *S. aureus* is higher than *P. aeruginosa* which is time dependent. DLF required higher concentrations to kill the bacteria that are beyond those clinically achievable with antibiotics, implying that DLF on its own could not effectively inhibit *P. aeruginosa* based infections. Dutta et al. [14] previously demonstrated that when DLF is used *in vitro*, it showed higher MIC values compared to conventional antibiotic drugs such as STP but *in vivo*, the amount of DLF required to protect an animal from *Mycobacterium* spp was much lower. This suggests that DLF might be used as adjuvant to current antibiotics to manage bacterial infections [13–15] as was done in this study.

For *S. aureus*, different ZOIs were observed which is attributed to the rate of diffusion of STP and DLF (films and wafers) and silver (commercial) from the dressings. Both POL-CAR; POL-SA films had significantly higher ZOI suggesting a synergistic action between both drugs compared to each individual drug (refer to Fig. 1). ZOI was ellipsoidal for POL-SA films due to the rapid initial swelling and disintegration of the polymer matrix and rapid diffusion of STP and DLF through the free flowing swollen gels (Fig. 4E & F). Bajpai & Sharma [26] explained that the more rapid swelling of SA is due to the mannuronate block where $Ca^{2+}$ binds to the poly gluconate units which starts to disintegrate the swollen matrix [26]. Differences in the ZOI of POL-CAR-DL and POL-SA-DL formulation could be related to the two different polymers (CAR and SA), their percentage ratios used and their different swelling mechanisms (surface wetting, hydration, hydrogel formation and erosion) [17] which subsequently affects rate of drug diffusion through the matrix and onto the bacterial colonies.

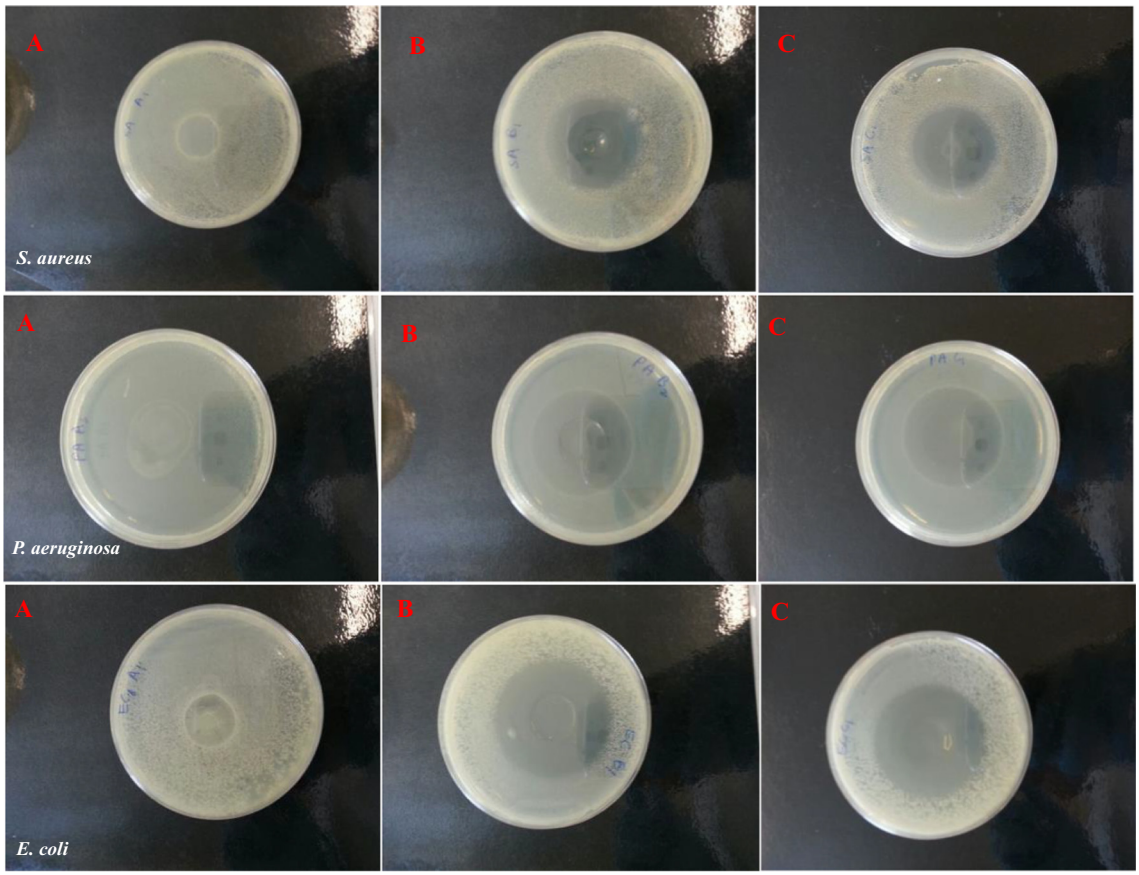


**Fig. 3.** The digital images of ZOI of (A) POL-CAR-BLK, (B) POL-CAR-DL, (C) POL-CAR-DL-20%GLY films observed for *S. aureus*, *P. aeruginosa* and *E. coli* (mean ± SD, $n = 3$).

Maximum ZOI was observed for POL-SA-DL and POL-SA-DL-9%GLY films due to rapid swelling and subsequently rapid diffusion of both STP and DLF from the swollen matrix. This supports the swelling and drug release data from previous studies [16,17].

All the DL films showed greater antibacterial activity compared to wafers which was interesting. Wafers generally have a higher loading capacity, faster hydration and cumulative percent drug release compared to films due to their generally more porous nature [27]. However, it was observed that higher drug loading in the wafers resulted in the formation of greater amounts of sodium sulphate which decreased the hydration capacity [16,17] of DL wafers subsequently affecting drug diffusion with a consequent decrease in ZOI compared to films but greater than the marketed dressings.

From a pharmaceutical perspective, these differences could be associated with the total amounts of polymer present in films and wafers which resulted in the different hydration rates and eventually different ZOIs. For example, the weights ranged from 22.1 mg and 30.3 mg for POL-CAR DL films and wafers respectively. This was also true for the POL-SA DL films and wafers (17.9 mg and 24.6 mg for films and wafers respectively). It should be noted that though both formulations had similar diameters, their contents were different as the films were cut out directly from a bigger sheet due to difficulty of removing a film with small diameter while the wafers were cast directly into 2 cm diameter moulds due to ease of removal. It is very difficult to effectively cut a relatively thick wafer into circular discs without damaging the structure due to their soft and porous nature.

More interestingly, the formulated film and wafer dressings, showed greater antibacterial efficacy than marketed silver based antibacterial dressing which showed either lower or absence of ZOIs for all three different microorganisms even though the area directly under the discs showed no microbial growth. This may be due to two reasons: (i) the lower amounts of silver present in these dressings (Fig. 6) relative to the combined concentrations of STP and DLF present in the composite films and wafers and (ii) most likely due to STP and DLF present in both films and wafers acting synergistically to kill the bacteria and potentially inhibiting biofilm formation and resistance of the bacteria. DLF consists of a secondary amino group and a phenyl ring, both ortho positions of which are occupied by chlorine atoms. This causes an angle of torsion between the two aromatic rings, which presents structural similarities with phenothiazine and this is responsible for its antibacterial activity against microorganisms such as *E. coli*, *S. aureus* and *P. aeruginosa* [14,15]. DLF's antibacterial activity involves the inhibition of bacterial DNA synthesis whereas STP acts by binding to 30S ribosomal subunits in the microorganisms and disrupting the initiation and elongation steps in protein synthesis. On the other hand, silver in the presence of moisture, such as wound exudate, readily ionises to release silver ions ($Ag^{+}$) which is involved in oxidation reactions by catalysing reactions between oxygen present in the cell and hydrogen from thiol groups. This results in disulphide bond formation, ultimately inhibiting cell function due to changes in protein structure, resulting in protein denaturation and enzyme inhibition [28]. The increased antibacterial activity of the film and wafer dressings suggests a potential application in chronic wound management. Formulations administered for systemic use usually have to overcome the challenges to drug absorption, metabolism, distribution and elimination before the drug reaches the target sites for activity, hence such systemic formulations tend not to always have direct *in vitro-in vivo* correlations. For formulations such as wound dressings, intended for direct application, where the drug (s) are in direct contact with the target tissues, a high positive *in vitro – in vivo* correlation tends to exist due to minimal pharmacokinetic barriers.

Silver is a widely used anti-microbial agent effective against infection causative wound pathogens which are responsible for delayed wound healing and can be added to a range of composite dressings

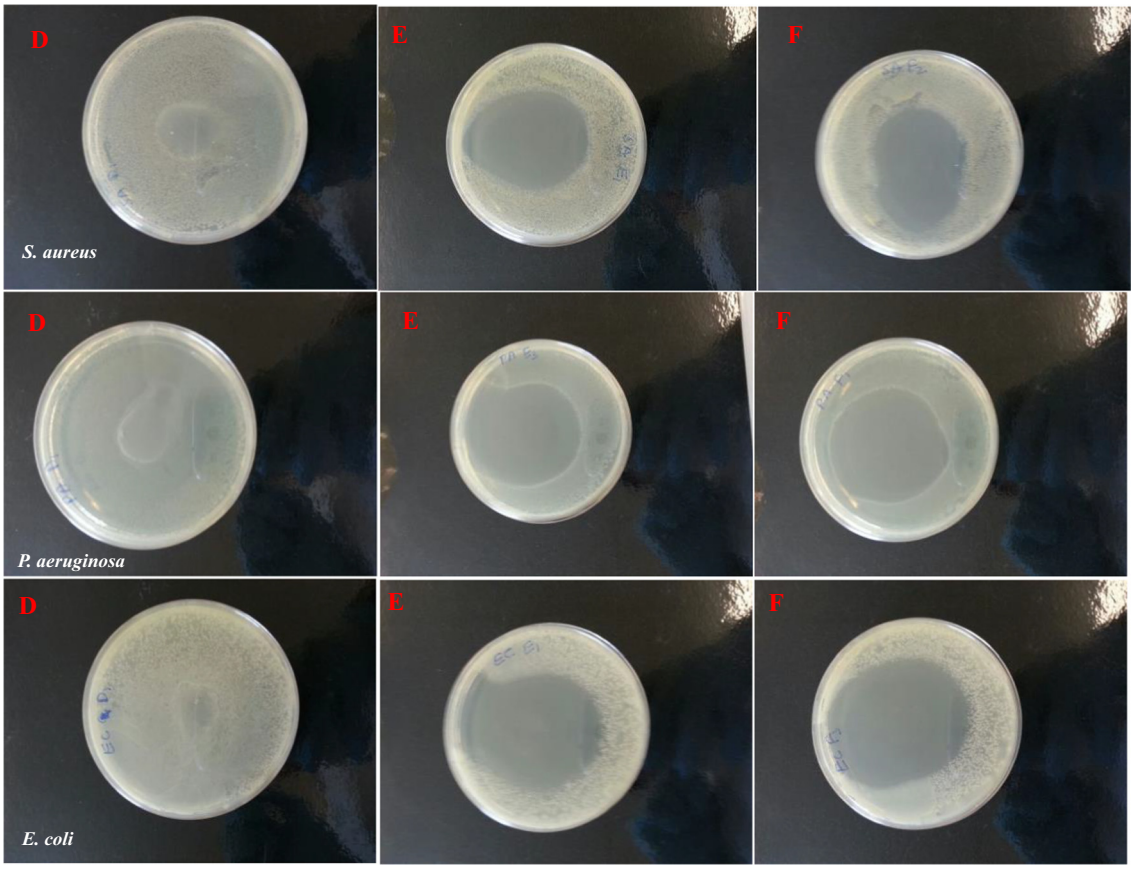


**Fig. 4.** The digital images of ZOI of (D) POL-SA-BLK, (B) POL-SA-DL, (C) POL-SA-DL-9%GLY observed for *S. aureus*, *P. aeruginosa* and *E. coli* (mean ± SD, $n = 3$).

[29]. Silver containing wound dressings release silver ions which vary due to the different forms (silver sulfadiazine, ionic silver nanoparticles containing scaffolds, nanofiber containing silver nanoparticles, silver-containing activated carbon and fibres) and the amount of the silver present [1,30]. Although there are important questions raised by Modak et al. [31] in regards to the use of silver in infected wounds and

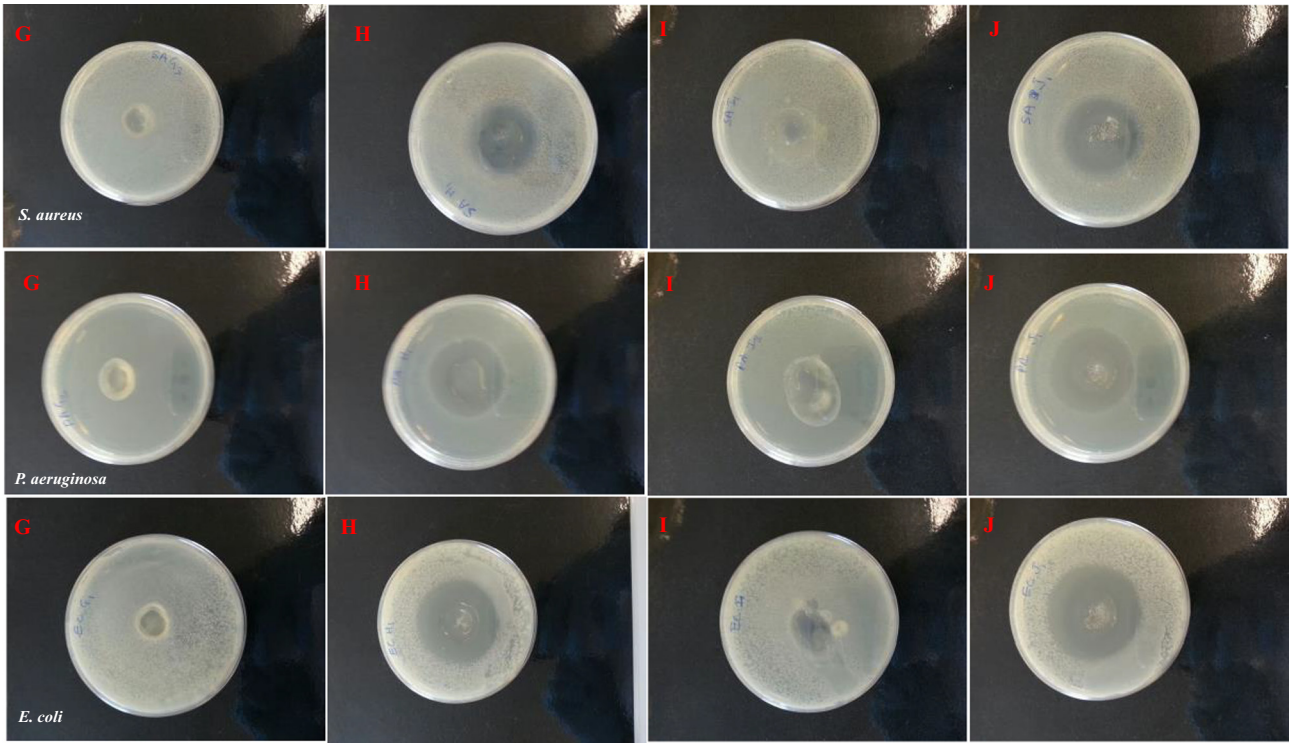


**Fig. 5.** Digital images of ZOI of (G) POL-CAR-BLK-An, (H) POL-CAR-DL-An, (I) POL-SA-BLK-An, (J) POL-SA-DL-An, observed for *S. aureus*, *P. aeruginosa* and *E. coli* (mean ± SD, $n = 3$).

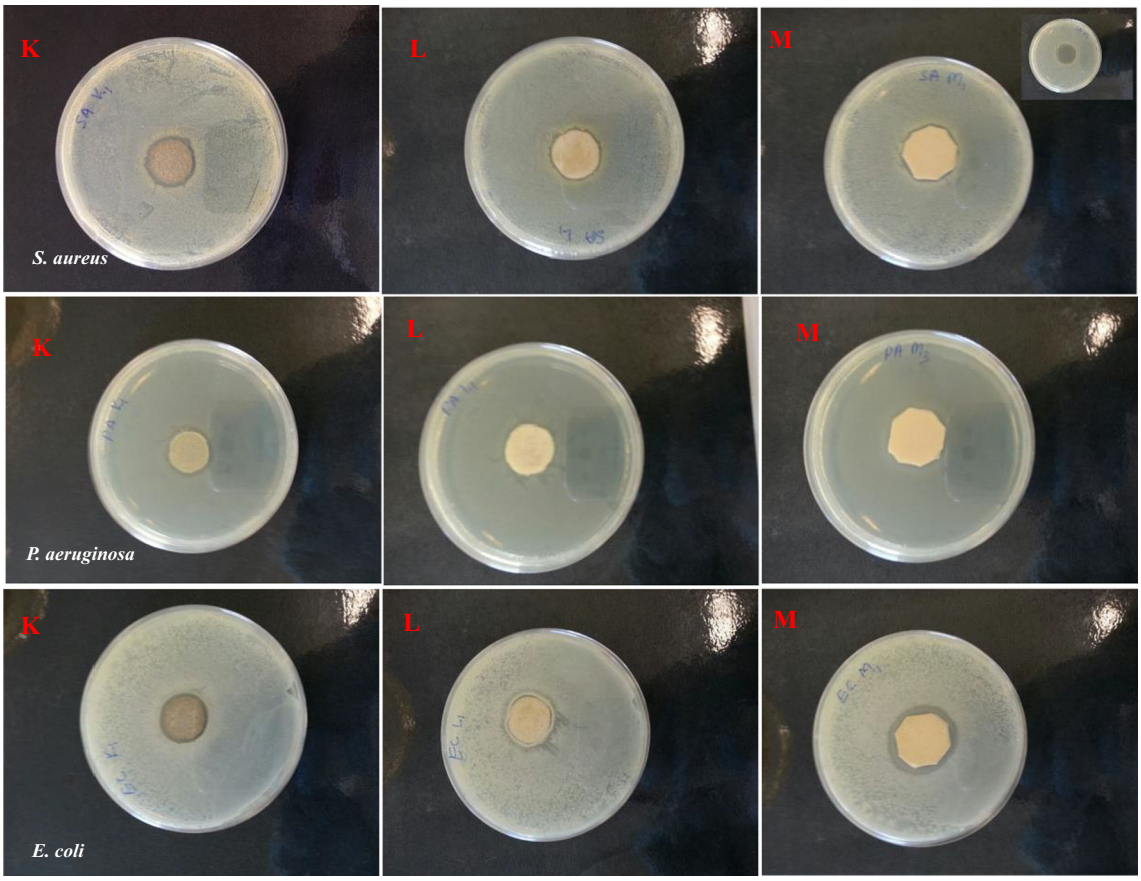


**Fig. 6.** Digital images of ZOI observed for *S. aureus*, *P. aeruginosa* and *E. coli* by (K) Aquacel® Ag; (L) Melgisorb® Ag; and (M) Allevyn® Ag. Inset shows the absence of bacteria in the immediate applied area of the dressing (mean ± SD, $n = 3$).

formation of biofilms by the microorganisms, the versatile effect of silver carries a low risk of resistance even though some studies in burn wounds have shown bacterial resistance to silver sulfadiazine and silver nitrate by *Pseudomonas* spp [31]. Moreover, the antimicrobial effect of silver incorporated in a number of dressings depends on the release rate of silver ions which influences the overall antimicrobial effect [32]. In comparative antimicrobial efficacy studies, it was reported that certain types of methicillin resistant strains among *S. aureus*, *P. aeruginosa*, and *E. coli* were less sensitive to Urgotul SSD®, Bactigras®, Acticoat®, Askina Calgitrol Ag® and Aquacel Ag® antimicrobial dressings [33]. Furthermore, *in vivo* silver can bind to proteins present in biofilms instead of binding to the bacterial cell walls, resulting in reduced antimicrobial effect against the bacteria [34]. Another potential concern is that silver does not act specifically against bacteria but also acts on any host proteins. Therefore, if very few bacteria counts are present at the wound site, then the effect on host tissue is greater which could slow down healing [35]. Concentrations above 1 mg/L (1 part per million) of silver reacts with wound exudate and could cause transient skin staining [36]. Li and co-authors suggested that bacterial resistance could be induced when low concentrations of silver were used [37]. There is therefore the possibility of these silver containing dressings inducing resistance from *S. aureus* and *P. aeruginosa* which are known to be able to form biofilms in an infected chronic wound environment [38]. However, because there was absence of bacteria in the immediate application area beneath the marketed dressings, it implies the silvers dressing were effective to kill the bacteria in only the applied area of a wound and could also potentially limit or completely prevent infection from external sources.

**Table 5**
Description of the silver containing dressings used for antimicrobial study (Hamberg et al., 2012) (mean ± SD, $n = 3$).

| Product | Formulation details | Silver content ($mg/cm^2$) |
|---|---|---|
| Aquacel® Ag | Sodium carboxymethylcellulose with ionic silver | 0.08–0.09 |
| Melgisorb® Ag | Alginate dressing with silver sodium hydrogen zirconium phosphate | 0.08 |
| Allevyn® Ag | Polyurethane foam dressing with soft gel adhesive and silver sulphadiazine | 0.90 |

## 5. Conclusion

Composite polymer based dressings containing STP and DLF appear to show significantly higher inhibition of the three bacterial strains compared to silver containing commercial dressings. STP can help to reduce bacterial infection by its known antimicrobial action and potentially in synergy with DLF while the latter can also help to reduce the swelling and pain associated with injury due to its anti-inflammatory action. However, these will, require further investigations in an *in vitro* cell culture (for cell viability and cell migration/proliferation) and *in vivo* animal study.

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## Declarations of interest

None.

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