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A Systematic Comparison of Antimicrobial Wound Dressings using a Planktonic Cell and an Immobilised Cell Model.

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Running title: Comparison of antimicrobial dressings

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Abstract

Aim: The aim of the study was to evaluate the ability of in-vitro planktonic and immobilised cell models for determining the antimicrobial efficacy of common antimicrobial wound dressings.

Methods and Results: Five strains of *A.baumannii*, *P.aeruginosa* and *S. aureus* (MRSA) were tested against four antimicrobial wound dressings containing silver, honey or PHMB, using both a planktonic and immobilised cell model. Across all species and models used, the NSCD demonstrated the best antimicrobial activity being as good if not better than all the other dressings. The planktonic cell model was less effective at differentiating the dressings on antimicrobial performance as the immobilised cell model indicating that a diffusion barrier had a significant impact on the performance of some dressings. In the presence of the diffusion barrier antimicrobial impact of the Honey and PHMB dressings was significantly reduced particularly in the case of *A. baumannii*. Activity was at least an order of magnitude lower in the immobilised cell model vs. the planktonic cell model.

Conclusions: The use of a planktonic cell model within standard tests may overestimate the efficacy of honey and PHMB. The use of an immobilised cell model provides a more demanding test for antimicrobial dressings allowing dressing to dressing and pathogen to pathogen differences to be more clearly quantified.

Significance and Impact of study: The introduction of planktonic and immobilised cell models as part of testing regimens for wound dressings will provide a more thorough understanding of their antimicrobial and antibiofilm properties.

Key words: Antimicrobial, Dressings, Biofilm, Planktonic, Wound.

Introduction:

Chronic wounds such as leg and pressure ulcers are commonly seen in primary care settings and are often infected or heavily colonised with pathogenic bacteria. Healing of these wounds depends on the interplay between the patient's defence mechanisms and the pathogenic organisms present in the wound environment (Stephen-Haynes 2004). Within a wound environment, microorganisms may exist in various states i.e. planktonic (free floating phenotypic state), or as part of a biofilm (attached phenotypic state) (Thomas et al. 2011). A biofilm is defined as a highly organized community of microorganisms attached to each other, or to biotic (living) or non-biotic surfaces enclosed in a polymeric matrix composed of complex polysaccharides, proteins, nucleic acids and glycoproteins (Donlan and Costerton 2002). Biofilms provide a protective environment that allows microorganisms to survive harsh environmental conditions such the presence of biocides and antibiotics (Donlan and Costerton 2002). *Pseudomonas aeruginosa* and methicillin resistant *Staphylococcus aureus* (MRSA) are common biofilm forming pathogenic organisms that have been observed within the clinical setting (Barrett et al. 1968; Lindsay and Von Holy 2006). *Acinetobacter baumannii* is an emerging biofilm forming pathogen, which has become a common source of infection within areas of conflict (Turton et al. 2006; Sebeny et al. 2008; O'Shea 2012).

Effective wound management is a multifaceted process that requires the control of excessive wound exudate, the elimination of excessive odours, the debridement of necrosis, the management of the microbial load and the promotion of tissue regeneration (Thomas et al. 2011). In response to these challenges, wound dressings have evolved from simple natural materials that covered and concealed the wound, through the use of materials that facilitate moisture management, to more modern dressings that attempt to actively manage the microbial load and encourage healing (Ovington 2007). Advancements in the scientific understanding of wound infections have allowed the development of a wide variety of dressings with increased antimicrobial efficacy

(Abdelrahman and Newton 2011). However, the formation and presence of biofilms within wounds represents a significant challenge to wound management, due to the inherent increase in resistance to both antibiotics and chemical treatments associated with biofilm formation.

There are a large range of antimicrobial wound dressings available with varying claims of antimicrobial efficacy. Due to the intimate nature of the interface between a wound and the dressing, careful selection of antimicrobial additives is of paramount importance. Silver and polyhexamethylene biguanide (PHMB) are now established in the market as antimicrobial chemical components of wound dressings (Fong and Wood 2006; Castellano et al. 2007; Eberlein et al. 2012); alongside these chemical agents honey is also available as a natural antimicrobial agent (Stephen-Haynes 2004; Mullai and Menon 2007; Bradshaw 2011). Silver has been used medically for thousands of years (Thomas et al. 2011); within wound care it is generally used in a nanocrystalline or ionic form. Silver is thought to act through the inhibition of cellular transport and respiration mechanisms or the disruption of transcriptional processes through denaturing of nucleic acids (Fong and Wood 2006; Asavavisithchai et al. 2010). In contrast, PHMB is a synthetic polymer mixture which is reported to affect cell membrane integrity resulting ultimately in the lysis of cells (Moore and Gray 2007). A range of honeys with antimicrobial properties is available including Manuka (New Zealand), Heather (UK) and Khandikraft (India) and these can vary substantially in their antimicrobial activity. The antimicrobial nature of honey is related to a number of properties including its pH, high osmolarity and the presence of hydrogen peroxide and phytochemicals (Stephen-Haynes 2004). However, it is thought that the principal mode of action is through the presence of hydrogen peroxides and phytochemicals, the mechanisms of which are poorly understood (French et al. 2005; Mullai and Menon 2007; Olaitan et al. 2007).

Previous procedures for the evaluation of the antimicrobial activity of wound dressings have varied significantly in terms of media used, inoculum and sample size (Tkachenko and Karas 2012), making the direct comparison of the antimicrobial efficacy of dressings difficult (Chopra 2007). Recent developments have seen the publishing of a draft standard test method for assessing the antimicrobial activity of wound dressings (BSI 2014). The draft standard currently allows the selection of either a direct contact (Gallant-Behm et al. 2005), shaking (Parsons et al. 2005) or a two compartment method (Agren and Mirastschijski 2004) employing planktonic cells of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* prepared in a simulated wound fluid containing foetal bovine serum, with no guidance as to which approach is preferred.

However, for the effective eradication of microorganisms within a biofilm, higher concentrations of antimicrobial agent are often required when compared with their planktonic or free floating non-biofilm counterparts (Thomas et al. 2011). Often the antimicrobial concentrations required for the removal can be 100-fold greater than that required for removal of micro-organisms in the planktonic state (Rasmussen and Givskov 2006). Consequently, a model system able to simulate the diffusion barrier created by an established wound biofilm would complement the existing testing approaches based on planktonic cells (BSI 2014); such a system would provide a model for the treatment of chronically infected wounds where biofilms are more prevalent. The aim of the following study was to determine if the presence of a diffusion barrier has a significant impact on the performance of antimicrobial wound dressings when compared to an approach employing planktonic cells. The data generated from this study may aid the development of the current draft test method (BS EN16756:2014) in order to determine whether strain selection or the incorporation of a diffusion barrier are worthy of consideration for the final version of the standard.

Materials and methods

Bacterial strains

Five strains of *Pseudomonas aeruginosa*, methicillin resistant *Staphylococcus aureus* (MRSA) and *Acinetobacter baumannii* were selected as candidate organisms for testing. Of the five strains selected from each group, a mix of type- and clinical/community-acquired strains were selected and are summarised in table 1.

Dressings

Four commercially available dressings were selected for evaluation: a Manuka honey based dressing (Actilite, Advancis medical, UK), a polyhexamethylene biguanide (PHMB) based dressing (Suprasorb, Lohmann and Rauscher, Germany), a nanocrystalline silver coated dressing (NSCD; Acticoat absorbent, Smith & Nephew Medical Ltd, UK) and an ionic silver coated dressing (ISCD; Aquacel Ag, Convatec, UK).

Planktonic cell model

A bacterial suspension of $1.0 - 1.5 \times 10^8$ CFU ml⁻¹ was prepared in maximum recovery diluent (MRD; Lab M) as per previous methods (BSI 2009; White et al. 2012). From this bacterial stock, a $1.0 - 1.5 \times 10^5$ CFU ml⁻¹ suspension was prepared in simulated wound fluid (SWF; 50% v/v foetal calf serum (Sigma, UK) in MRD) and used as an inoculum for subsequent testing. A 16 cm² portion of dressing was placed on a sterile plastic sheet in the base of a sterile Petri dish, 400 µl of inoculum was added to the surface of the dressing before being covered with a second layer of sterile plastic. The inoculated dressings were then incubated for 24 hours at 37°C and 90 % humidity. Following incubation the top and bottom plastic layers, alongside the dressing were each transferred to a sterile stomacher bag containing 10 ml of a validated neutraliser, sealed, and stomached for 20 minutes. A range of dilutions of the neutralisation mixture were then prepared in MRD and plated out

onto tryptone soya agar (TSA; LabM, UK). In addition, the stomached dressing and plastic layers were aseptically removed and transferred to Petri dishes and immersed with molten TSA, prior to incubation at 37°C for 24-48 hours. Suitable controls were carried out in line with previously described testing methods (BSI 2007); here the active dressing was replaced with a piece of sterile plastic and processed as described above. The impact of the dressings was determined as a log reduction factor (per cm²) calculated by comparison with the number of bacteria recovered from controls (BSI 2007).

Immobilised cell model

In order to determine the efficacy of dressings in the presence of a diffusion barrier, an *in vitro* immobilised cell model mimicking the presence of a biofilm in a chronic wound was selected based on previous methods (Brackman et al. 2011). Matrices of polymerized rat-tail collagen type I (BD Biosciences UK) were prepared in 15 ml tubes following the manufacturer's protocol for eukaryotic cell culturing. To prepare 10 ml of collagen matrix solution (3.8 g l⁻¹), 1ml of phosphate buffer saline (PBS) was mixed with 0.019 ml of NaOH (1 mol l⁻¹) and 0.711 ml of inoculum (prepared in SWF) and kept on ice. Finally 8.27 ml of collagen from cold collagen stock (4 g l⁻¹) was added and, after mixing, 1ml of collagen matrix was added in each well of polystyrene microtiter plate (Nunc surface 24 well plates, Fisher Scientific, UK). Following polymerisation for 1 hour at 37°C, dressing was added to the top of the matrix and incubated for 24 hours at 37°C and 90% humidity. In order to enumerate the surviving bacteria, dressings were removed following incubation and 1ml of collagenase solution added to the wells (1 g l⁻¹ in PBS from *Clostridium histolyticum* (Sigma Aldrich, UK)), the suspension was then completely mixed and incubated at 37°C until the collagen was completely digested (60-80 minutes). The contents of the well were then added to 10 ml of validated neutraliser and plated out onto

TSA alongside the dressings, controls were carried out in the same manner and the surviving fraction of bacteria calculated as per the planktonic cell model.

Statistical analysis

All statistical analysis was carried out on IBM SPSS Statistics 20 for Windows. One-way between groups ANOVAs were conducted to evaluate the effect of dressing type (independent variable) on number of bacteria (dependent variable) for each model, with the Games-Howell *post hoc* test used to identify significant differences between variables. Because the data were not normally distributed and, in most cases, the assumption of homogeneity of variance was violated, the more robust Welch *F*-ratio is reported. The difference in Log reduction between the two models was evaluated using independent *t*-tests.

Results

The mean Log number of organisms per cm² recovered from control pieces can be seen in supplementary table S1. In the case of both *Acinetobacter* and *Pseudomonas* species, there was no obvious difference between the yield of microorganisms observed within either the planktonic or immobilised cell model. Within the MRSA isolates, a greater Log recovery of microorganisms was observed using the immobilised cell model rather than the planktonic model, suggesting that the growth of the selected MRSA strains was enhanced through the immobilisation within collagen.

The mean antimicrobial activity demonstrated by each wound dressing against each species, for the planktonic [*A. baumannii* $F(3, 58.33) = 792.54, p < 0.001$; *P. aeruginosa* $F(3,75.10) = 6.54, p = 0.001$; MRSA $F(3,38.35) = 8.35, p < 0.001$] and immobilised cell models [*A. baumannii* $F(3, 55.70) = 3244.80, p < 0.001$; *P. aeruginosa* $F(3,91.31) = 41.40, p < 0.001$; MRSA $F(3, 57.25) = 37.08, p < 0.001$], can be seen in table 2. In both the

planktonic and the immobilised cell model, there were significant differences by dressing within each species (see Figures 1a and 1b). A comparison of the two models can be seen in Figure 2; the planktonic model demonstrated a significantly greater Log reduction in viable counts compared with the immobilised cell model, with the exception of the ISCD dressing, which was more effective in the immobilised cell model against *A baumannii*.

There was considerable difference between individual strains in the Log reductions observed (see Figures 3, 4 & 5). Within both models, *A. baumannii* strain 822 was less susceptible to the silver dressings than the other isolates (Figure 3). The NSCD dressing resulted in a total kill in both models for the other strains of *A. baumannii*; the significant difference in Log reduction observed in Figure 2 appears to be due to the greater Log reduction of strain 882 in the planktonic model. The honey dressing was much less effective against strain 882 compared with the other strains of *A. baumannii* in the planktonic model, but was more susceptible in the immobilised cell model.

A total kill was observed with the *P. aeruginosa* Type strain in the immobilised cell model for the PHMB, ISCD, and NSCD dressings, and in the planktonic model for the NSCD dressing (Figure 4). The dressings were considerably less effective against the other strains for *P. aeruginosa* when tested using the planktonic model, although the difference in effectiveness was less for the NSCD dressing compared with the other dressings.

Within the MRSA testing, greater Log reductions were seen across the planktonic model compared with the immobilised cell model for most strains of MRSA, with the notable exceptions of strain 6538 and 7F/C7 when treated with the silver coated ISCD dressing (Figure 5).

Discussion

A wide variety of test methods have been employed to evaluate antimicrobial wound dressings, from simple zone of inhibition testing, to more complex broth based methods (Chopra 2007; Tkachenko and Karas 2012). In order to standardise the methods by which these dressings are compared, a draft standard for testing has been devised using broth based methods against planktonic cells (BSI 2014). In this study we have assessed a range of antimicrobial dressings against both Gram positive and negative pathogens using both a planktonic model similar to those suggested in the current draft standard and an immobilised cell model chosen to mimic the diffusion barrier generated by the presence of a biofilm. In particular, the immobilised cell model provides a consistent wound environment with which to determine the anti-biofilm forming properties of wound dressings which greater reflects the *in vivo* than the planktonic model. The method is reproducible and allows for simple, cost effective enumeration through plating methods in comparison to other published strategies using fluorescence (Brackman et al. 2013).

The immobilised cell model presented here is also capable of yielding results within 48 hours. Previous studies have required up to 48 hours to prepare a suitable biofilm for study against antimicrobials (Thorn et al. 2009; Hill et al. 2010; Kucera et al. 2014), as such the immobilised cell model provides an alternative that may improve timescales in which candidate dressings are tested at a clinical level (Durante 2012).

Within the planktonic model, the greatest Log reductions in viable cell counts was observed with the nano-crystalline silver dressings, with total kills observed with four of the *A. baumannii* strains, one of the *P. aeruginosa* strains and two of the MRSA strains within 24 hours. In a similar fashion, previous authors have also found that a complete kill was observed within 24 hours when testing against NSCD and ISCD against Gram positive and negative isolates using a similar planktonic cell testing method (Ip et al. 2006; Tkachenko and Karas 2012). Within our planktonic model, no complete kills were observed with ISCD;

however, when tested with the immobilised cell model, ISCD produced comparable results to NSCD against *A. baumannii* and MRSA isolates. The efficacy of the honey and PHMB dressings were most impacted by the use of the immobilised cell model across all three isolates, which agreed with previous work suggesting that honey was more effective against planktonic isolates over those within biofilms (Alandejani et al. 2009; Merckoll et al. 2009). In particular, *P. aeruginosa* and *A. baumannii* were less susceptible to honey and MRSA less susceptible to PHMB, within the immobilised cell model. The reduced susceptibility of *P. aeruginosa* to Honey when compared to *S. aureus* has recently been reported (Lu et al. 2013). This reduced susceptibility may be due to the catalase activity of these species reducing the impact of the hydrogen peroxide generated by the Honey dressing. The impact of catalase activity on the biocidal impacts of hydrogen peroxide have been specifically reported for *A. baumannii* (Herruzo et al. 2014). Previous work has suggested that PHMB is effective against MRSA within a wound environment when applied directly (Wild et al. 2012), and as such this result may be a reflection of the diffusion of PHMB from the dressing into the biofilm rather than of the activity of the chemical itself. Clinical trials investigating the usage of PHMB dressings also suggest that beneficial effects against biofilms required prolonged use of up to 28 days to achieve results against biofilms of critically colonised patients (Fong and Wood 2006).

Strain selection for any potential testing method was also investigated. In both the planktonic and immobilised cell model, variation was seen with isolates of the same species, suggesting that strains selection may be important when finalising a standard test method. Of particular note, within the immobilised cell model (Figures 3, 4 & 5) Type strains were more susceptible to the active ingredients than clinical isolates. This result agrees with the previous data using an *in vitro* model employing zone of inhibition tests, which found no significant differences between the dressings tested, but found that differences occurred between strains (Du Toit and Page 2009). These observed

differences between clinical and type strains will contribute to patient to patient variations in the efficacy of antimicrobial dressings and emphasise the caution required when translating in-vitro data into clinical settings.

In summary, our findings indicate that an immobilised cell model provides a more rigorous testing strategy for antimicrobial wound dressings than the planktonic models reported in the literature and proposed in the draft standard (BSI 2014). The immobilised cell model evaluated here was more effective at differentiating between antimicrobial dressings and highlighting those most susceptible to the presence of a diffusion barrier (PHMB and Honey), which may in turn indicate that they are less suitable for the prevention of chronic biofilm formation. In addition, the variation observed between strains suggests that clinical identification of strains present within a wound may still play an important role in wound management.

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Conflict of interest

None to declare

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Species	Name	Isolate	Source
<i>Pseudomonas aeruginosa</i>	10421	Type	NCIMB
	I	Clinical	Isolated from a wound dressing.
	II	Clinical	
	III	Clinical	
	IV	Clinical	
<i>Acinetobacter baumannii</i>	19606	Type	ATCC
	OXA - 24 Clone I	Clinical	Provided by LHCAI, HPA Colindale, London.
	OXA - 24 Clone II	Clinical	
	Hospital	Clinical	Provided by Dr M. Wren, UCLH, London.
	CIP 106882	Culture Collection	Pasteur Institute, Paris, France.
Methicillin resistant <i>Staphylococcus aureus</i>	6538	Culture Collection	ATCC
	43300	Culture Collection	ATCC
	13142	Culture Collection	NCTC
	7F/C7	Community acquired	Previous Study, White et al 2012.
	9B/F6	Community acquired	Previous Study, White et al 2012.

Table 1: Bacterial strains used in this study.

	Dressing								P
	NSCD		ISCD		Honey		PHMB		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Planktonic cell model									
<i>A. baumannii</i>	9.09	1.01	6.53	0.54	7.69	2.51	3.95	0.73	<0.001
<i>P. aeruginosa</i>	7.70	0.65	6.93	1.05	7.10	1.10	6.42	2.05	0.001
MRSA	6.97	0.41	5.55	1.86	6.58	0.48	6.12	0.79	<0.001
Immobilised cell model									
<i>A. baumannii</i>	8.26	0.87	8.18	1.03	3.10	1.09	2.93	0.35	<0.001
<i>P. aeruginosa</i>	6.90	0.74	5.95	1.33	3.55	1.24	5.23	1.79	<0.001
MRSA	6.20	1.33	6.22	1.30	5.09	0.39	3.71	0.44	<0.001

Table 2. Mean (SD) difference between control and test, where a larger number indicates more bacteria killed

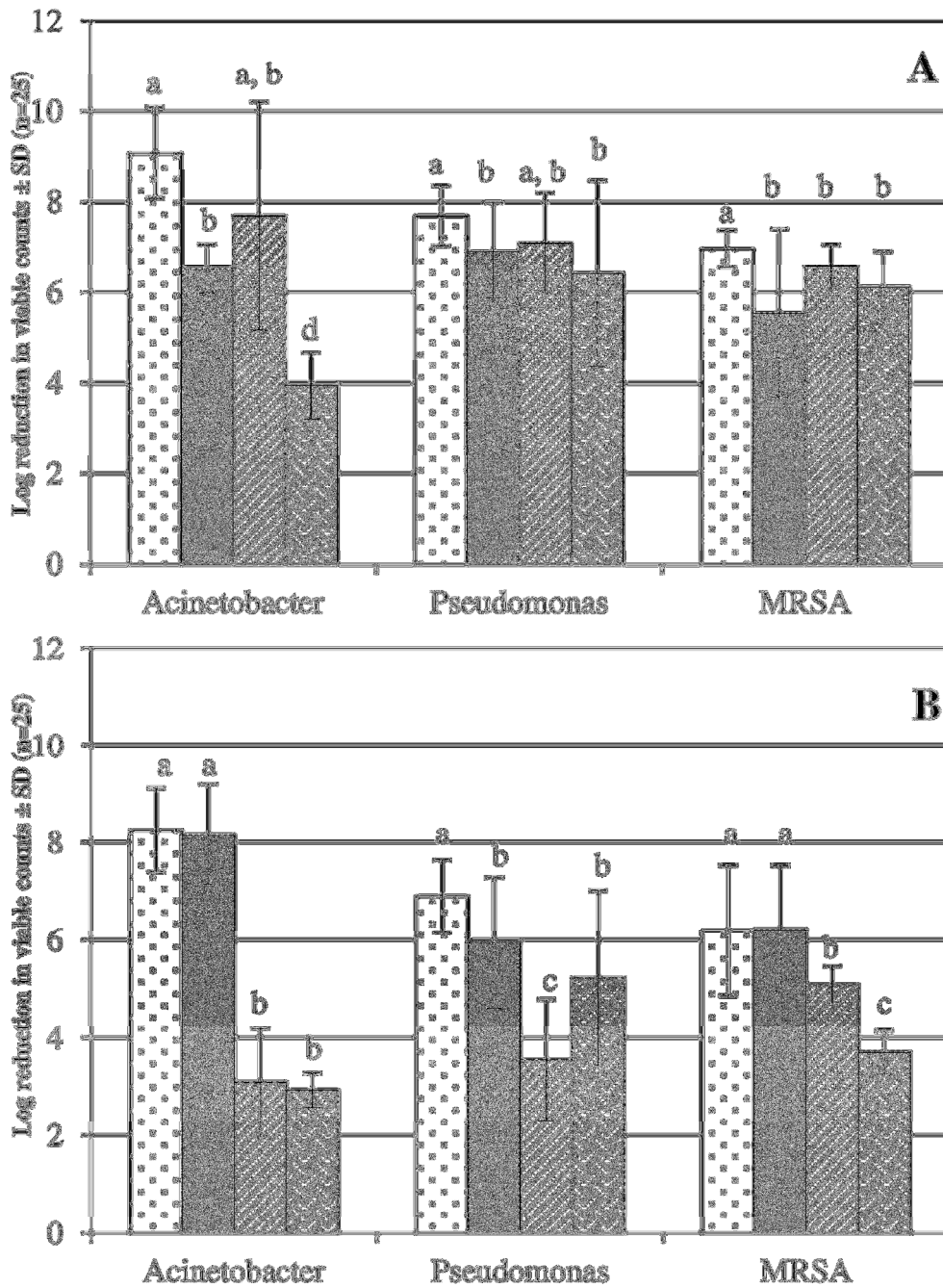


Figure 1 Mean antimicrobial activity of NSCD (▤), ISCD (■) Honey (▨) and PHMB (▧) wound dressings, against five strains of *A. baumannii*, *P. aeruginosa* and MRSA isolates using the planktonic model (A) and the immobilised cell model (B). One-way between groups ANOVAs with Games-Howell *post hoc* tests were used to identify significant differences in the impact of dressing within species; different letters indicate significant differences between dressings within species.

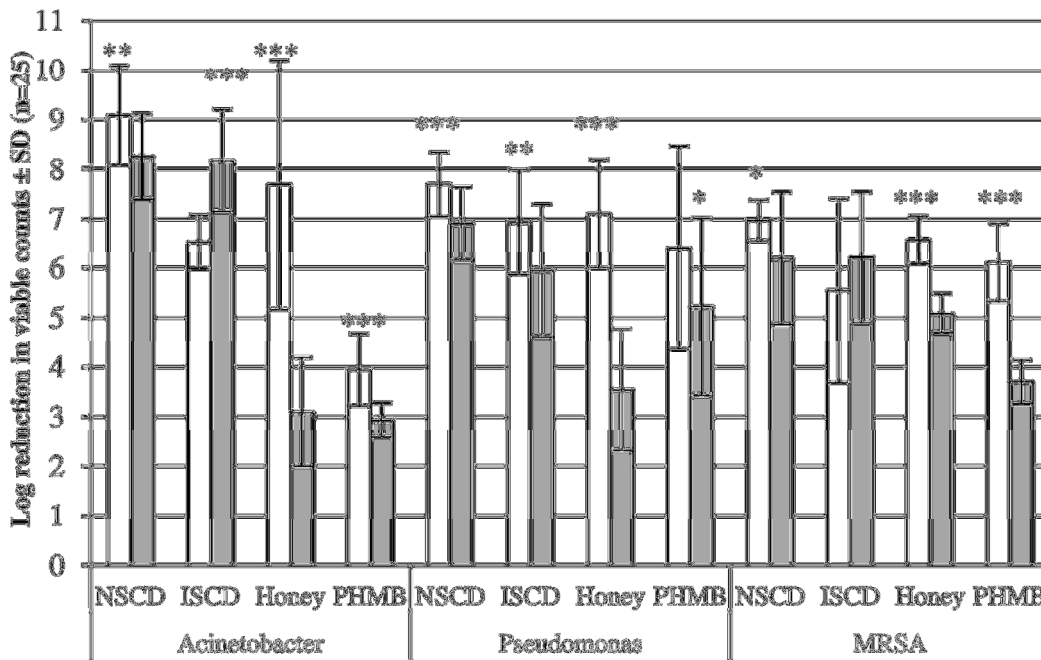


Figure 2: Collective comparison of planktonic (white bars) and immobilised cell models (grey bars) of each dressing tested for each species tested, error bars represent the standard deviation (n= 25); The difference in Log reduction between the two models was evaluated using independent t-tests, * p<0.05, ** p < 0.01, *** p < 0.001.

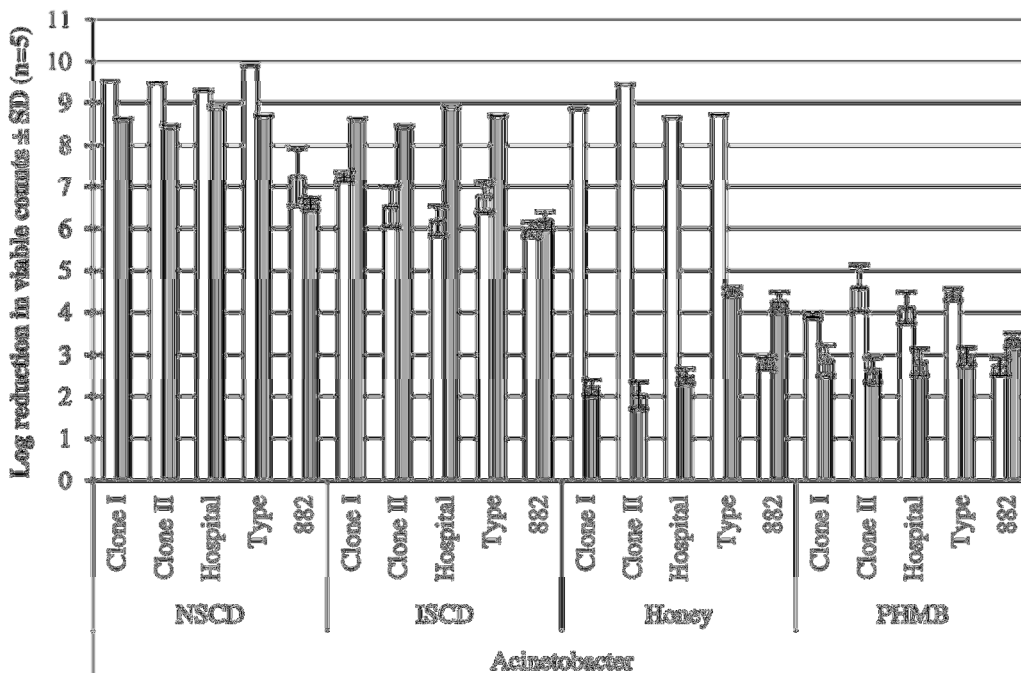


Figure 3. Comparison of Log reduction between the planktonic (white bars) and immobilised cell (grey bars) models by dressing and bacterial strain for *A. baumannii*.

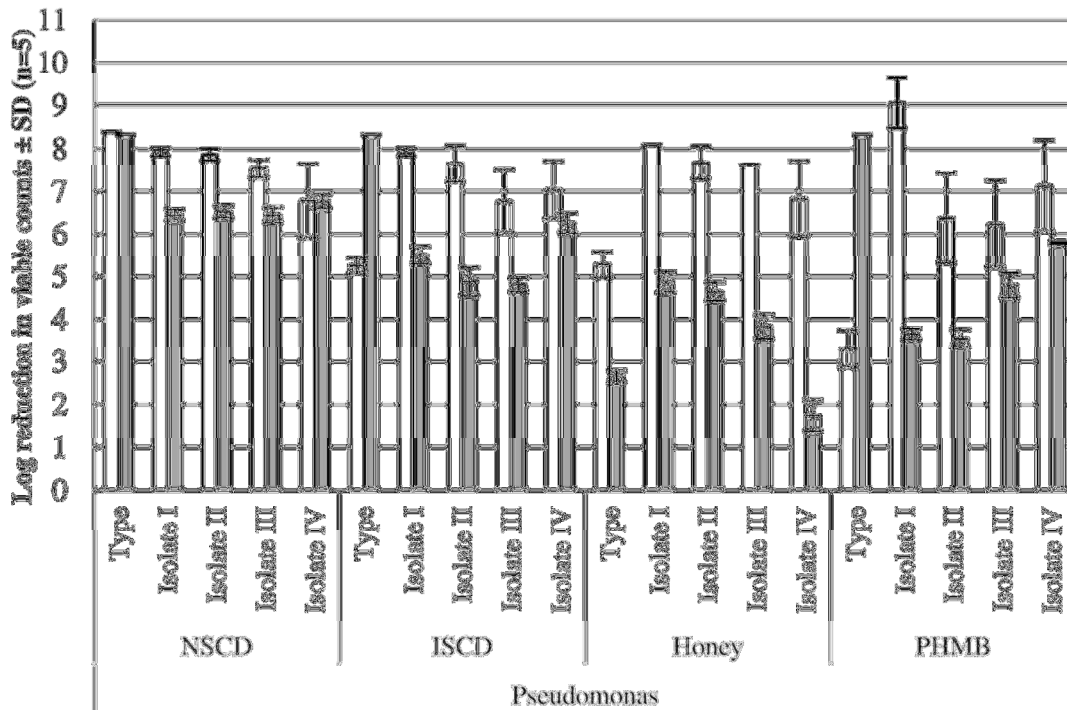


Figure 4. Comparison of Log reduction between the planktonic (white bars) and immobilised cell (grey bars) models by dressing and bacterial strain for *P. aeruginosa*.

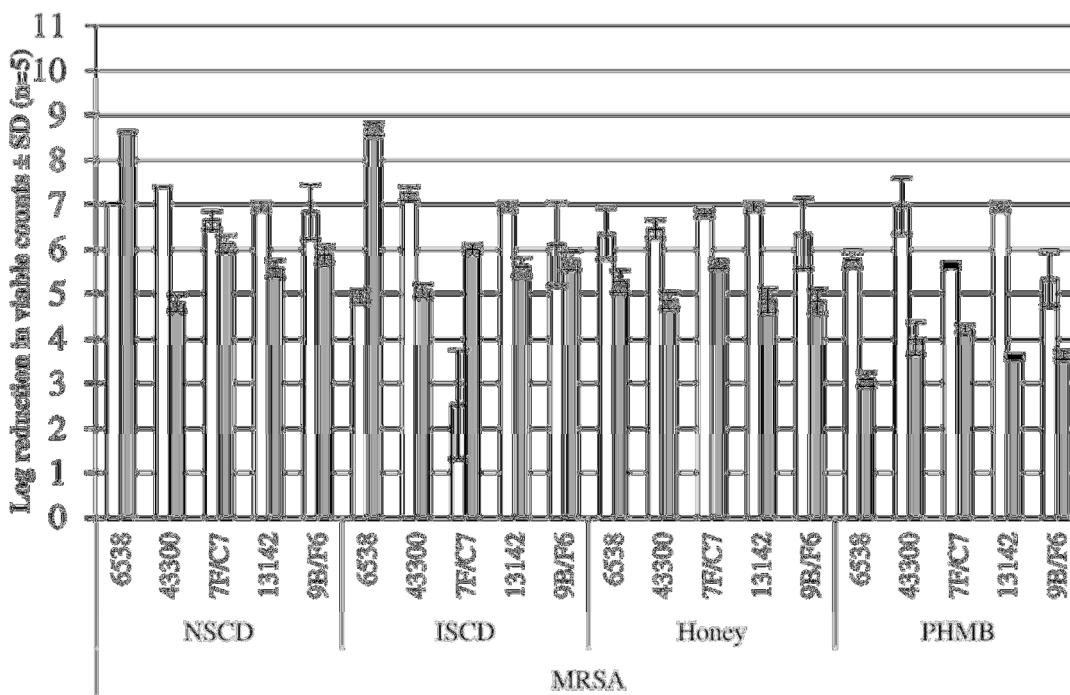


Figure 5. Comparison of Log reduction between the planktonic (white bars) and immobilised cell (grey bars) models by dressing and bacterial strain for MRSA.