Developing novel topical antimicrobial agents for the treatment of biofilm infections

By

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i Acknowledgements

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ii Index of published work for consideration

The following four papers make up the body of work for this thesis. Refer to Appendix 2 for attested statements of contribution for the candidate for each paper.

Paper	Reference	Pages
1	**Halstead FD**, Rauf M, Moiemen NS, *et al.* (2015). The Antibacterial Activity of Acetic Acid against Biofilm-Producing Pathogens of Relevance to Burns Patients. PLoS ONE 10(9): e0136190.	**69-86**
2	**Halstead FD**, Rauf M, Bamford A, *et al.* (2015). Antimicrobial dressings: Comparison of the ability of a panel of dressings to prevent biofilm formation by key burn wound pathogens. *Burns,* 41:1683-1694.	**87-98**
3	**Halstead FD**, Webber MA, Rauf M, *et al.* (2016). *In vitro* activity of an engineered honey, medical-grade honeys, and antimicrobial wound dressings against biofilm-producing clinical bacterial isolates. *Journal of Wound Care*, 25(2).	**99-107**
4	**Halstead FD**, Thwaite JE, Burt R, *et al.* (2016). The antibacterial activity of blue-violet light against nosocomial wound pathogens growing planktonically and as mature biofilms. *Applied and Environmental Microbiology*, 82(13): 4006-4016.	**108-119**

iii Statement of ethical considerations

There are no ethical considerations for the body of work submitted, since all of the individual studies are classified as research, and all experiments were *in vitro*.

iv Submission declaration

I declare that the thesis is my own work, based on collaborative research.

I declare that the submitted material as a whole is not substantially the same as published or unpublished material that I have previously submitted, or am currently submitting for a degree, diploma, or similar qualification at any university or similar institution.

The views expressed in this thesis are those of the author, and not necessarily those of the funders (the NHS, NIHR SRMRC, or University of Birmingham).

v Word count

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Revised thesis: 13,076 words (exclusions as above)

vi Abbreviations

AA	Acetic Acid
AMDs	Antimicrobial dressings
BL	Blue light
CLED	Cysteine lactose electrolyte deficient
CV	Crystal violet
EPS	Extracellular polymeric substance
EtOH	Ethanol
ISO	Iso-sensitest
LED	Light-emitting diode
LB	Lysogeny Broth
MBEC	Minimum Biofilm Eradication Concentration
MBIC	Minimum Biofilm Inhibitory Concentration
Med	Medihoney
MIC	Minimum Inhibitory Concentration
MHB	Muller-Hinton broth
MH	Manuka honey
MOA	Mechanism of action
MTT	Microtiter Tray
nAMD	Non antimicrobial dressing
OD	Optical Density
QEHB	Queen Elizabeth Hospital Birmingham
QS	Quorum Sensing
RCT	Randomised controlled trial
ROS	Reactive oxygen species
SH1	SurgihoneyRO
SOC	Standard of care
TAA	Test Antimicrobial Agent
TBI	Test Bacterial Inoculum

0 SUMMARY

Background
Bacterial wound infections (especially those involving biofilms) represent a major challenge to healthcare, and are responsible for significant morbidity and mortality. Owing to the rise in antimicrobial resistance, there is renewed interest in alternative antimicrobial agents for treatment of wound infections, where prevention of colonisation largely relies on topically-applied biocides.

Objectives
The aim was to investigate the antibacterial activity of acetic acid (AA), following on from preliminary testing, and small-scale use on burns patients. This led on to the testing of additional products (SurgihoneyRO (SH1) and blue light (BL)), for which no prior evaluation (against biofilms) had been performed.

Methods
In vitro experiments were performed to test the antimicrobial activity of the agents against bacteria growing planktonically and as biofilms. Comparisons were also made to a range of commercially-available antimicrobial dressings (AMDs) and medical honeys. Results were assessed through measurement of biofilm biomass, and biofilm seeding using a crystal violet assay.

Results
All agents were effective against biofilms of a large panel of clinically important nosocomial wound pathogens. AA could prevent biofilm formation at concentrations of ≤0.31%, and eradication of mature biofilms was observed after 3 hours of exposure. SH1 prevented biofilm formation of 16 bacterial isolates at dilutions (from neat) of 1:2 to 1:128. Mature biofilms were highly susceptible to BL, with significant reduction in seeding observed for all isolates.

Conclusions
All of the test antimicrobial agents have shown promise in *vitro* for the treatment and eradication of biofilm infections caused by a range of important wound pathogens. However, there are still some unanswered questions. Clinical trials are planned, and it remains to be seen whether the *in vitro* findings will translate to the *in vivo* setting, where there is a complex interplay between host and pathogen, and many other factors that influence biofilm presence and persistence.

1 BACKGROUND

Bacteria may exist in one of two growth states; planktonically (free-floating), or aggregated (in a biofilm) [1]. Cells within a biofilm can be attached to each other (in multicellular aggregates which may themselves not be directly adhered to a surface), or adhered to a surface [2]. This trait enables bacteria to adapt to environmental change, and affords higher resistance to adverse physiological conditions [3]. Consequently biofilms are abundant in natural and engineered environments, where they can have both beneficial and detrimental effects (from an anthropocentric point of view).

The beneficial aspects of biofilms include their use in wastewater treatment plants (as filters and bioreactors in the treatment process) [4], bioremediation (e.g. biofilms can metabolise organic pollutants) [5], and promotion of plant growth (owing to the presence of growth-promoting rhizobacteria in plant-associated biofilms) [6]. They are also important in marine environments, with bacteria often the first microorganisms to colonise a surface, leading to the rapid succession of colonisation by higher organisms including barnacles. Biofilms can regulate the settlement/attachment of higher sessile organisms, thus potentially reducing the chance of biofouling [7]. Since they readily colonise both biotic and abiotic surfaces, biofilms can also be detrimental, causing bio-deterioration in engineered systems, slowing down ships (due to the accumulation of biofilm on the hull), and the bio-fouling of food processing equipment [3].

Although it can be argued that the majority of our beneficial bacteria may exist as biofilms in the body (e.g. the protective microbiome found in the large intestine), biofilms are also responsible for the majority of chronic infections [8]. These infections, typically of mixed microbial aetiology [9] are important clinically for a range of conditions (e.g. foreign-body related, otitis media, chronic wounds, and lung infections) [10], and it is estimated that at least 60% of chronic infections involve a biofilm [11,12]. The longer a biofilm persists, the greater the risks are that the patient will develop invasive systemic infections [13], such as bacteraemia. Furthermore, a large evidence base now exists showing that biofilms exist in the majority of non-healing chronic wounds [14,15], and are associated with delayed healing [16,17] (Figure 1). Indeed, several authors [14,18,19] advocate that the presence of a biofilm should be considered for any non-healing wound.

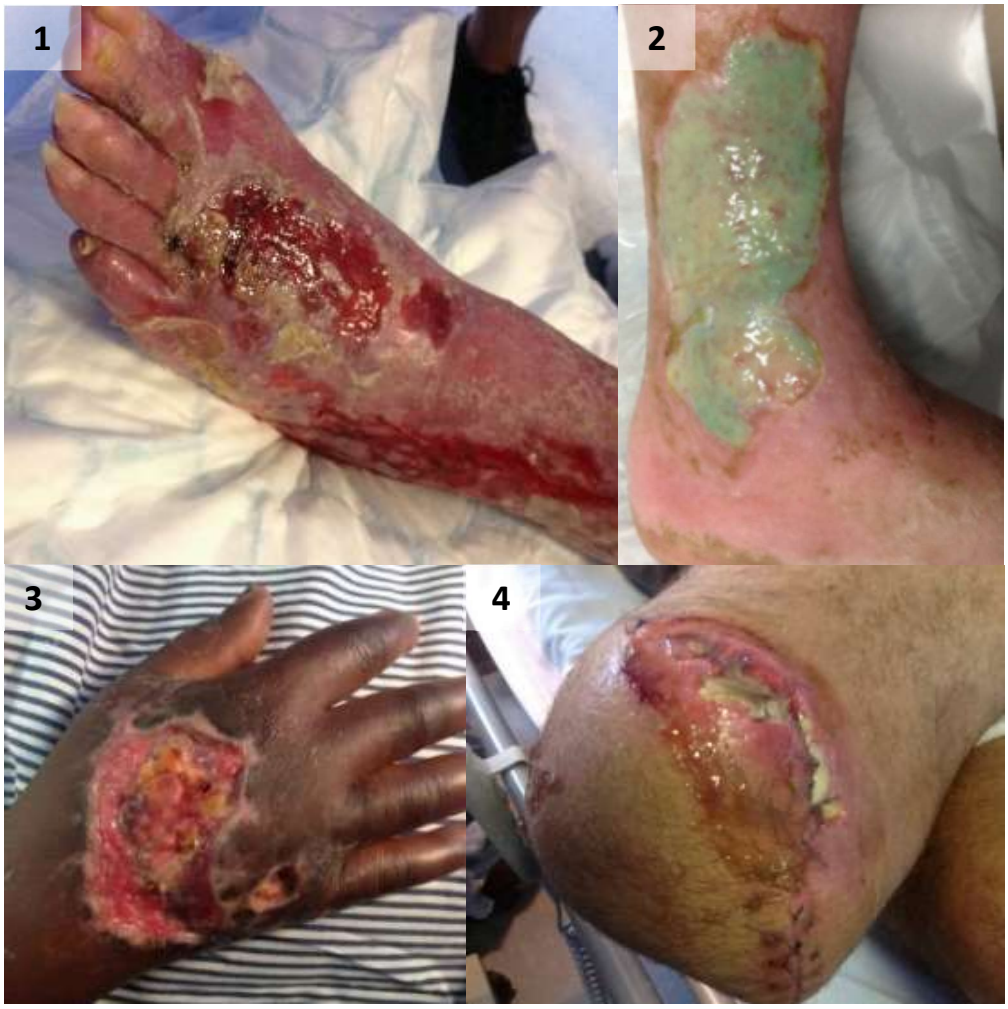

Figure 1: Panel of photographs showing clinical biofilm infections (all taken from [30]) **1:** Chronic foot ulcer infected with *P. aeruginosa*, **2:** large ischaemic foot ulcer infected with *P. aeruginosa* and coliforms, **3:** hand of a Ugandan woman with an infected deep traumatic wound (no microbiological results), **4:** Amputation site with *Streptococcus agalactiae* infection.

1.1 The biofilm formation process

The switch from planktonic to sessile existence (i.e. biofilm formation) in bacteria is controlled by a number of species-specific environmental and genetic factors, but generally begins with contamination and the initial adsorption and attachment of bacteria to a surface or to each other.

In *Pseudomonas aeruginosa*, biofilm formation can occur in just six hours [14], and established biofilms may be present by 24 hours [14]. Here, the initial reversible attachment of bacteria to a surface occurs due to the action of the flagella (which aid in the mobility of the bacterium), type IV pili, fimbria, extracellular DNA, and the Psl exopolysaccharide. The pilli are constructed from a single protein subunit (PilA), and mutants lacking the required genes to form flagella and pilli/fimbriae are poor at surface attachment and biofilm formation [20].

After reversible attachment, cells may undergo irreversible attachment (where they attach by more than just their 'poles'). This involves surface proteins (e.g. Lipopolysaccharide-A in *P.* aeruginosa which acts to increase hydrophobicity of the cell surface, which enhances adhesion to hydrophobic surfaces), as well as intracellular secondary messenger molecules such as bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) and cyclic adenosine monophsphate (cAMP) [21]. Once attached, the cells produce matrix, and the biofilm expands, matures and disperses (where planktonic cells are released from the mature biofilm to find new sites for colonisation) (Figure 2).

The matrix (referred to as the extracellular polymeric substance (EPS)), is made of polysaccharides, proteins, lipids and extracellular DNA [22], and enables the biofilm to withstand adverse conditions. The EPS layer also houses quorum sensing signalling (QS) molecules, extracellular enzymes, and metabolic products [21]. Alginate is a key component of the biofilm matrix in *P.* aeruginosa and is overproduced by isolates associated with cystic fibrosis (mucoid phenotype) [23]. Dispersal of planktonic cells from the mature biofilm is controlled through QS and three interconnected gene systems: the Las system; the Rhl system; and the Pqs system [24]. In order to encourage detachment of the bacteria and dispersal to new sites, *P.* aeruginosa also produces an alginate lyase enzyme which cleaves the alginate into short chain polymers, and resulting in detachment of planktonic cells [25]

The physiology of biofilm-containing cells differs considerably to the physiology of planktonic cells. The complex structure of biofilms affords them greater ability to withstand adverse conditions (e.g. high osmotic stress, low nutrients, low oxygen availability, antibiotics and the host immune response). Furthermore, bacteria contained within biofilms have reduced rates of metabolism [11], and consequently are harder to treat and eradicate than their planktonic counterparts [26], requiring concentrations of systemic antimicrobials 10-1,000 times higher than those needed to treat the same species of planktonic bacteria [27]. Despite the administration of antibiotics, treatment is often ineffective due to the high circulating levels of antimicrobial resistance in common wound pathogens, the existence of metabolically-dormant persister cells (which can recrudesce and repopulate the biofilm [28]), and the presence and mixed microbial aetiology of biofilms (resulting in imperfect antibiotic coverage) [14,29]. Furthermore, chronic wounds often have poor blood supply [14,30], leading to poor delivery of systemic antimicrobials.

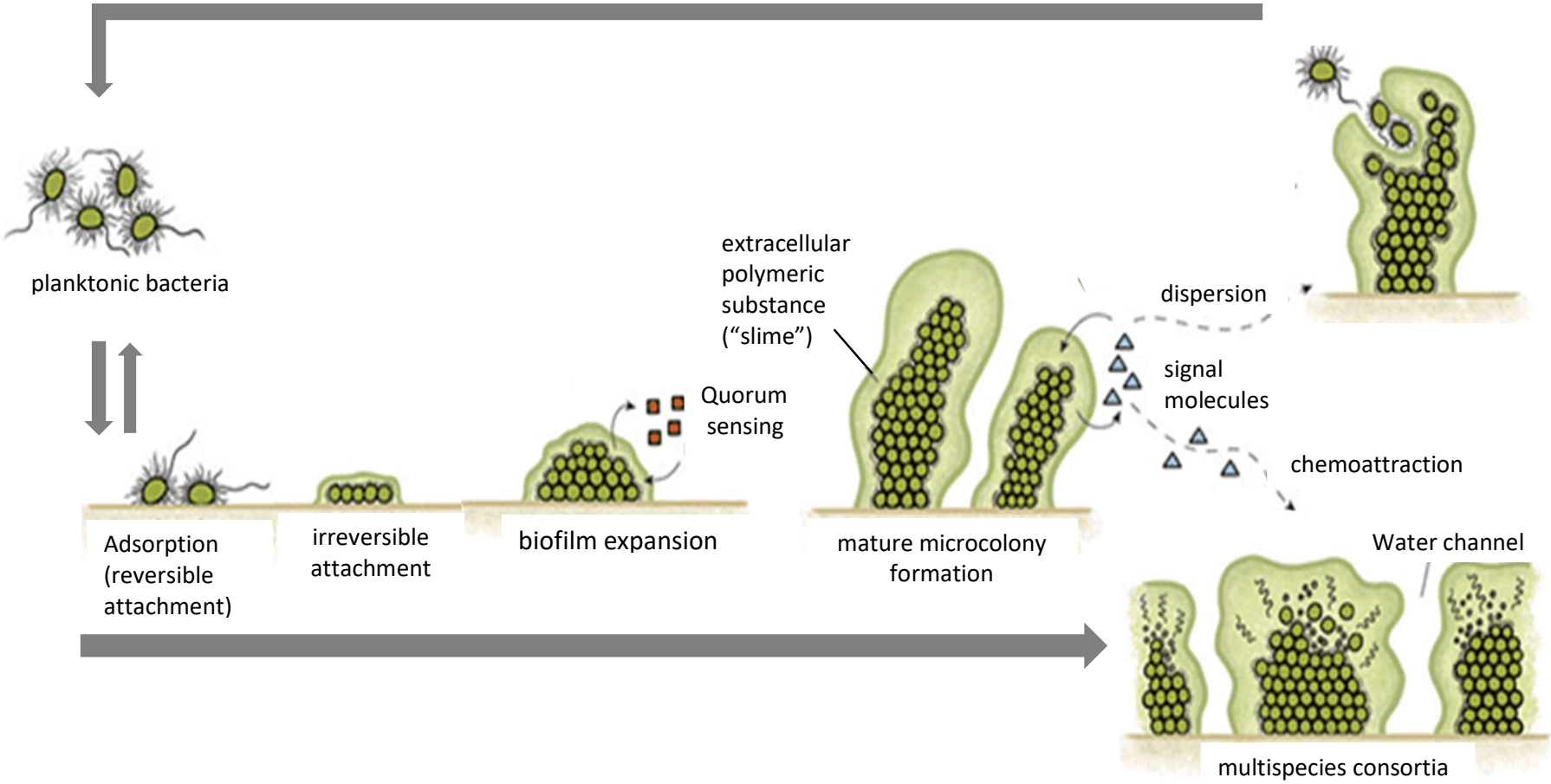

Figure 2: The stages of biofilm formation, maturation and dispersion (modified from the original version [31] by rearranging the figure, and adding text)

1.2 Therapeutic anti-biofilm strategies

Therapeutic anti-biofilm strategies intend to prevent, remove and kill bacterial biofilms (Table 1), and target the bacteria forming the biofilm, but also aim to suppress the biofilm through simultaneous use of other strategies, in what is referred to as 'biofilm-based wound care'. Below is a review of a few of these strategies.

To prevent biofilms, the contamination and adhesion/colonisation of wounds with bacteria must be prevented e.g. via aseptic surgical techniques, as well as the use of barrier dressings, which aim to keep the wound clean and free from bacteria (physical dressings), and in most cases contain an antimicrobial agent (more detail will be provided in the next section). Other preventative measures may involve the use of anti-deposition agents to interfere with EPS production, QS inhibitors e.g. hamamelitannin (although limited since QS signal molecules vary between bacterial species, and only 50% share the same autoinducer [31]), probiotics, and lytic bacteriophages (which carry enzymes to degrade EPS [32]) (Table 1). For example, DispersinB (a matrix-degrading enzyme of *Aggregatibacter actinomycetemcomitans*), can inhibit formation, induce detachment, and sensitize biofilms to antibiotics and host defences [33].

Once biofilm infection is established (which is the case seen with the majority of clinical wounds), most of the clinical management relies on physical debridement of the wound to remove the biofilm, in conjunction with systemic antimicrobials and topical antiseptics to kill the remaining bacteria. The terminology used to describe antimicrobial agents can be confusing. Antimicrobial agents include those effective against bacteria, fungi, viruses, parasites and spores. Within this umbrella term lie the antibiotics ('naturally occurring or synthetic organic substances which inhibit or destroy selective bacteria, generally at low concentrations'), the biocides ('broad-spectrum chemical agents that inactivate microorganisms'), and the antiseptics ('biocides or products that destroy or inhibit the growth of microorganisms in or on living tissue')[34].

Table 1: Anti-biofilm strategies for wound care which aim to prevent, remove and/or kill biofilms (self-drawn)

Aim	Description
Prevention of biofilm formation	
Prevent contamination	
- Aseptic (surgical) procedures	The risks of contamination can be reduced through careful attention to aseptic techniques in surgery, and the use of barrier wound dressings (to mimic the function of intact skin and keep the wound sterile)
- Barrier dressings	
Prevent colonisation	
Prevent biofilm development/EPS expression via	
- Anti–deposition (anti-attachment) agents	e.g. Lactoferrin and xylitol. • Lactoferrin has been demonstrated *in vitro* to prevent the adhesion of *P. aeruginosa* to a surface [35]. • Some evidence exists that xylitol (a sugar alcohol) can inhibit attachment and biofilm growth of oral biofilm bacteria [36].
- Quorum sensing (QS) inhibitors	QS signals can be degraded (using quorum quenching enzymes) or inhibited (using QS analogues). For example, the N-acyl homoserine lactone (AHL) signalling molecule can be degraded by specific lactonases acylases [37].
- Probiotics	*Lactobacillus* has been shown to outcompete and inhibit pathogenic activity of *P. aeruginosa* and *Staphylococcus aureus* [38]
- Lytic bacteriophages	Bacteriophages (viruses which infect bacteria) have been demonstrated to be effective against biofilms through disruption of the EPS, and lysing of the biofilm-associated cells [39].
Removal of biofilms	
Debridement	
- Physical	The biofilm, along with infected and non-infected tissue can be removed in a variety of ways. • Sharp debridement uses sharp blades to remove the infected plus healthy tissue [40]. • Ultrasound (pulsed) and pulsed electrical fields can also be used to disrupt and remove biofilms, although these are not widely used [40].
- Chemical	Three types of biofilm matrix degrading enzymes can be used to inhibit biofilm formation, and induce biofilm dispersion e.g. polysaccharide-degrading enzymes (such as alpha-amylase which has been shown to reduce cell aggregation in *S. aureus* biofilms [41], nucleases (e.g. DNase I), and proteases (which can inhibit biofilms by degrading the EPS, pili, fimbriae and surface adhesins [36]).
Killing of biofilms	
- Systemic antibiotics	Use of antimicrobial agents (often after debridement) to kill any remaining bacteria that would otherwise reform a biofilm.
- Topical antiseptics	

1.3 Optimal treatment for wound/topical biofilms

Systemic antibiotics are not optimal for treating wound biofilms owing to factors mentioned previously, combined with allergy, toxicity, and the development of resistance in non-target organisms. Therefore, wound biofilm management largely relies on the use of debridement followed by the application of topically applied biocides or antimicrobial agents. Debridement aims to physically disrupt the biofilm and reduce the biomass of bacteria down to small numbers, which then results in a 2-3 day window of time where the bacterial levels are low, and antibacterial agents may be more effective against the biofilm [42].

Antimicrobial dressings (AMDs) account for a quarter of all dressings prescribed in primary care in England [43], and may contain a range of antimicrobial agents (e.g. silver, iodine, honey, and chlorhexidine). The use of AMD and silver-dressings (which are classed as 'advanced' dressings) has risen in recent years, with £25 million spent on silver dressings in 2006/7 [30]. Silver is a potent antimicrobial. Although silver-containing dressings vary in their composition, they act by a combination of i) absorbing wound exudates and killing the microorganisms drawn into the dressings, and/or ii) releasing active silver onto the wound bed. These biologically active ions then bind to negatively charged proteins, RNA, and DNA and damage bacterial cell walls, inhibit replication and reduce metabolism and growth [44].

They are marketed as effective against a broad range of bacteria (growing as biofilms), and are indicated for a variety of wounds. Since they are considered to provide an ancillary action on the wound, they are classified as medical devices [45], and hence there are lesser requirements in terms of robust data to support safety and efficacy. A Cochrane Review from 2010 looking at the use of topical silver products (dressings and creams) for wound care, concluded that there was 'insufficient evidence to support the use of silver containing dressings or creams, as generally they did not promote wound healing or prevent wound infections' [46], and furthermore there is little data available in peer-reviewed literature [47]. Topical AMDs may also be associated with toxicity, bacterial resistance, and relative high cost.

Biocides offer a possible alternative treatment and preventative measure for biofilm infections, and a number have been investigated in *in vitro* studies in this regard (including silver, (povidone) iodine, polyhexamethylene biguanide, ethylenediaminetetraacetic acid, octenidine and chlorhexidine). Generally they have broader spectrum activity and are less

selective in their actions than antibiotics [14,19], and therefore toxicity on host cells must be considered.

1.4 The clinical need for alternative topically applied antimicrobial agents

The body of work for this PhD was initiated by a Burns plastic surgeon based at the Queen Elizabeth Hospital, Birmingham (QEHB), who recognised in the course of daily clinical practice, that i) infection is a significant concern in patients who survive an initial burn insult (and accounts for over 75% of the mortality [48]), ii) burns patients are especially susceptible to infection owing to the injury removing the protective barrier provided by the skin, combined with general immunosuppression, the presence of endogenous microflora, prolonged hospital stays, and invasive diagnostic and therapeutic procedures, and iii), prevention and treatment of bacterial colonisation are key parts of burn wound care [47]. Despite the standard of care for such patients being the early excision of necrotic tissue, followed by the covering of the wound with an AMD, and administration of systemic antimicrobials (if infection is detected), there was some scepticism over the effectiveness of this strategy.

Three topical antimicrobial agents (acetic acid, SurgihoneyRO, and blue light) have been investigated (*in vitro*) against key bacteria involved in burn and chronic wound infections. Although a diverse range of genera and species can be recovered from clinical biofilms, important pathogens include Gram-positive bacteria (e.g. *S. aureus*), and nosocomial Gram-negatives (e.g. *P. aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli*), most of which are often resistant to multiple drugs.

Background information on these topical antimicrobial agents will be provided in subsequent sections.

2 AIMS & OBJECTIVES

Despite the importance of biofilms to chronic wounds, the majority of *in vitro* testing that has been performed on antibiotics and antimicrobial wound care products has focussed on bacteria existing in the planktonic phase. Owing to the prevalence and recurrence of chronic wounds, and the recognition that biofilms are harder to treat than planktonic cells, biofilm testing of the novel topical agents was deemed of high importance.

The aim of this work was firstly to evaluate the antibacterial activity of acetic acid (AA) against biofilms, following on from preliminary antimicrobial testing performed by Fraise *et al.* [49], and small-scale use of AA clinically on burns patients in QEHB. This led on to the testing of additional products (SurgihoneyRO (SH1) and blue light (BL)), for which no prior testing against biofilms had been performed.

The specific objectives of the work were as follows:

1. To test the *in vitro* antimicrobial activity of the agents against a large panel of clinically important nosocomial wound pathogens when growth is in the form of a biofilm.
2. To compare the activity of one or more of the agents to conventionally used products

This project focused on potential translation of these therapies and examination of activity, rather than examination of the mechanism of action of the agents. A mechanistic study of the three diverse classes of antimicrobial would be challenging and was considered beyond the scope of this work.

3 METHODS

A series of simple, high-throughput *in vitro* experiments were conducted with a panel of organisms (Table 1, appendix 4) to determine the activity of the three topical test antimicrobial agents (TAA) against planktonic and biofilm growth. Not all assays were performed for each TAA, as detailed in Table 2 (appendix 4).

The strains comprised well-characterised control strains and clinical isolates (including those from burn patients at QEHB), and were selected because of their relevance to infection in the burn unit setting (*P. aeruginosa* and *A. baumannii*), or their relevance as nosocomial wound pathogens (*E. coli*, *Proteus mirabilis*, *S. aureus, Klebsiella pneumoniae* and *Enterobacter cloacae*). Control strains were chosen to represent major globally relevant clonal complexes of the two species (ensuring the results are likely to be generally applied to each species as a whole), and also included control strains of *S. aureus* (NCTC 10788, NCTC 12493) and *P. aeruginosa* (NCTC 6749, ATCC 27853), since these are recognised test strains in the EN standards for assessing the efficacy of chemical disinfectants (e.g. EN 13727 [50]).

I tested diverse isolates (in preference to large numbers of related strains), and included recent isolates from burns patients to ensure no differences were seen in isolates from typical patient specimens. All *P. aeruginosa* and *A. baumannii* isolates were genotyped prior to the study through variable number tandem repeat analysis and pulsed-field gel electrophoresis, respectively by the relevant UK reference laboratories of Public Health England, to ensure that adequate levels of diversity were represented. Isolates were stored at -80°C on Protect™ beads, and were routinely cultured on cysteine lactose electrolyte deficient (CLED) agar prior to each experiment.

The *in vitro* assays were designed to test the antimicrobial activity of the TAA against planktonic bacteria in terms of the 'minimum inhibitory concentration' (MIC), and to assess whether the TAA could prevent the formation of a biofilm, and eradicate a pre-formed, mature biofilm. All assays involved the creation of an overnight bacterial culture, followed by quantification and dilution.

3.1 Preparation of Test Bacterial Inocula

Fresh overnight Lysogeny Broth (LB) [Oxoid, Reading] cultures of the organisms were made by inoculating 3-5 colonies (off an 18-24 hour agar plate) into 5mls of sterile LB and incubating overnight at 37°C with gentle agitation. The overnight cultures were then diluted in fresh antibiotic-free Iso-sensitest (ISO) broth [Oxoid] (for the MIC assay), or Muller-Hinton (MHB) broth [Oxoid] (for the biofilm assays), to an optical density (OD) at 600nm (OD_{600}) of 0.1. This will herein be referred to as the 'test bacterial inoculum' (TBI), and equates to approximately 10^5 colony-forming units per ml.

3.2 Preparation of the Test Antimicrobial Agents

3.2.1 Acetic Acid

Acetic acid supplied at 5% w/v [Tayside Pharmaceuticals, Dundee, UK], was used as a stock for all experiments, and was diluted in sterile water to the following concentrations: 2.5%, 1.25%, 0.63%, 0.5%, 0.4%, 0.31%, 0.16%, 0.1%, 0.09%, 0.08%, 0.06%, 0.05%, 0.04%, 0.03%, 0.02%, and 0.01%. This range was chosen to reflect the concentration used in clinical practice (2.5%), and those below, so that the lowest effective concentration could be identified.

3.2.2 Antimicrobial dressings (AMDs)

A range of AMDs were prepared for testing by carefully cutting the sterile AMD into a number of $1cm^2$ pieces (sufficient for 1 piece per test well) using a sterile scalpel or a pair of ethanol-dipped flame sterilised scissors.

3.2.3 SurgihoneyRO (SH1)

SH1 and the other comparator honeys were diluted in sterile, autoclaved water to produce a range of test dilutions, since it was not possible to standardise the inoculum when they were neat. The strongest dilution was made by placing 6ml of honey into a universal tube and adding 14ml of sterile, autoclaved water to make a total volume of 20mls.

NB: This was referred to in the paper as 1:3, but in hindsight this is misleading as this suggests 1 part honey to 3 parts water, when in reality it was 1 part honey to approximately 2 parts water. For clarity, this dilution should have been referred to as 1 in 3,

or 1:2 relative to the honey component. I will use 1:2 from now on. Table 3 (Appendix 4) shows the ratios as published, and the revised ratios used in this thesis.

The strongest dilution (1:2) was then serially double diluted down in water until 1:4096 was reached. Dilutions were made just prior to assay set up, to avoid any premature release of antimicrobial products from the honey.

3.2.4 Blue light

No preparation was needed for the blue light (BL) TAA. High-intensity 400nm BL was provided by a light-emitting diode (LED) flood array [Henkel-Loctite, Hemel Hempstead, UK] which gave a reproducible irradiance of 60mW/cm^2 when the LED array was positioned 15.5cm above the test area. The emission spectrum of the array was determined using a USB2000 spectrophotometer (Ocean Optics, Oxford, United Kingdom), and calibration was performed using a PM100D radiant power meter (Thorlabs, Newton, NJ).

3.3 Planktonic MIC Assay

Susceptibility of planktonic bacteria to the TAA was assessed by placing 50µl of the prepared TAA into wells of a 96-well microtiter tray (MTT), alongside 50µl of the TBI, and a further 50µl of ISO. Controls were included per assay, and comprised 50µl of the TBI alongside 100µl of ISO (for the positive control). The negative control comprised 150µl ISO alone (Figure 3). In line with the ESCMID-EUCAST guidelines of for the determination of MICs using a broth microdilution method [51], MTTs were incubated for 18-20 hours statically at 37°C, after which, the results were manually read. The MIC was recorded as the lowest concentration of the TAA for which growth was prevented.

3.4 Biofilm 'MBIC' assay: preventing formation

The impact of the TAAs on biofilm formation was assessed using a crystal violet (CV) biofilm formation assay, as described by Baugh *et al.* [52], with the endpoint measurement being the 'minimum biofilm inhibitory concentration' (MBIC). Wells of the MTT were loaded with 100µl of the TBI, alongside either 100µl of TAA (for the test wells), or 100µl of sterile MHB (for the positive control wells), to a total volume of 200µl. Negative controls were included,

comprising of 200µl MHB only. MTTs were sealed and incubated statically for 72 hours at 33°C (the average temperature of the surface of a wound [53]) (Figure 4).

3.4.1 MBIC CV assay

After 72 hours, the liquid was removed from the wells, and the MTTs rinsed in tap water to remove any unbound cells. Any existing biofilms were then visualised through staining with 200µl of 1% CV [Sigma Aldrich, Poole], further rinsed (as above) to remove unbound CV, and dye solubilised by the addition of 200µl of 70% ethanol. The OD_{600} of the solubilised CV solution was then measured with a FLUOstar Optima [BMG Labtech] to assess the biomass of the biofilms.

The positive and negative controls for each test MTT were examined and if within a normal range, the rest of the data were analysed for statistical significance by comparing values for the TAA treated wells to the untreated (control) wells, using the Student's 't' test.

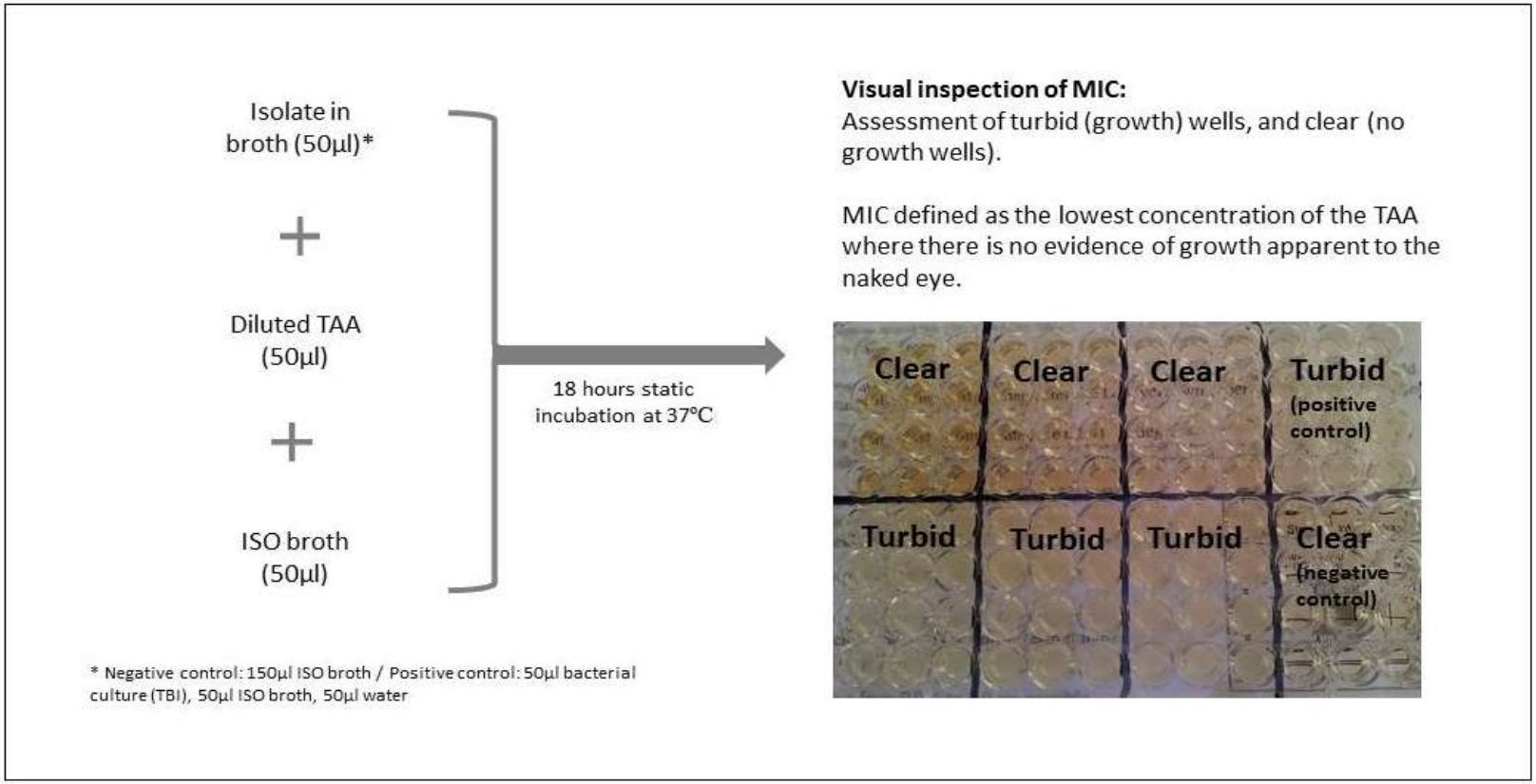

Figure 3: Flow diagram showing the stages involved in the minimum inhibitory concentration (MIC) assay

50µl each of bacterial inoculum (isolate in broth), diluted TAA, and ISO broth were added to wells of a 96 well MTT, alongside a negative, and a positive control (for composition, see * in figure). The MIC was manually read after 18 hours of incubation. [Diagram and photo candidate's own].

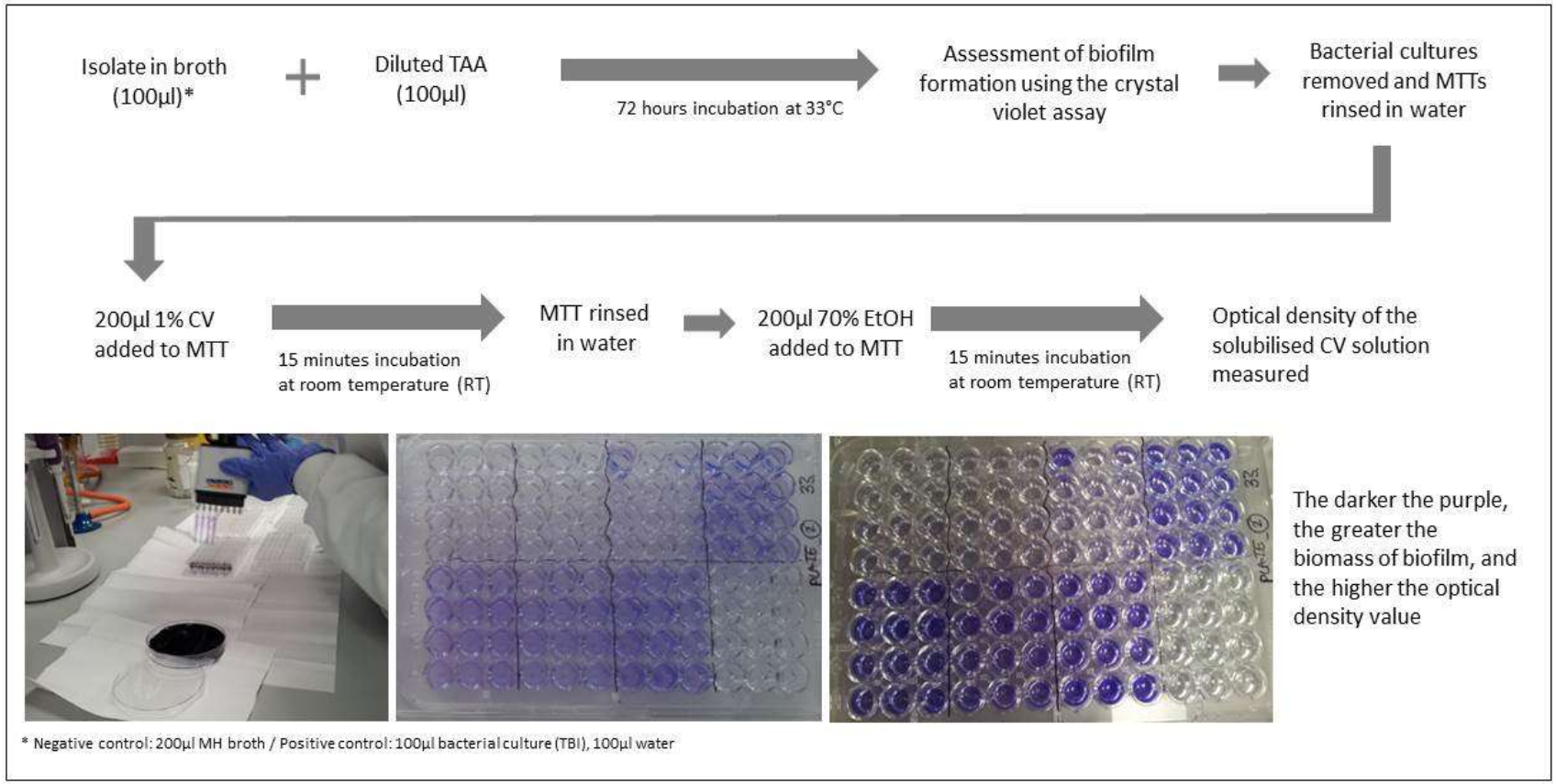

Figure 4: Flow diagram showing the stages involved in the minimum biofilm inhibition concentration (MBIC) assay

100µl of bacterial inoculum (isolate in broth), was added to an equal volume of the diluted TAA in a 96 well MTT, alongside a negative, and a positive control (for composition, see * in figure). Assessment of biofilm formation was performed using a crystal violet assay, resulting in an ethanol-solubilised crystal violet solution that was measured in terms of optical density [Diagram and photo candidate's own].

3.5 Biofilm 'MBEC' assay: eradication of pre-formed biofilms

The antibacterial activity of the TAAs against pre-formed biofilms was assessed by conducting 'minimum biofilm eradication concentration' (MBEC) experiments per isolate, as per Ceri *et al.* [54]. To produce a 'transferable' mature biofilm, 200µl of the TBI were loaded into wells of a MTT, and a 96 well polypropylene plate [Starlabs, UK] placed into the MTT, so that each well contained a 'peg' on which biofilms could form. Once positive and negative control wells were included, the assembly was sealed with cling film, and statically incubated at 33°C (the average temperature of the surface of a wound [53]) for 72 hours (Figure 5).

After this time, the pegs were washed in a MTT containing sterile water (to remove any unbound cells), and the peg plate then placed either into a further MTT containing the TAA (AA or SH1) at a range of concentrations, or underneath the BL LED light array. The exposure time differed per TAA (and was arbitrarily chosen), with pegs exposed to AA for 3 hours, SH1 for 24 hours, and BL for 1 hour maximum (15 minute intervals).

To assess the viability of the exposed peg biofilm, the peg plate was removed from the TAA, washed as before and placed into a MTT containing 200µl sterile 'reporter' broth (MHB) for overnight incubation. After 18 hours, the OD of the reporter broth was measured to assess the viability (seeding) of the biofilms following the exposure to the TAA, and to determine the minimum concentration which prevented any seeding of the reporter broth (and by inference had killed some of the cells in the biofilm).

3.5.1 MBEC CV assay

To demonstrate the presence of biofilms on the pegs, CV assays were performed on the pegs after the OD of the reporter broth had been measured. This involved placing the pegs into MTTs containing 200µl of 1% CV, followed by washing and subsequent solubilisation in 200µl of 70% ethanol.

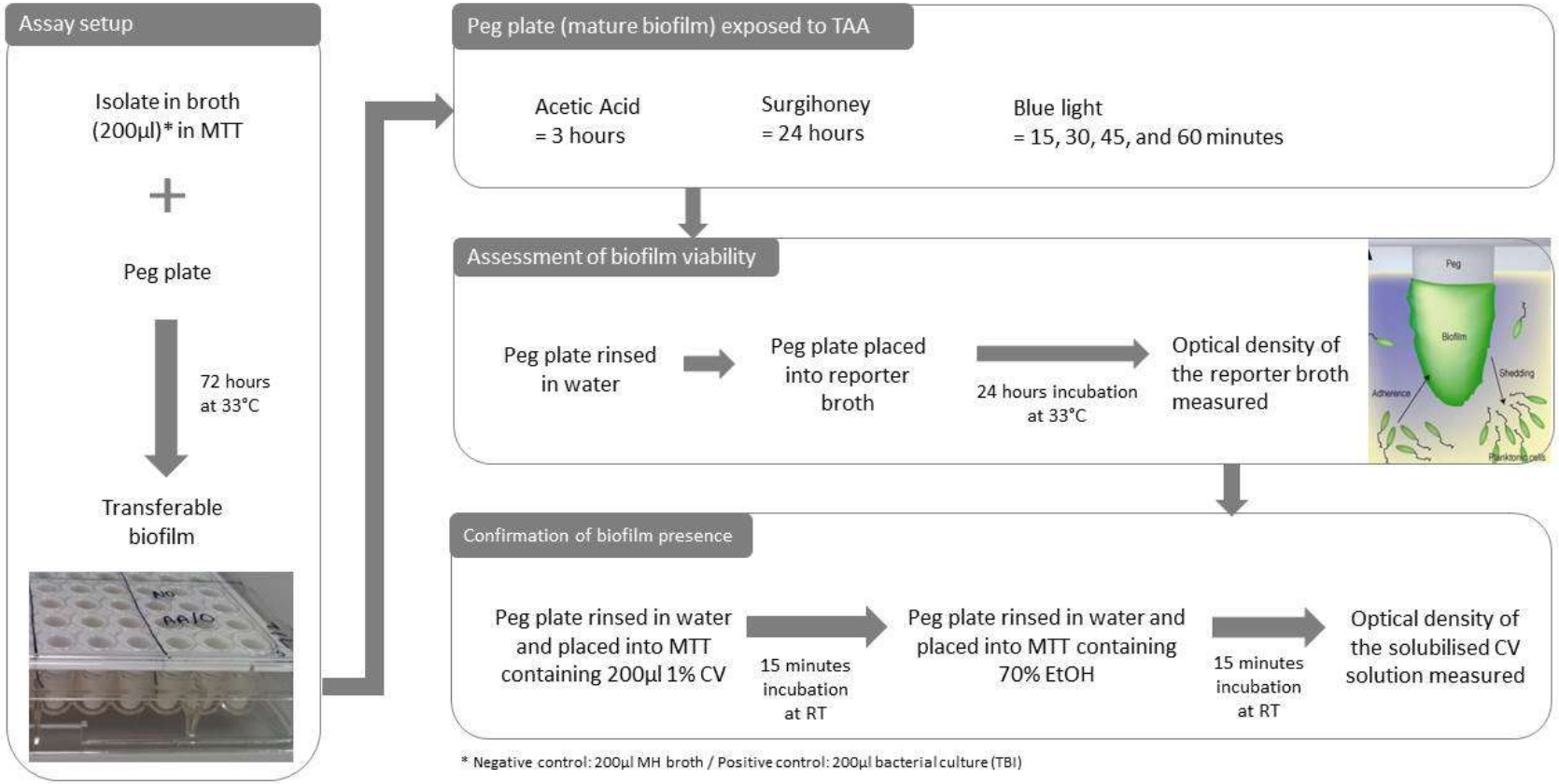

Figure 5: Flow diagram showing the stages involved in the minimum biofilm eradication concentration (MBEC) assay

200µl of bacterial inoculum (isolate in broth), was added to wells of a 96 well MTT, alongside a negative, and a positive control (for composition, see * in figure). A peg plate was then added and incubated for 72 hours at 33°C so that 'transferable' biofilms were formed. Biofilms were then exposed to the TAA, and the viability (seeding) of the exposed biofilms was assessed. As a final stage, a crystal violet assay was performed to determine whether biofilms had been present on the exposed test pegs. [Diagram and photo: candidate's own].

3.6 Polymicrobial biofilm assays

To investigate the antimicrobial activity of the TAAs on polymicrobial biofilms, a small number of MBEC experiments were performed using AA, and two Gram-positive (*S. aureus* and *Enterococcus faecium*), and two Gram-negative (*P. aeruginosa* and *A. baumannii*) isolates. These bacterial species were selected owing to their easily-distinguishable colony morphologies, which made it possible to identify which bacteria were present by visual inspection, and without the need for any identification tests.

To make a polymicrobial biofilm, I initially added equal volumes (50µl) of both the Gram-positive and Gram-negative bacteria to the MTT (with 96 well polypropylene plate inserted), at the same time, at the start of the experiment. The composition of the biofilm was assessed (through plating on solid agar) after 72 hours, and demonstrated that the Gram-negative bacteria had completely outcompeted the Gram-positive.

Following a literature search, a new protocol was devised. This loosely followed that of Guggenheim *et al.* [48], and involved growing pure and mixed Gram-positive biofilms on peg plates for 41.5 hours, before adding in the Gram-negative bacteria and incubating for a further 23 hours, so that the peg plates contained polymicrobial biofilms. The mature biofilms (64.5 hours old) were then exposed to AA for 3 hours, before the viability of the exposed biofilm was assessed (as per 3.5).

To confirm the existence of a polymicrobial biofilm, serial dilutions of the seeded reporter broth were plated out onto standard blood agar and colonies inspected.

3.7 Data analysis

All experiments were repeated on separate occasions and at least two biological and three technical replicates were used (per concentration) to ensure reproducible data sets were obtained. Data analysis was performed as detailed in the individual published papers. In general the positive and negative controls were examined and if within a normal range (according to OD readings), the rest of the data were analysed for statistical significance by comparing values at each concentration of TAA to untreated (positive) controls using the Student's 't' test.

4 SUMMARY OF THE PUBLISHED WORKS

4.1 Investigation of the antibacterial activity of acetic acid (paper 1)

This paper investigated the antibacterial activity of acetic acid (AA), against 29 clinical and control bacterial isolates (growing both planktonically and in biofilms) of relevance to the nosocomial setting (e.g. the QEHB Burns Unit). This was prompted by initial observations on the antibacterial planktonic activity of AA as reported by Fraise *et al.* [49], and through a QEHB Burns plastic surgeon who had observed AA (of concentration 2.5% w/v), to be well-tolerated, and have good clinical outcomes when applied topically within dressings to patients with heavily colonised wounds. This work was further justified by the paucity of literature surrounding the antibacterial activity of AA.

AA, or vinegar, has been used sporadically in medicine for 6000 years [55,56], with evidence of successful elimination of *P. aeruginosa* from war wounds [57], and treatment of the plague, and ear, chest, and urinary tract infections [58–60]. Historically, several small-scale studies have been performed which have shown effectiveness of AA against wound infection [55,56,61,62]. Sloss *et al.* [61] recruited 16 patients with *P. aeruginosa*-infected burns or ulcers, and treated them with sterile gauze soaked in 1-5% AA, applied for 15 minutes twice daily, for 14 days. Over the study period, swabs were taken to assess the elimination of organisms from the wounds, and the MIC of AA needed to inhibit the growth of each isolate. Of 16 patients, *P. aeruginosa* was eliminated from ten within seven days and from five more within 14 days.

Ryssel *et al.* [55] assessed the activity of 3% AA against a range of Gram-negative and Gram-positive bacterial strains isolated from patients in their Burns Unit. Overnight cultures of the organisms were exposed to 3% AA for 5, 30, and 60 minutes at 37°C before being diluted and covered with agar. After 48 hours incubation, the numbers of colony-forming units were counted, and analysis revealed good activity of AA, with the majority of the organisms (*Proteus vulgaris*, *P. aeruginosa*, *A. baumannii*, ß-haemolytic *Streptococci* A and B, *S. epidermidis*, *S. aureus*, and *Enterococcus faecalis*) eradicated after just 30 minutes of exposure. Furthermore Madhusudhan [63] tested the efficacy of 1% AA for the treatment of chronic wounds infected with *P. aeruginosa*. Thirty two patients were enrolled and randomised to receive twice daily dressing changes with AA or saline. *P. aeruginosa* was eliminated seven days earlier with AA treatment compared to saline.

A recent paper by Bjarnsholt *et al.* [64] tested the *in vitro* ability of 0.5% or 1% AA to eradicate pre-existing biofilms of *P. aeruginosa* or *S. aureus* during 24 hour exposures. They found that *P. aeruginosa* biofilms were completely eradicated by 0.5% AA, but *S. aureus* required the higher dose of 1% AA for complete eradication.

Although these clinical studies provide evidence in support of the clinical utility of AA, the small sample sizes, heterogenous nature of the studies, and majority focus on planktonic growth, make it difficult to draw conclusions. The current study was undertaken to further investigate the *in vitro* antibacterial activity of AA against important burn wound colonising organisms growing planktonically and as sessile biofilms.

Experiments measured the MIC, MBIC and MBEC of AA (at concentrations from 5% to 0.01%), and the results were highly reproducible. AA was antibacterial against planktonic growth, with an MIC of 0.16% (9 isolates) or 0.31% (20 isolates). The difference in MIC for different strains of the same species was not considered significant, and was not linked to any differences in antibiogram.

In terms of biofilms, AA was able to prevent formation of biofilms (at all concentrations from 5% down to 0.31%) for the 23 isolates tested (6 isolates were omitted due to poor or unreliable biofilm formation in the control wells). Five organisms had MBICs of <0.10%, 10 of 0.16%, and the remaining eight had MBICs of 0.31% (Table 2). There was no observable pattern between MBICs and species of bacteria, with isolates of *P. aeruginosa* with a variety of MBICs. Reduced seeding of mature biofilms was observed for all 22 isolates (seven isolates were not tested as they were unreliable biofilm producers) after 3 hours of exposure. The MBECs of AA ranged from ≤0.10% to 2.5%, and for 11 isolates, there was statistically significant reduction in seeding at all dilutions of AA.

Example graphs are shown for the MBIC and MBEC results in Figures 6 and 7, respectively.

This work has revealed that AA is antibacterial against a wide range of bacterial species, and is active at a far lower concentration than currently used at the hospital, and those previously reported in the clinical trials. This adds a lot of information to the AA story, since such comprehensive testing has not been done before, and previous authors were using far higher concentrations of AA (e.g. 2-5%). Based on these data, I feel these high concentrations are unnecessary and could prove to be uncomfortable to patients. Owing to current concerns of the reducing efficacy of systemic antibiotics, AA therefore offers great promise as a cheap and effective measure to treat infections in burns patients.

Table 2: showing the results for the tests performed on the 29 isolates in terms of minimum inhibitory concentration (MIC), minimum biofilm inhibition concentration (MBIC) and minimum biofilm eradication concentration (MBEC) of acetic acid (AA).

Study Identifier	Organism	Tests performed[a] (inhibitory % of AA)								
		MIC		MBIC			MBEC			
		(0.16)	(0.31)	(≤0.1)	(0.16)	(0.31)	(≤0.1)	(0.16–0.5)	(1.25)	(2.5)
PS_PA01	*P. aeruginosa*	✓				✓	✓ [b]			
PS_6749	*P. aeruginosa*		✓		✓		✓ [b]			
PS_27853	*P. aeruginosa*		✓			✓	**Not performed**			
PS_919	*P. aeruginosa*	✓		✓					✓ [b]	
PS_927	*P. aeruginosa*	✓				✓	✓			
PS_1054	*P. aeruginosa*	✓		✓				✓ [b]		
PS_1586	*P. aeruginosa*		✓			✓		✓ [b]		
PS_1587	*P. aeruginosa*		✓			✓			✓ [b]	
AB_19606	*A. baumannii*	✓		✓					✓	
AB_17978	*A. baumannii*		✓	**Unreliable biofilm production**			**Not performed**			
AB_1a	*A. baumannii*		✓		✓			✓ [b]		
AB_53	*A. baumannii*		✓	✓			✓ [b]			
AB_AYE	*A. baumannii*	✓			✓			✓		
AB_C58	*A. baumannii*		✓	**Poor biofilm formed**			**Not performed**			
AB_C59	*A. baumannii*	✓			✓				✓ [b]	
AB_C60	*A. baumannii*	✓			✓				✓ [b]	
EC_073	*E. coli*		✓		✓			✓ [b]		
EC_042	*E. coli*		✓		✓					✓
PM_421	*P. mirabilis*		✓			✓			✓	
MSSA_10788	*S. aureus*		✓			✓			✓	
MRSA_12493	*S. aureus*		✓	**Poor biofilm formed**			**Not performed**			
MRSA_F475	*S. aureus*	✓		**Poor biofilm formed**			**Not performed**			
MRSA_F473	*S. aureus*		✓	**Poor biofilm formed**			**Not performed**			
MDR_A	CPE *K. pneumoniae*		✓		✓			✓		
MDR_B	CRE *K. pneumoniae*		✓			✓		✓		
MDR_C	ESBL *E. coli*		✓	✓					✓	
MDR_D	VIM *P. aeruginosa*		✓		✓			✓		
MDR_E	CRE *E. cloacae*		✓	**Unreliable biofilm production**			**Not performed**			
MDR_F	CRE *E. cloacae*		✓		✓				✓	
		9	20	5	10	8	4	8	9	1

[a] ✓ denotes that the test was performed and the result.
[b] denotes that there was statistically significant reduction in seeding at all concentrations of AA (t-test, $p<0.05$)

The numbers in brackets refer to the inhibitory % of AA (in terms of weight/volume), and the tick symbol denotes that the test was performed and the result. All results derive from 12 technical replicates per isolate and per dilution of AA. The superscript b denotes that there was statistically significant reductions in seeding at all concentrations of AA (according to a Student's t-test with n-1 degrees of freedom)

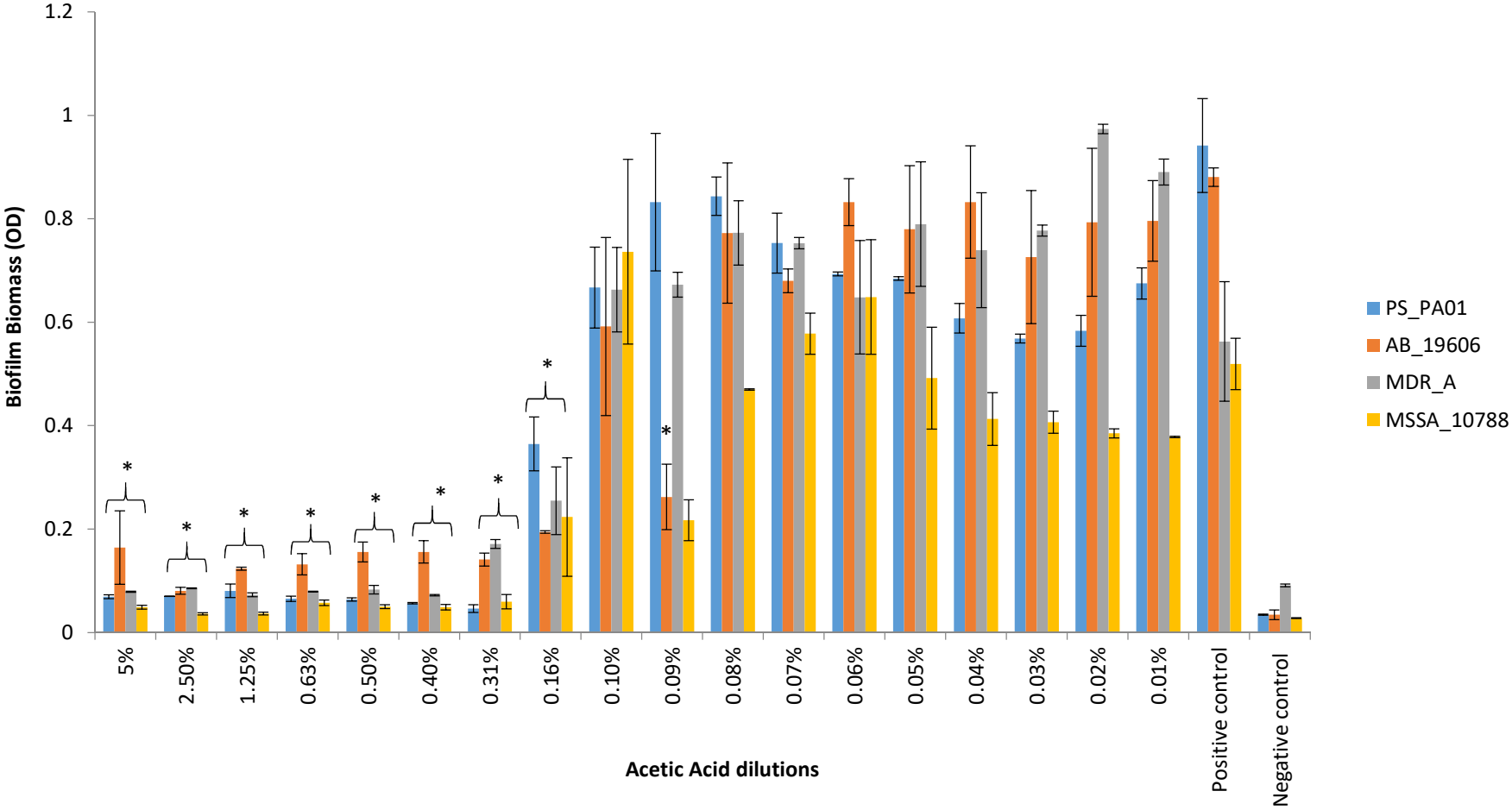

Figure 6: Graph showing the mean average biomass of the biofilms produced by isolates of *P. aeruginosa* (PS_PA01), *A. baumannii* (AB_19606), *K. pneumoniae* (MDR_A), and *S.* aureus (MSSA_10788) when planktonic cells were incubated with a range of dilutions of acetic acid for 72 hours. Optical density (OD) on the y axis refers to the average biofilm biomass for the isolates when tested against the range of AA dilutions shown on the x axis. Each data bar represents the mean average biofilm biomass from 12 technical replicates. Standard error bars have been included, alongside asterisks (*) which denote statistically significant reductions in biofilm biomass according to the Student's t–test with n-1 degrees of freedom, and a significance threshold of 0.05. The positive control represents the average biomass of biofilm produced in the absence of acetic acid. The negative control (composed of broth only) represents the background OD levels for the experiment.

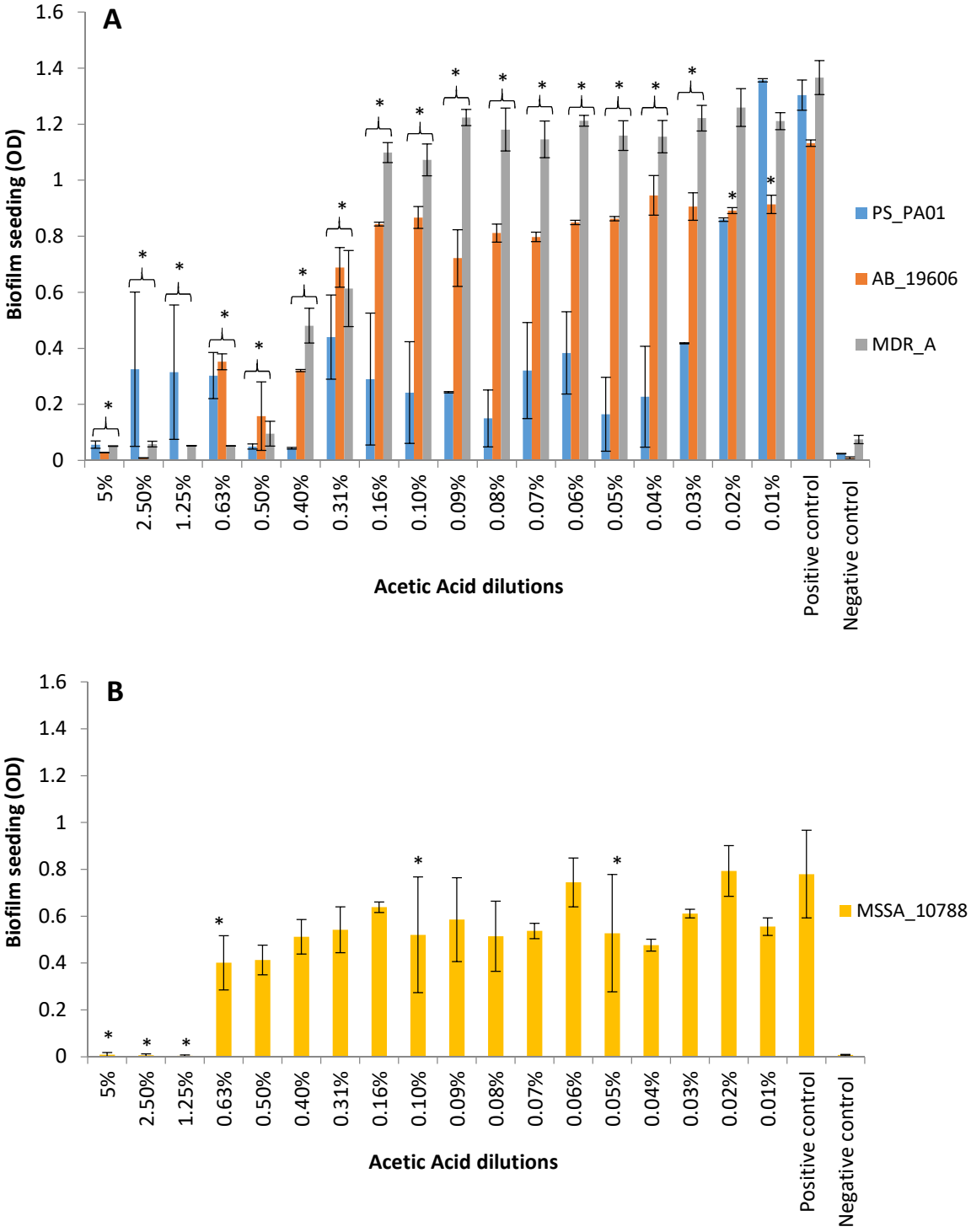

Figure 7: Graph showing the mean average biofilm seeding results when 72 hour old biofilms produced by PS_PA01, AB_19606, MDR_A (A) and MSSA_10788 (B) were exposed to dilutions of acetic acid (AA) for 3 hours. Biofilm seeding is shown on the y axis for the isolates at the range of AA dilutions tested (x axis). Each data bar represents the mean average biofilm seeding from 12 technical replicates. Standard error bars have been included, alongside asterisks (*) which denote statistically significant reductions in biofilm seeding (compared to the positive control) according to the Student's t–test with n-1 degrees of freedom, and a significance threshold of 0.05. OD: Optical density, PS_PA01: *Pseudomonas aeruginosa*, AB_19606: *Acinetobacter baumannii*, MDR_A: *Klebsiella pneumoniae*, MSSA_10788: *Staphylococcus aureus*.

4.2 Comparison of the ability of a panel of antimicrobial dressings (AMDs) to prevent biofilm formation (paper 2)

The NHS spent £110 million (in 2014-2015) on advanced wound dressings and antimicrobial dressings (AMDs) [65]. Although AMDs may contain a range of different antimicrobial agents (e.g. silver, iodine, honey, and chlorhexidine), the majority prescribed in England contain silver. Typically the antimicrobial agent is contained within a commercially marketed wound dressing, which may be used prophylactically (to prevent colonisation of the wound and subsequent biofilm formation), and in the treatment of established infection [66]. This paper compared the ability of a range of 11 AMDs to prevent biofilm formation of four important burn wound pathogens. It was prompted from the work conducted on AA, since it was necessary to demonstrate (as part of an undergraduate placement-year project), how AA compared in terms of antimicrobial activity, to the standard of care (SOC) treatments used in the Burns Unit at QEHB.

AMDs are used heavily for burns patients, where infection is a large risk, where prevention and treatment of bacterial colonisation are key parts of wound care, and where systemic antimicrobials are not thought to be effective [26,30,67]. They are marketed as broad-spectrum antimicrobials; effective against both Gram-positive and Gram-negative bacteria [68] growing as biofilms, as well as viruses [69], and fungi [70]. However, provided that the antimicrobial agent only provides an ancillary action on the wound, the majority of AMDs are classified as medical devices [45]. This means that there are lesser requirements for robust data (e.g. from randomised controlled trials (RCTs)), and literature reviews and commercial company-led research are often deemed acceptable for licensing. Consequently, there is little data available in peer-reviewed literature concerning their activity. Opinions on silver dressings are divided in clinical practice, with some clinicians believing them to have a role in preventing infection in burns patients [71–73], and others not endorsing their use owing to a lack of evidence of effectiveness [30,74], and a non-supportive Cochrane Review [75].

I sought to assess and compare the antibiofilm properties of AA versus a representative selection of the AMDs currently used in the Burns Unit at QEHB, with the aim to help guide clinical practice at this centre and others.

Four organisms were tested (two *A. baumannii* and two *P. aeruginosa*) against 11 AMDs and two non-antimicrobial dressings (nAMDs). Here the MBIC assay was performed, with

slight modifications; 24 well MTTs were used, and were filled with a total of 2ml of liquid (1ml TBI plus 1ml AA (as comparator), or 1ml water (AMD test wells)). Additionally, the AMD test wells contained 1cm^2 pieces of the 11 AMDs and two nAMDs (dressing volumes were not considered). The MTTs were sealed, and statically incubated at 33°C for 72 hours. Experiments were performed using at least two biological replicates, and at least four technical replicates of each isolate.

The CV assay was performed (scaling up to 2mls), and the data analysed in terms of the percentage change in biofilm biomass between the test wells (containing the TAA), and the (untreated) positive control wells. The results are shown for the *A. baumannii* isolates (Table 3 and Figure 8). The Student's t-test was used to compare the percentage changes, and adjustments for multiple comparisons were made (to control for the family-wise error rate) using Holm's method [76] to obtain a p-value of statistical significance.

This study was not designed to demonstrate shortcomings in the performance of the AMDs. However, the data indicates that (in this experiment and in the *in vitro* setting) there is a large variation between the AMDs in terms of reducing/preventing biofilm formation. This is apparent between dressings containing the same antimicrobial agent (e.g. silver), and those containing different AM agents. For the seven silver AMDs, Mepilex®Ag, and Acticoat were highly effective, leading to 90-100% reductions in biofilm formation, compared to the Aquacel® dressings, where the reductions were modest, averaging 44% for PS_PA01, and 34% for PS_1586. The four non-silver AMDs gave varied results (Table 3), and in some cases were ineffective at preventing biofilm formation (e.g. biofilm formation of PS_PA01 and PS_1586 was increased compared to the positive control by 115 and 200% with L-Mesitran® Hydro, respectively). In contrast, the AA results were consistent, showing reductions of >90% for all concentrations of AA and for all isolates and replicates.

This work demonstrates that the antibacterial activity of AA compares favourably to the best-performing AMDs, and that clinicians should be wary using of AMDs (if intended to prevent or treat infections) in the absence of robust data showing anti-biofilm efficacy.

Table 3: Average percentage (%) change in biofilm biomass for each of the *A. baumannii* isolates (ACI_AYE and ACI_721) when planktonic cells were coincubated with each of the antimicrobial dressings (AMDs), non-antimicrobial dressings (nAMDs), or acetic acid (AA) for 72 hours, when compared to an untreated (positive) control.

	Dressing/agent	*A. baumannii* ACI_AYE			*A. baumannii* ACI_721		
		Percentage change	Number of replicates	Adjusted p-value	Percentage change	Number of replicates	Adjusted p-value
AMD (silver)	Mepilex® Ag	-95.9	8	0.001	-100	6	<0.001
	Aquacel® Ag	-39	8	1.000	-74	6	0.006
	Aquacel® Ag Foam	-66	8	0.295	-74	6	0.010
	Aquacel® Ag Burn	-94	6	0.031	-82	4	0.032
	UrgoTul® Silver	-20	10	1.000	-4	8	1.000
	Acticoat	-96	8	0.002	-100	6	<0.001
	PolyMem Silver®	-61	8	0.090	-75	6	0.001
AMD (iodine)	Inadine®	+3	8	1.000	-6	6	1.000
AMD (honey)	L-Mesitran® Net	-10.4	6	1.000	+33	4	0.385
	L-Mesitran® Hydro	-1	8	1.000	-62	6	0.038
AMD (chlorhexidine)	Bactigras	-59	8	0.148	-68	6	0.038
nAMD	UrgoTul® plain	-7	6	1.000	-27	4	0.405
	PolyMem® plain	-66	6	0.054	-74	4	0.003
AA	Acetic acid 5%	-92	10	<0.001	-90	8	<0.001
	Acetic acid 2.5%	-93	10	0.001	-92	8	<0.001
	Acetic acid 1.25%	-93	10	<0.001	-93	8	<0.001
	Acetic acid 0.63%	-93	10	<0.001	-96	8	<0.001
	Acetic acid 0.31%	-93	10	<0.001	-96	8	<0.001
	Acetic acid 0.16%	-90	10	0.001	-94	8	<0.001
	Acetic acid 0.08%	-28	10	1.000	-95	8	<0.001
	Acetic acid 0.04%	+10	10	1.000	+5	8	1.000
	Acetic acid 0.02%	+6	10	1.000	+13	8	0.787
	Acetic acid 0.01%	-30	4	1.000	+7	4	1.000

The number of replicates, and the p-values from the Student's t-test (performed with n-1 degrees of freedom and adjusted for multiple comparisons using Holm's method) are also shown. Negative values (denoted by -) in the 'percentage change' column denote reductions in biofilm biomass compared to the untreated positive control. Positive values (denoted by +) denote increases in biofilm biomass compared to the untreated positive control.

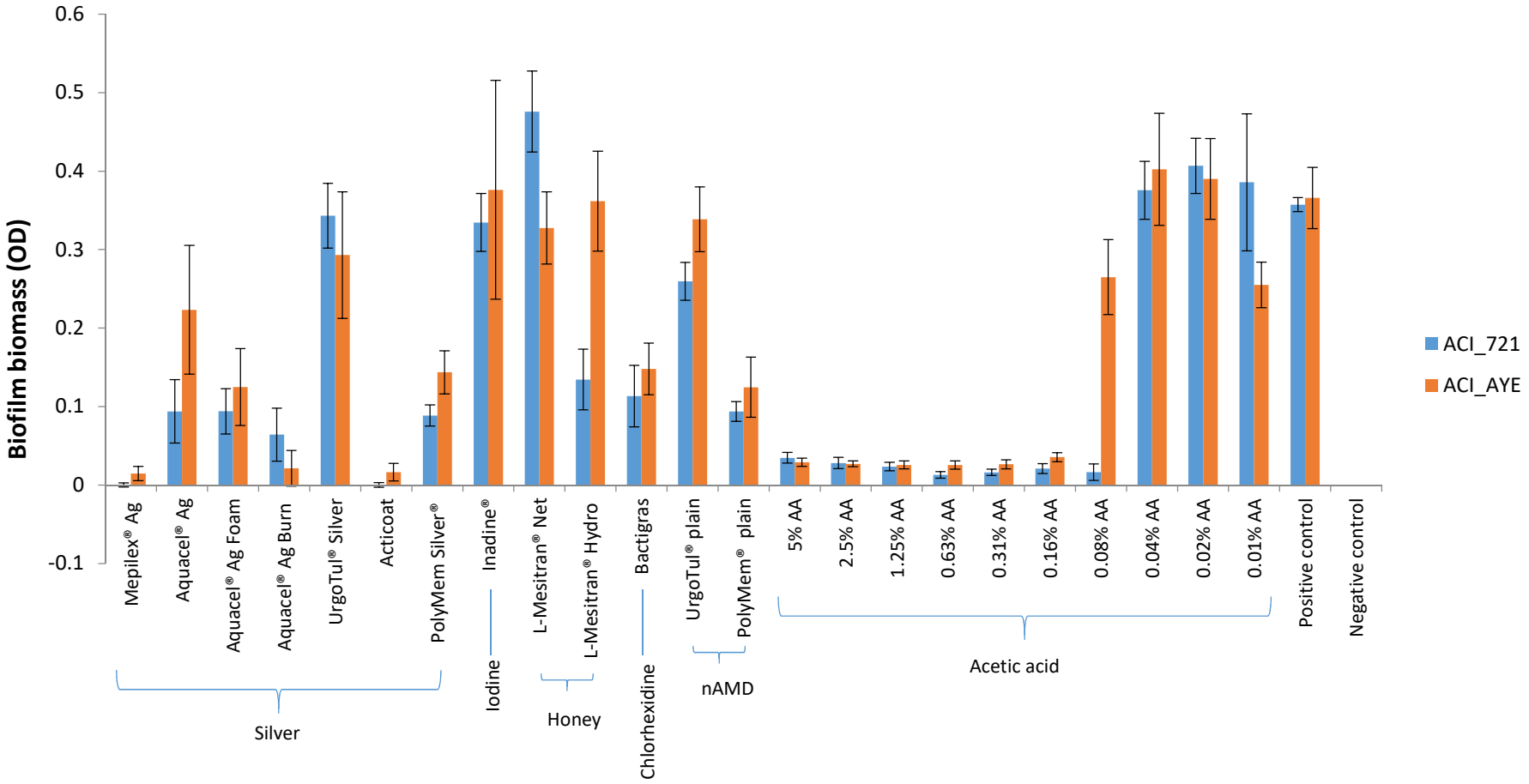

Figure 8: Graph showing the mean average biomass of the biofilms produced by the two *A. baumannii* isolates that were produced when planktonic cells were incubated a range of antimicrobial dressings, non-antimicrobial dressings, or acetic acid for 72 hours.

Optical density (OD) on the y axis refers to the average biofilm biomass for the *A.baumannii* isolates when tested against the range of agents shown on the x axis. Each data bar represents the mean average biofilm biomass from 4-10 technical replicates (see Table 3), and standard error bars have been included. The positive control represents the average biomass of biofilm produced in the absence of any of the test agent. The negative control (which has been subtracted) provides a baseline OD value for the assay in the absence of bacteria or test agents.

4.3 Investigation of the antibacterial activity of an engineered honey, medical-grade honeys, and antimicrobial wound dressings (paper 3)

SH1 is a newly licensed sterile product which has been developed for wound care, and as a prophylactic dressing agent (Figures 9A & B). It has been engineered so that it produces consistently high levels of reactive oxygen species (ROS), and consequently is thought to have enhanced antimicrobial activity compared to other medical honeys. This *in vitro* study was performed to assess whether SH1 has antibacterial activity against biofilms, in terms of preventing formation. Direct comparisons were also made to competitor honeys and honey and silver-based dressings.

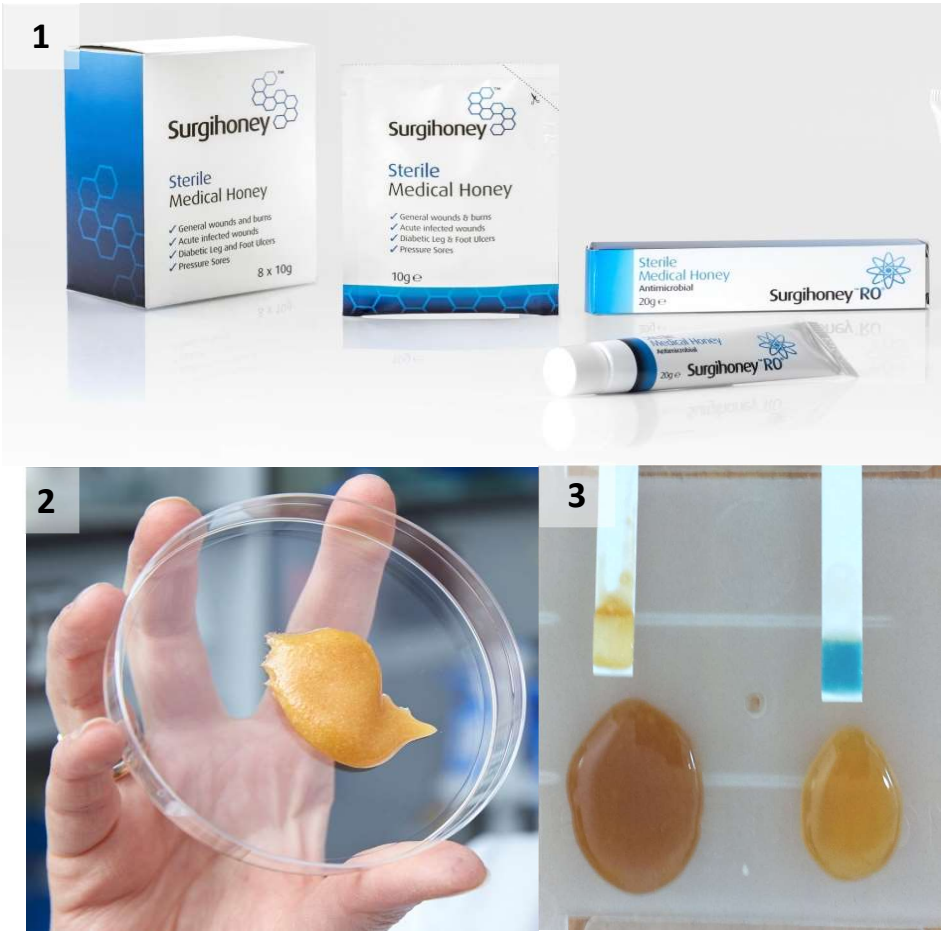

Figure 9A: Composite figure: SurgihoneyRO. 1: SurgihoneyRO sachets (as used in the experiments) and other formulations, **2:** Neat SurgihoneyRO, **3:** H_2O_2 negative reaction of MH (left) and positive reaction of SH1 (right) (all self-taken).

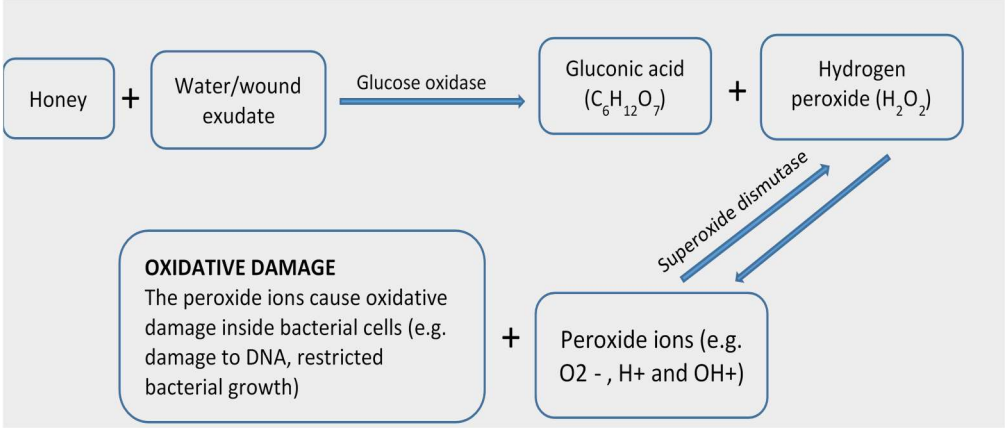

Figure 9B: SurgihoneyRO: proposed mechanism of action of SurgihoneyRO (self-drawn)

Honey has a long history as a wound care agent. In addition to wound healing properties [77], honey has broad spectrum antibacterial activity, with 37 genera of bacteria shown to be susceptible [78–80], and a multifactorial mechanism of action. The antimicrobial components of the honey are thought to be the preparation itself (e.g. the physical properties including pH and hyperosmolarity), and innate antibacterial compounds including methylglyoxal, bee defensin-1, and ROS such as hydrogen peroxide (H_2O_2) [81].

A limitation of natural honeys is that the production depends on a large number of factors (e.g. floral source, species of bee, geographical location, harvesting process, and subsequent storage conditions) [81], and consequently the honey may not be of predictable and consistent quality. Medical honeys are generally standardised in preparation.

SH1 differs from medical honey, in that it has enhanced production of ROS, does not require a single floral source, and can be scaled up or down in terms of potency [82]. Pilot *in vitro* tests performed by Dryden *et al.* [82] measured the antimicrobial activity against 48 Gram-positive and Gram-negative bacterial isolates using an agar diffusion method. Zones of inhibition around the honey preparation were observed after 18-24 hours incubation, and SH1 formulations had larger zones of inhibition (and therefore higher antibacterial activity) than the other honeys tested. Clinical results (using SH1 topically) have also been favourable [83].

Sixteen bacterial isolates were tested using the MBIC assay against three medical honeys (SH1, Medihoney® (Med), and Activon® tube 100% medical grade Manuka honey (MH)), and a subset then tested against the five AMDs. Three of the AMDs were honey-containing (Actilite®, L-Mesitran® Net, and L-Mesitran® Hydro), and were chosen from a number of honey dressings used at QEHB to act as comparators. The silver dressings (Aquacel® Ag and Aquacel® Ag+) were included since they represent the most commonly used dressings in the Burns Unit.

The MBIC was performed as previously, with the MBIC defined as the lowest dilution of honey where there was both statistical significance in the t-test ($p<0.05$), and a prevention of biofilm biomass accumulation ≥50% compared to the positive control.

All honeys were antibacterial and able to prevent biofilm formation, but SH1 was the most potent, with MBIC values ranging from 1:4 to 1:128 (Table 4), and potency at lower dilutions than both of the other honeys for five isolates; and equivalent dilutions for a further six. For the remaining isolates, SH1 was either the only effective honey (PS_PA01), was one of two effective honeys, or gave discordant/concordant results compared with the other honeys. MH prevented biofilm formation for 14 of the 16 isolates, but was repeatedly ineffective for two isolates, with no statistically significant reduction in biofilm biomass even at the strongest concentration. Similarly, Med was effective for 15 of the 16 isolates (ineffective for PS_PA01), and had MBICs ranging from 1:2 to 1:1024. Sample data is shown in figure 10.

Four of the *P. aeruginosa* isolates were additionally tested against SH1 (chosen owing to the higher potency[1]) and the AMDs. Percentage change in biofilm biomass was calculated for all the results (since MBIC values were not possible for the AMDs) (Table 5). There was a large variation in the ability of the test agents to prevent biofilm formation (Figure 11). SH1 (at a dilution of 1:2) was effective for all isolates, leading to significant reductions ranging from 79.8% (PS_1586) to 94.1% (PS_PA01). These results furthermore compared favourably with the reductions observed with the silver dressings. The performance of the honey-containing dressings was disappointing, with increases in biofilm biomass seen with all dressings for three of the isolates, and an increase of 62.3% seen with Actilite® and PS_PA01.

[1] And since it was not logistically possible to test all honeys

This study has demonstrated that the medical honeys tested were effective at reducing biofilm formation in this *in vitro* model, and that SH1 was superior in potency. Additionally, it has shown that a number of honey dressings currently marketed as being antimicrobial, have poor levels of antimicrobial activity *in vitro.* These findings have furthermore informed a translational clinical trial being undertaken at QEHB into the use of SH1 for the treatment of chronic, non-healing ulcers.

Table 4: Showing the minimum biofilm inhibition concentration (MBIC) data for each of three honeys (SH1, MH and Med) and for the 16 isolates included in the study

Isolate	SH1			MH			Med		
	MBIC	n	p-value	MBIC	N	p-value	MBIC	n	p-value
PS_PA01	**1:4**	6	<0.001	**>1:2**	n/a	n/a	**None**	n/a	n/a
PS_1054	**1:8**	6	<0.001	**1:8 ***	6	<0.001	**1:8 ***	6	<0.001
PS_1586	**1:4**	6	<0.001	**1:2 ¤**	6	0.02	**1:8 ^**	6	0.019
PS_6749	**1:8**	6	<0.001	**1:8 ***	6	<0.001	**1:1024^**	6	0.009
ACI_AYE	**1:8**	6	<0.001	**1:8 ***	6	<0.001	**1:2 ¤**	6	<0.001
ACI_C59	**1:8**	6	<0.001	**1:2 ¤**	6	<0.001	**1:2 ¤**	6	<0.001
ACI_C60	**1:8**	6	<0.001	**1:2 ¤**	6	<0.001	**1:2 ¤**	6	<0.001
ACI_19606	**1:8**	6	<0.001	**1:4 ¤**	6	<0.001	**1:4 ¤**	6	<0.001
MDR_B	**1:32**	6	<0.001	**1:4 ¤**	6	<0.001	**1:16 ¤**	6	<0.001
MDR_C	**1:128**	6	<0.001	**1:128 ***	6	<0.001	**1:128 ***	6	<0.001
MDR_D	**1:16**	6	<0.001	**1:2 ¤**	6	0.023	**1:2 ¤**	6	0.043
EC_042	**1:64**	6	<0.001	**1:64 ***	6	<0.001	**1:64 ***	6	<0.001
MRSA_F475	**1:32**	6	0.04	**None**	n/a	n/a	**1:16 ¤**	6	0.036
MSSA_10788	**1:16**	6	0.04	**1:16 ***	6	0.001	**1:16 ***	6	<0.001
MRSA_F483	**1:128**	6	<0.001	**1:128 ***	6	<0.001	**1:128 ***	6	<0.001
MSSA_F77	**1:32**	6	<0.001	**1:32 ***	6	0.006	**1:32 ***	6	0.02

Abbreviations: MBIC – Minimum biofilm inhibition concentration, SH1 – SurgihoneyRO, MH – Manuka Honey, Med – Medihoney.
Symbols: * MH or Med MBIC equivalent to the SH1 MBIC, ¤ MH or Med MBIC is a stronger dilution of honey than SH1, ^ MBIC for MH or Med is a weaker dilution of honey than SH1.
The sample size (n), and the p-values from the Student's t-test (performed with n-1 degrees of freedom) are also shown.

Table 5: Table showing the average percentage (%) change in biofilm biomass for each of the *Pseudomonas aeruginosa* isolates when coincubated with SH1 and each of the antimicrobial dressings for 72 hours, when compared to an untreated, positive control.

Dressing/agent	*P. aeruginosa* PS_PA01			*P. aeruginosa* PS_1054		
	% change	n	T-test p-value	% change	n	T-test p-value
Aquacel® Ag	-38.4	3	0.33	-94.1	2	0.001
Aquacel® Ag + Extra	**-95.3**	**4**	**0.007**	-86.8	4	<0.001
Actilite	+62.3	4	0.063	+87.2	4	0.048
L-Mesitran® Net	-27.8	4	0.132	+129.7	4	0.047
L-Mesitran® Hydro	-15.8	4	0.488	+43.1	4	0.431
SH1 1:2	**-94.1**	**6**	**0.005**	**-86.7**	**6**	**0.01**
SH1 1:4	**-82.8**	**6**	**0.01**	**-76.6**	**6**	**0.029**
SH1 1:8	-34.9	6	0.16	**-57.08**	**6**	**0.005**
SH1 1:16	+22	6	0.25	**+7.5**	**6**	**0.02**
SH1 1:32	+4.5	6	0.81	+22	6	0.12
SH1 1:64	-1.6	6	0.93	+9.7	6	0.37
SH1 1:128	-32.1	6	0.68	-25.4	6	0.5
SH1 1:256	-34.2	6	0.184	-25.5	6	0.29
SH1 1:512	-19.6	6	0.39	-14.2	6	0.64
SH1 1:1024	-11.4	6	0.58	+6.03	6	0.67

Notes: % change values refer to the percentage increase (denoted by +), or decrease (denoted by -) in biofilm biomass for the *P.aeruginosa* isolates treated with the dressings or SH1, compared to an untreated positive control. SH1: SurgihoneyRO, n: number of replicates, T-test p-values: values obtained from the Student's t-test performed between treated and untreated data points with n-1 degrees of freedom.

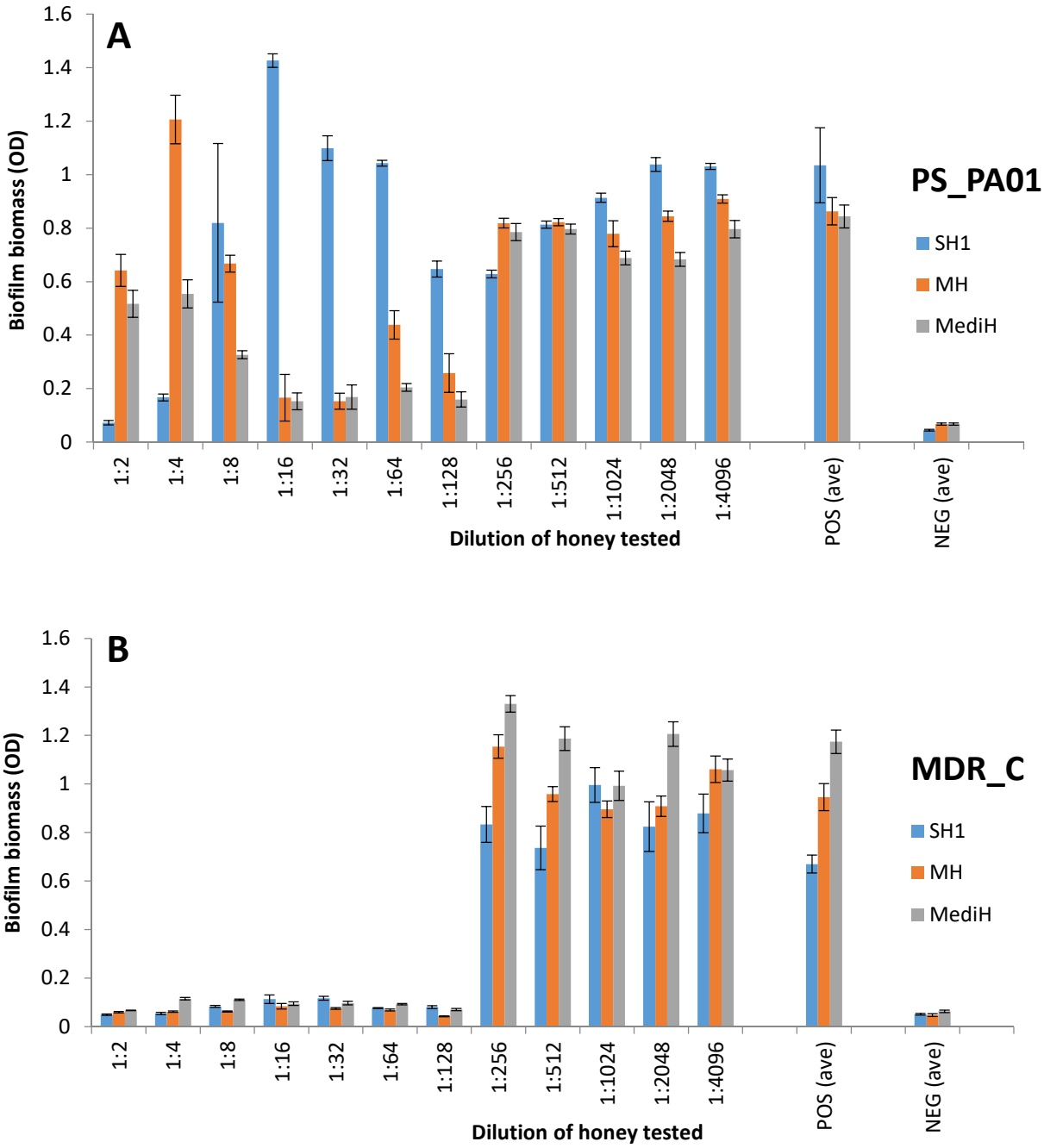

Figure 10: The mean average biomass of the biofilms of *P. aeruginosa* isolate PS_PA01 (A), and ESBL positive *E. coli* MDR_C (B) that were produced when planktonic cells were incubated with three types of honey (SH1, MH and Med) for 72 hours. Optical density (OD) on the y-axis refers to the average biofilm biomass for the isolate and the range of honey dilutions tested. Each data bar represents the mean average biofilm biomass from 6 technical replicates. The standard error of the mean is also plotted. POS (ave) is the positive control and represents the average biomass of biofilm produced in the absence of honey. NEG (ave) is the negative control.

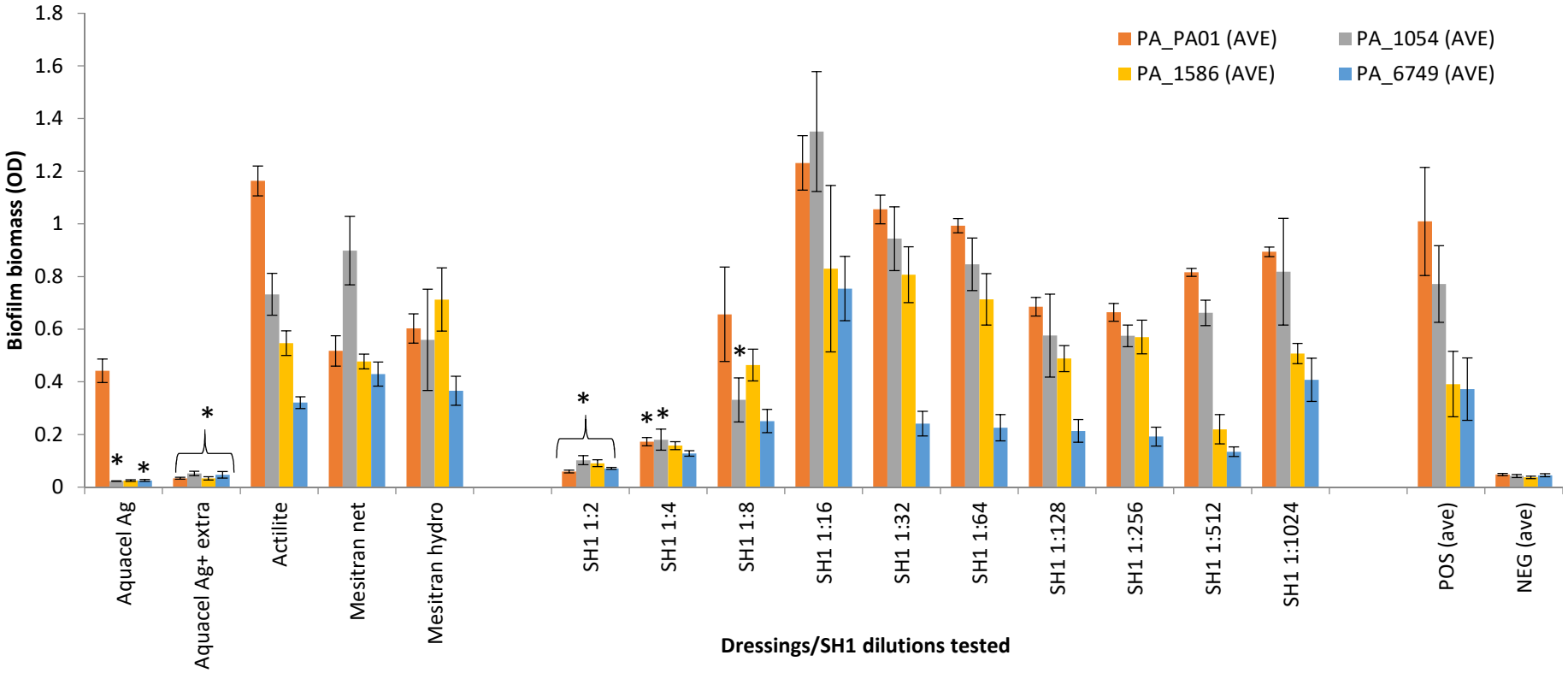

Figure 11: Graph showing the mean average biomass of the biofilms produced by the four *P. aeruginosa* isolates that were produced when planktonic cells were incubated with five different antimicrobial dressings (AMDs) or Surgihoney (SH1) for 72 hours. Optical density on the y axis refers to the average biofilm biomass for the *P. aeruginosa* isolates when tested against the range of agents shown on the x axis. Each data bar represents the mean average biofilm biomass from four technical replicates (for the AMDs) and from six replicates for the SH1 dilutions. Standard error bars have been included, alongside asterisks (*) which denote statistically significant reductions in biofilm biomass according to the Student's t–test with n-1 degrees of freedom, and a significance threshold of 0.05. POS (ave) is the positive control and represents the average biomass of biofilm produced in the absence of any test agent. NEG (ave) is the negative control.

4.4 Investigation of the antibacterial activity of blue light against nosocomial wound pathogens (paper 4)

The blue wavelengths (400-470nm) within the visible light spectrum are intrinsically antimicrobial, and can photodynamically inactivate the cells of a wide spectrum of Gram-positive and Gram-negative bacteria, as well as fungi [84,85]. This occurs due to photoexcitation of intracellular porphyrins [86], which leads to energy transfer, and the production of cytotoxic ROS (e.g. singlet oxygen) [87–89] (Figure 12). Maximum porphyrin absorption occurs at 405nm [90], and successful inactivation (and killing) by blue light (BL) has been demonstrated at this wavelength *in vitro* against a range of nosocomial pathogens [91–93]. Furthermore, BL therapy has been shown to significantly reduce the bacterial burden of wounds infected with *P. aeruginosa* [94], MRSA [95], and *A. baumannii* [96].

Currently BL is used topically to treat acne vulgaris [97], and internally as a 'light string' to treat *Helicobacter pylori* [98]. It has also been incorporated into a hospital disinfection strategy called the HINS-light environmental decontamination system [86,99], which delivers BL constantly (as part of a light fitting), and is suitable for patient occupied settings. Evaluation studies have shown that this can reduce numbers of cultivable Staphylococci spp. on surfaces by 90% when used for 24 hours in an unoccupied room [100], and by 56-86% when used in rooms occupied by MRSA positive patients.

I sought to investigate the antibacterial activity of BL against mature biofilms formed by nosocomial wound pathogens. This was warranted since the majority of research on BL has involved bacteria in the planktonic phase, and for treating or preventing wound infections it is necessary to demonstrate anti-biofilm activity.

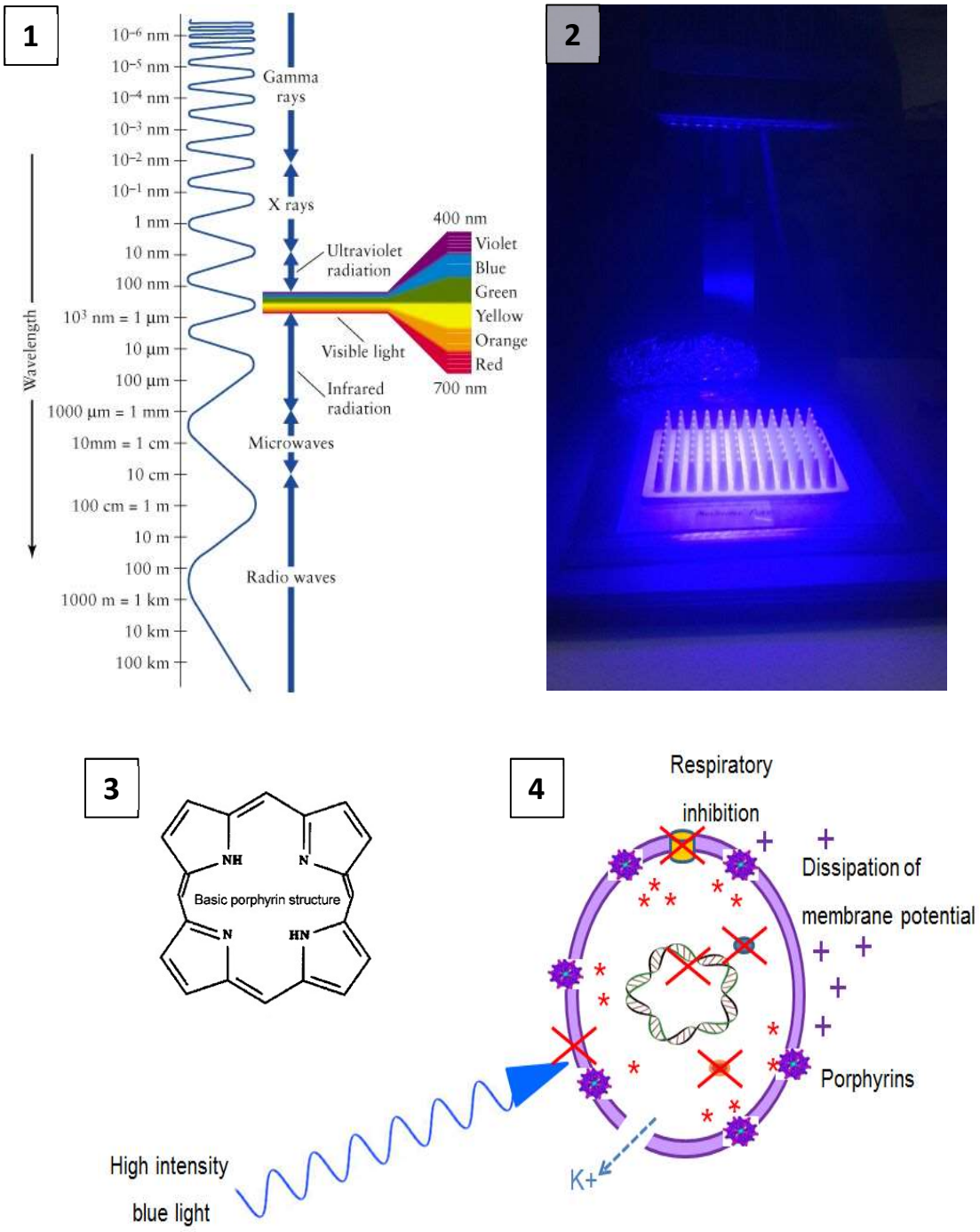

Figure 12: Composite figure: Blue light. 1; the electromagnetic spectrum at 400-700nm (google images), **2;** biofilms on pegs being exposed to blue light (own photo), **3;** basic porphyrin structure (google images), **4;** mechanism of action of blue light showing damage (indicated by red X) to DNA, cell membranes and components within the cell (J Thwaite – Dstl collaborator)

Experiments were performed on 34 bacterial isolates in both planktonic and biofilm modes of growth. The panel comprised a range of control and clinical isolates, but mostly focussed on *A. baumannii* owing to their importance as nosocomial pathogens, the high levels of antimicrobial resistance, and their relevance to the QEHB setting (a large protracted outbreak had occurred involving 65 patients from July 2011 to February 2013). All planktonic-phase bacteria were susceptible to BL treatment, with the majority (71%) demonstrating ≥5 $\log_{10}$ decrease in viability after 15-30 minutes exposure (54 J/cm^2 to 108 J/cm^2). This testing was performed by a collaborator at Dstl (Defence Science and Technology Laboratory), and will not be mentioned further.

The MBEC assay was performed on 72 hour mature biofilms by placing peg-plates (containing the biofilms) under the BL LED array for 15, 30, 45 and 1 hour timepoints (this corresponded to BL doses of 54, 108, 162 and 216 J/cm^2, respectively). A positive and negative control plate, covered in foil and placed under the array, was included per timepoint in order to control for any impacts of heating or drying on the viability of the biofilms. After exposure, the peg-plates were placed into reporter broth, and the viability/seeding of the biofilms assessed after 18 hours incubation.

BL treatment resulted in reductions in biofilm seeding for all isolates tested (Figure 13), and reductions were statistically significant ($p<0.05$) for all except one isolate at one timepoint (MSSA_10788 at 15 minutes). The Gram-negative isolates were the most susceptible, with >80% reductions in biofilm seeding for 11/28 isolates after 15 minutes, rising to 26/28 isolates at 60 minutes (Table 6). Interestingly, the Gram-positive isolates were less susceptible to BL treatment (literature states that Gram positive organisms are generally more susceptible than Gram negative [84]), with only two isolates (33%) achieving at least 90% reductions in biofilm seeding at 60 minutes. However, this may be an artefact of the small sample size. There were two particularly interesting results which were repeatable and were seen in a number of replicates. One of the *Enterobacter cloacae* isolates (ENTCL_804) responded well to BL treatment with reductions at 30, 45 and 60 mins (of 46.6, 88.2 and 87.8%, respectively), but demonstrated increased biofilm seeding at 15 minutes of 18.7%.

There was additionally one isolate of *S. aureus* (MSSA_10788), for which the maximum reduction in seeding was only 36% at 45 minutes (162 J/cm^2). This result was repeatable, and it was noted that this isolate was highly pigmented, appearing orange on solid agar, compared to the yellow colouration of the other *S aureus* isolates. Additional planktonic

tests showed that the LD_{90} values were significantly higher in the orange pigmented strains than their yellow counterparts ($p=0.003$).

This work has demonstrated that BL can be used to inactivate a wide range of clinical pathogens existing both planktonically and as mature biofilms. This technology therefore has real promise as a new antimicrobial agent for the healthcare setting and could ameliorate opportunistic infections indirectly by reducing the bacterial load on environmental surfaces, and directly within wounds. Although future studies are required (especially to investigate whether the exposure times could be reduced, and to test a larger panel of *S. aureus* strains), this study has already informed a significant part of a grant proposal investigating the use of BL for the treatment of civilian blast injuries.

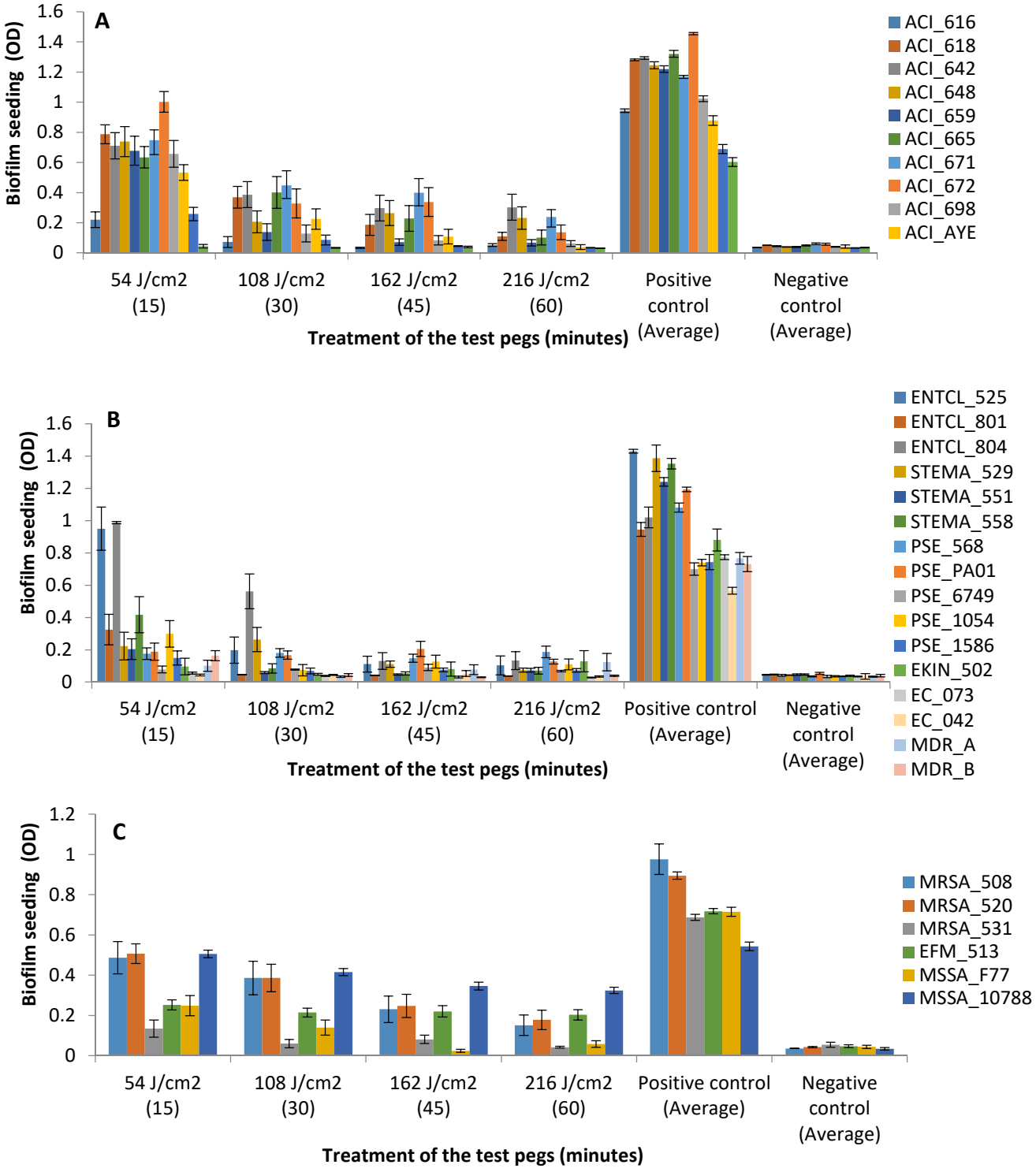

Figure 13: Graph showing the mean average biofilm seeding results for all isolates when they were tested with blue light (BL) for 15, 30, 45 and 60 minutes. Optical density (OD) on the y-axis refers to the mean average biofilm seeding for the isolates tested after exposure to blue light at the range of durations tested (in minutes) on the x-axis. A) *A. baumannii* isolates B) comparator Gram negative isolates, C) Gram positive isolates.

Each data bar represents the mean average biofilm seeding from 24 technical replicates (NB:ENTCL_8004 and MSSA_10788 are averages of 48 technical replicates). The standard error of the mean is also plotted. The positive control represents the average biofilm seeding that occurred per isolate in the absence of BL treatment across all timepoints. The negative control represents the background level of optical density for the reagents used in the assay. ACI: *A. baumannii*, ENTCL: *E. cloacae*, STEMA: *S. maltophilia*, PSE: *P. aeruginosa*, EKIN: *E. meningoseptica*, EC: *E. coli*, MDR A: carbapenemase-producing *K. pneumoniae*, MDR_B: carbapenem resistant *K. pneumoniae*, MRSA: methicillin-resistant *S. aureus*, MSSA: methicillin-sensitive *S. aureus*, EFM: *E. faecium*

Table 6: Table showing the average percentage (%) change in biofilm seeding in isolates exposed to blue light for 15, 30, 45 or 60 minutes compared to unexposed dark-incubated positive control biofilms

Isolate	n	Average change in biofilm seeding (%) with blue light exposure as follows			
		15 min (54 J/cm^2)	30 min (108 J/cm^2)	45 min (162 J/cm^2)	60 min (216 J/cm^2)
ACI_616	24	-75.9	-92.4	-96.5	-94.5
ACI_618	24	-39	-71.1	-85.6	-91.3
ACI_642	24	-45.6	-69.8	-77.3	-76.3
ACI_648	24	-43.7	-83	-78.2	-81.4
ACI_659	24	-47.1	-88.6	-94.1	-94.3
ACI_665	24	-53.9	-69.9	-82.5	-92
ACI_671	24	-37.4	-60.6	-65.7	-79.9
ACI_672	24	-31.1	-77.7	-76.5	-90.7
ACI_698	24	-36.7	-87.3	-92.2	-93.7
ACI_AYE	24	-41.9	-76.2	-86.7	-95.5
ACI_C60	24	-60.4	-89	-93.3	-94.8
ACI_19606	24	-93.5	-94.6	-93.2	-94.3
ENTCL_525	24	-34.9	-86.1	-92.2	-92.6
ENTCL_801	24	-61.3	-94.6	-95.6	-96.4
ENTCL_804	48	+18.7	-46.6	-88.2	-87.8
STEMA_529	24	-80.7	-81	-92.4	-95.1
STEMA_551	24	-84.5	-95.1	-96.2	-94
STEMA_558	24	-71	-93.3	-96.2	-94.7
PSE_568	24	-83.9	-82.8	-87.2	-81.8
PSE_PA01	24	-83.7	-86.2	-82.8	-89.5
PSE_6749	24	-88.9	-90.3	-87.1	-88.9
PSE_1054	24	-58.3	-90.7	-83.2	-84.3
PSE_1586	24	-80.3	-92.0	-89.4	-88.8
EKIN_502	24	-85.8	-94.8	-91.6	-86.5
EC_073	24	-93.0	-94.6	-96.2	-96.2
EC_042	24	-92.1	-91.3	-92.1	-93.4
MDR_A	24	-87.4	-96.0	-89.2	-82.4
MDR_B	24	-75.3	-95.0	-95.8	-94.3
MRSA_508	24	-59.5	-58	-73.7	-83.3
MRSA_520	24	-44.5	-57.7	-73.2	-78.8
MRSA_531	24	-81.6	-91.2	-88	-93.7
MSSA_10788	48	-5.0 ^	-30.9	-36.3	-34.6
MSSA_F77	24	-67.8	-79.6	-96.4	-92.0
EFM_513	24	-66.3	-69.3	-68.2	-72.2

Notes: % change values refer to the percentage increase (denoted by +), or decrease (denoted by -) in biofilm seeding for all the isolates exposed to blue light, compared to an unexposed dark-incubated positive control. Shading denotes reductions of at least 80% in biofilm seeding compared to the positive control. The ^ denotes the single isolate and single timepoint where there was not a significant change in biofilm biomass according to the Student's t-test performed between treated and untreated data points with n-1 degrees of freedom.

5 DISCUSSION

5.1 Findings

Using a series of high-throughput assays, the *in vitro* antimicrobial activity of a range of topical antimicrobial agents was assessed. All agents were effective against a large panel of clinically important nosocomial wound pathogens, in terms of their ability to prevent biofilm formation (all agents), and eradicate mature biofilms (NB: SH1 and AMDs were not tested in the MBEC assay). AA could prevent biofilm formation at concentrations of ≤0.31%, and eradication of mature biofilms was observed for all isolates after 3 hours of exposure. SH1 prevented biofilm formation of 16 bacterial isolates at dilutions (from neat) of 1:2 to 1:128. Mature biofilms were highly susceptible to BL, with significant reduction in seeding observed for all isolates at all levels of exposure.

In line with the aims and objectives, experiments were also conducted to compare the antimicrobial activity of the novel agents to conventionally used products (e.g. AA activity was compared to silver based AMDs used for burns patients, and SH1 compared to other honey-based wound care products). These demonstrated that the novel agents are not inferior (and in most cases were superior) to the conventional products in the *in vitro* setting, and therefore are promising antimicrobial agents, despite their humble and unassuming roots.

5.2 New Insights

In terms of new insights, antimicrobial activity against biofilms had not previously been assessed for SH1. The published findings therefore have been invaluable for the marketing of SH1, enabling a claim to be made of the product's 'antibiofilm' properties (appendix 5.1), a patent application (appendix 5.2), and furthermore informing a clinical trial (appendix 5.3).

There was also limited data regarding the antimicrobial activity of BL against biofilms (especially those formed by wound pathogens). The published findings have prompted a large multi-centre collaboration investigating the use of visible light for the treatment of civilian blast injuries. Although as yet unpublished, the quantitative data also indicates that the BL treatment has anti-biofilm effects. The optical density measurements of the CV dye from the exposed biofilms was compared to the CV values of the positive control, and there

were statistically significant reductions (according to a paired Wilcoxon test) in the biomass of biofilm in the BL exposed biofilms. These data are shown in a series of box and whisker plots in figure 2 (appendix 5.4), with the statistical analysis (performed using R v3.3.2 [101] and other packages [102–104]) shown in table 1 (appendix 5.4). This is an important finding, as it suggests that the reductions in seeding seen following BL treatment most likely do represent some form of damage to the biofilm (instead of just a transient reduction in seeding).

In terms of AA, the historical literature represents a series of case reports and poorly designed studies investigating the *in vivo* and *in vitro* antimicrobial activity for a range of bacteria and clinical conditions. Generally they report on the use of 1-5% AA for bacterial killing/eradication, and do provide some evidence in support of the clinical utility of AA [55,56,61,62]. However the small sample sizes, heterogeneous study designs, and limited information on the antibacterial nature of AA, makes it difficult to draw any conclusions. Although Fraise *et al.* [49] showed that AA can prevent the growth of important nosocomial pathogens, and that AA was effective at lower concentrations (e.g. 0.16% AA) than previous studies, a key limitation was the focus on planktonic growth. Bjarnsholt *et al.* [64] in part addressed this, contemporaneously to this study, showing that AA could eradicate preformed biofilms when used at 0.5% for *P. aeruginosa* and 1% for *S. aureus*.

My published study confirmed that in the *in vitro* setting, AA can inhibit biofilm formation and eradicate pre-existing biofilms when used at very dilute concentrations (e.g. down to ≤0.1%). Furthermore, through comparison studies with a range of AMDs, I have shown AA to be superior or equivalent in efficacy to market leaders [105].

It is reasonable to envisage that there will be lower toxicity with lower concentrations of AA, and hence this study demonstrates that AA is a potential alternative to antibiotics and AMDs for preventing colonisation of burns, and may have a role in the management of burns in both developed and developing countries.

5.3 Considerations

The limitations of the *in vitro* assays must be acknowledged. The assays were conducted on a range of abiotic surfaces (e.g. plastic), using standardised growth conditions, and hence are unlikely to mimic biofilm formation and persistence in the *in vivo* setting. Malone *et al.* [17] address this in an elegant review, highlighting the complexities of biofilms, and the

large variation and influences on biofilm architecture from *in vitro* to *in vivo* settings (e.g. the host immunological response). Furthermore, it is considered by some [106] that the morphology and behaviour of biofilms *in vivo* are at least as different from *in vitro*, as the differences between biofilms and planktonic bacteria. Whilst these are valid concerns, I feel it is necessary and ethical to evaluate activity of agents using available tests before they are introduced into patient care (which currently occurs on an *ad hoc* basis often without any direct laboratory investigation). Therefore, I chose to attempt to demonstrate some *in* vitro antimicrobial activity to inform clinical colleagues who are choosing AM agents to use on an *ad hoc* basis in patients. This has also allowed progression towards clinical trials. Due to the difficultly in setting up clinical trials (and the medical device classification negating them as necessary), case reports are unfortunately abundant in the medical device field. A lot of trust is placed in these findings, although there is no guarantee that the reported clinical improvement was due to the AM agent in question, instead of being explained by some other form of treatment.

For the *in vitro* tests, additional limitations concern how the endpoints were measured. CV binds to bacterial biomass, but does not differentiate between live and dead cells [107]. The OD of CV was used to assess the extent of biofilm formation, but cannot be used to assess viability, or whether cells persisting after exposure could then disperse. Furthermore, it was raised by a reviewer that interpretation of reduced seeding in the MBEC assay may only represent death of the outermost bacterial biofilm cells (and not the biofilm *per se*). A small sonication experiment was performed, demonstrating that the whole biomass of the biofilm is effectively killed with AA exposure (unpublished) (appendix 5.5). Similar (unpublished) data exists for SH1 and for BL.

Additionally, it is possible that the Student's t-test performed to assess significance of differences between TAA-exposed wells compared to unexposed positive control wells may not have been entirely appropriate. This is because the test assumes that the data follows an approximately normal distribution, and has equal standard deviations between data sets. The suitability of this test however appears to be a grey area, with one statistician (an author for two of the papers) happy with this choice, and another independent statistician suggesting that a non-parametric test would have been better. Given the small and highly significant p-values obtained, I felt reassured that the significance would not be affected, however to address this, a subset of the data was reanalysed using non-parametric tests (which accept that the data are not normally distributed); the Mann Whitney U test, and the Wilcoxon Rank Sum test (performed using PRISM (version 7.0 [108]). These data are

shown in tables 2-5 in Appendix 5.6. Generally the p-values closely match those obtained using the Student's t-test, and there is only one instance (MDR_D, MH, 1 in 3) where the p-value has shifted from being significant ($p<0.05$) to insignificant. I am therefore satisfied that conclusions from the papers remain valid.

As a simple assessment of antibacterial activity, all studies were performed using monomicrobial biofilms, although it is well recognised that the majority of clinically relevant biofilms are polymicrobial, and involve interactions that may affect overall biofilm function, physiology and general properties (e.g. enhanced resistance to antimicrobials in polymicrobial biofilms compared to monomicrobial) [109]. In recognition of this limitation, some additional experiments were performed to assess the antibacterial activity of AA on a range of polymicrobial biofilms. Using a method modified from Guggenheim *et al.* [48], mono- and polymicrobial biofilms of *P.* aeruginosa, *A. baumannii*, *E. faecium*, and *S. aureus,* were generated over a period of 72 hours, and the mature biofilms subjected to AA at a range of concentrations from 5% to 0.08%. AA was still effective when biofilms were polymicrobial, but the MBEC shifted to a value intermediate between the Gram-negative MBEC (0.63%), and Gram-positive (2.5%) (appendix 5.7). Of interest, fungi are also susceptible to AA, as revealed through a single (unpublished) experiment (data not shown), and more extensive testing from collaborators at the University of Birmingham [110].

An additional limitation is that this work did not investigate the mechanism of action (MOA) for the test agents. There is some evidence of a possible biphasic response in some of the data, whereby exposure of the bacteria to sub-inhibitory levels of the TAA has resulted in increased biofilm production (for the MBIC experiment), or enhanced biofilm seeding (for the MBEC). Figure 10 shows the biphasic response seen when isolate PS_PA01 was co-incubated with SH1 and MH. For both, there is an antimicrobial effect at the stronger dilutions of honey, followed by enhanced biofilm production at the strong (but sub-inhibitory) dilutions of honey (e.g. 1:16 for SH1), before biofilm production reduces to positive control levels. This response is also apparent in some of the AA data. To investigate this further, the biphasic response from two isolates (MSSA_10788 and ACI_19606) was analysed, and is shown in appendix 5.8. For ACI_19606 there does appear to be enhanced biofilm production for all replicates at 0.09% AA, however for MSSA_10788 the biphasic response appears to be more random, only occurring in 6 of the replicates, and absent from the other 6 (NB: these represent two separate experiments). For one isolate (ENTCL_804), there was also enhanced seeding of biofilms following treatment with BL. This has been observed elsewhere [111,112] and is likely to represent a bacterial response

to a low level of stress which results in increased biofilm formation at some sub-lethal concentrations of an antimicrobial agent. This potential pro-biofilm impact should be considered when choosing concentrations of active agents for incorporation in dressings etc, as exposure to low levels may promote unintended consequences. Interestingly however, this may not be entirely detrimental from a treatment point of view, as in some case such as BL-treated organisms there was a switch to being susceptible to an antibiotic, when previously they were resistant. This is described generally in the literature [113], but the presence/absence of this response should be investigated further prior to clinical translation of the TAAs.

Although MOAs are proposed in the literature for SH1 and BL, the MOA of weak organic acids is thought to involve a variety of mechanisms and is not just a consequence of their acidity [114,115]. Hughes & Webber [116] propose the MOA to be due to the lowering of the internal cytoplasmic pH, and an increase in acetate levels interfering with central metabolism of the cells [115,116].

Bjarnsholt *et al.* [64] took 24-hour cultures of *P. aeruginosa* and treated them with 0.5% AA solutions with varying levels of pH (modified through the addition of NaOH). The test solutions ranged in pH from 4.33 to 6.0 and they found complete killing of all bacteria in the wells when the pH was ≤4.33. In my experiments, the pH of AA ranged from 2.48 at 5%, to 3.12 at 0.8%, with the lowest universally effective concentration of AA (0.31%) being at pH 2.9. To investigate pH effect further, a small MBIC experiment was performed to compare the antibacterial activity of AA (organic acid) vs hydrochloric acid HCl (inorganic acid), against two strains of *P. aeruginosa*, and two *A. baumannii*. The pH was matched for the neat solutions of each acid, and dilutions made as previously. The results indicate that at the same pH, HCl was unable to prevent biofilm formation, compared to AA which was effective at 0.31% (appendix 5.9).

It is possible that a range of other *in vitro* biofilm formation models could have been used, e.g. open systems such as the CDC reactor (where biofilms are formed on disks present in a chemotactic media), and continuous flow cell systems/drip flow reactors (where biofilms are grown on glass and supplied with a continuous source of media). Roberts *et al.* [2] elegantly reviewed a range of biofilm models and evaluated their ability to reflect chronic infections. They comment that models involving the growth of biofilms on a surface do not reflect chronic wound infections, and are only applicable for foreign body infections. For chronic wound infections, it is proposed that porcine *in vivo* models are beneficial, due to

similarities in immune response, tissue structure, and wound healing processes [117,118]. Additionally they suggest an improved *in vitro* method that better represents *in vivo* conditions to be the 'wound-like medium' model [119], where biofilms are grown in a static medium of plasma, blood cells and broth, but are not attached to a surface. Additionally, Thøstrup *et al.* [120] used a mouse burn wound model to visualise the development of *P. aeruginosa* biofilms using PNA_FISH. Although useful, these tests would have increased complexity and costs considerably, and I wanted a simple method to quickly assess a range of novel antimicrobial agents.

Although not considered vital for translation, it is important to understand the MOA of the antimicrobial agent, since this helps in the general acceptance of the agent as a concept, and so that the possibility of resistance development can be considered. There are reports in the literature of resistance developing to Manuka honey [121], and of bacterial pigmentation leading to reducing susceptibility to BL. In terms of the TAA, it may be that lower concentrations are better tolerated by patients, but that there are greater risks of resistance developing.

5.4 Translational outcomes

This body of work focussed on potential translation of novel antimicrobial agents from the bench to bedside, and to this end has prompted a number of clinical trials. Ethical approval for such trials would not have been possible without these *in vitro* data.

For AA, a pilot feasibility trial is planned which aims to investigate the optimal concentration of AA (0.5 vs 2%) for the treatment of burns patients, in terms of tolerability and antibacterial effect. Patients with small burns will be dressed with AA dressings twice daily, and assessments made of pain on application of the AA and at intervals afterwards. Swabs will be collected daily from the burn wound (so bacterial loads can be assessed), and AA will be retrieved from the used dressings to assess remaining antibacterial activity. Patients will be followed up for 5 days (appendix 5.10), and the team hope to start recruitment towards the end of 2017.

The trial planned for SH1 involves the recruitment of 15 patients with bilateral venous leg ulcers (of comparable size and shape) (appendix 5.3). Each ulcer would be dressed with either SH1 or an AMD, and then covered with a standard compression bandage. Dressings would be changed every 48 hours, and a microbiology swab collected (so that bacterial

loads can be assessed). Patients will be followed up for 6 weeks and ulcer healing compared between the treatment groups.

5.5 Barriers to future developments

It is hoped that these translational studies will address some of the uncertainties surrounding the delivery, dosage and routes of application of these agents. However, there are many barriers to overcome. These are elegantly covered by Malone *et al.* [17], who suggest a range of possible explanations for the slow progress in medically-relevant biofilm research.

Politics and regulatory matters in the medical arena make the planning and conducting of trials very time consuming and labour intensive. As a research centre, we have been trying to set up the AA clinical trial for 3 years, but have encountered delays due to the inefficient NHS IRAS system, lack of support from clinical trials teams, and classification of AA by the MHRA as an 'investigational medicinal product' (IMP), rather than a medical device. The majority of AMDs are classified as medical devices, since they only provide an ancillary action on the wound. Consequently, there are less stringent requirements for much data on effectiveness, and this can often be kept out of the public domain. Although I understand the reasons for such tight regulation in clinical trials research, I find it hard to accept that a trial using dilute vinegar (a foodstuff that the general population ingest regularly), could encounter so many delays given the large set of data from case reports and laboratory findings which strongly suggest that AA can be an extremely cheap, effective and well tolerated treatment for biofilms in wounds. The potential impact of this treatment across the NHS is very large and this is a solution which is globally applicable and requires little resource or expertise to apply.

Given the reduced requirements to demonstrate efficacy, little incentive exists for producers of medical devices to perform costly and complicated RCTs to test their product clinically, and many would be reluctant to fund a costly trial based on *in vitro* data. Furthermore the agent itself must be considered. Although a patent does exist for the use of AA in dressings and devices [122], AA is not attractive from a business point of view, since it is unlikely to give good financial returns.

In addition to the confusing classification of biocides, there is no 'target' reference value which can be used to assess the 'effectiveness' of an anti-biofilm agent. This means that

claims of an agent being ‘anti-biofilm’ need to be carefully assessed and considered. Coupled to this, there is also no standard way to test anti-biofilm agents in the laboratory (therefore no consistency across research groups), and for *in* vivo trials, there is no gold standard way to monitor wounds for the presence of biofilms [123,124], or to monitor wounds for healing. All these factors result in vast heterogeneity, and make it very hard to design *in vivo* trials to adequately test new agents.

6 CONCLUSIONS

Given the many threats posed by antimicrobial resistance [125], and the reducing numbers of effective conventional antibiotics, it is clear that we really do need to investigate alternative antibacterial agents to combat infections. Although there is now a lot of interest in developing new antibiotics, topical treatments appear to be constantly overlooked. Topical biocides (such as AA) potentially represent antibiotic-sparing treatment options, but more research, funding and support is needed to help with initial early *in vitro* testing and translational studies, since the current system is chronically underfunded and represents a regulatory and political minefield.

Nevertheless, this body of work has highlighted that there are a number of alternative and simple antimicrobial agents that have shown promise in *vitro* for the treatment and eradication of biofilm infections caused by a range of important nosocomial wound pathogens. Clinical trials are planned for these agents, and it remains to be seen whether the *in vitro* findings will translate to the *in vivo* setting, where there is a complex interplay between host and pathogen, and many other factors that influence biofilm presence and persistence.

7 REFERENCES

[1] Percival SL, Finnegan S, Donelli G, Vuotto C, Rimmer S, Lipsky BA. Antiseptics for treating infected wounds: Efficacy on biofilms and effect of pH. Crit Rev Microbiol 2016;42:293–309.

[2] Roberts AEL, Kragh KN, Bjarnsholt T, Diggle SP. The Limitations of *In Vitro* Experimentation in Understanding Biofilms and Chronic Infection. J Mol Biol 2015;427:3646–61.

[3] Gambino M, Cappitelli F. Mini-review: Biofilm responses to oxidative stress. Biofouling 2016;32:167–78.

[4] Nicolella C, van Loosdrecht MC, Heijnen JJ. Wastewater treatment with particulate biofilm reactors. J Biotechnol 2000;80:1–33.

[5] Wu Y, Ding Y, Cohen Y, Cao B. Elevated level of the second messenger c-di-GMP in *Comamonas testosteroni* enhances biofilm formation and biofilm-based biodegradation of 3-chloroaniline. Appl Microbiol Biotechnol 2015;99:1967–76.

[6] Rudrappa T, Biedrzycki ML, Bais HP. Causes and consequences of plant-associated biofilms. FEMS Microbiol Ecol 2008;64:153–66.

[7] Mieszkin S, Callow ME, Callow JA. Interactions between microbial biofilms and marine fouling algae: a mini review. Biofouling 2013;29:1097–113.

[8] Bjarnsholt T, Alhede M, Alhede M, Eickhardt-Sørensen SR, Moser C, Kühl M, *et al.* The *in vivo* biofilm. Trends Microbiol 2013;21:466–74.

[9] Bowler, PG, Davies, BJ. The microbiology of acute and chronic wounds. Wounds 1999;11:72–8.

[10] Mihai MM, Holban AM, Giurcaneanu C, Popa LG, Oanea RM, Lazar V, *et al.* Microbial biofilms: impact on the pathogenesis of periodontitis, cystic fibrosis, chronic wounds and medical device-related infections. Curr Top Med Chem 2015;15:1552–76.

[11] Høiby N, Bjarnsholt T, Moser C, Bassi GL, Coenye T, Donelli G, *et al.* ESCMID guideline for the diagnosis and treatment of biofilm infections 2014. Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis 2015;21 Suppl 1:S1-25.

[12] Malone M, Bjarnsholt T, McBain AJ, James GA, Stoodley P, Leaper D, *et al.* The prevalence of biofilms in chronic wounds: a systematic review and meta-analysis of published data. J Wound Care 2017;26:20–5.

[13] Church D, Elsayed S, Reid O, Winston B, Lindsay R. Burn wound infections. Clin Microbiol Rev 2006;19:403–34.

[14] Metcalf, D, Bowler, P, Parsons, D. Wound Biofilm and Therapeutic Strategies. Microb. Biofilms - Importance Appl., 2016, p. 271–98.

[15] Kirketerp-Møller K, Jensen PØ, Fazli M, Madsen KG, Pedersen J, Moser C, *et al.* Distribution, organization, and ecology of bacteria in chronic wounds. J Clin Microbiol 2008;46:2717–22.

[16] Metcalf DG, Bowler PG. Biofilm delays wound healing: A review of the evidence. Burns Trauma 2013;1:5–12.

[17] Malone M, Goeres DM, Gosbell I, Vickery K, Jensen S, Stoodley P. Approaches to biofilm-associated infections: the need for standardized and relevant biofilm methods for clinical applications. Expert Rev Anti Infect Ther 2017;15:147–56.

[18] Westgate, S, Cutting, K. The role of microbial biofilms in chronic and acute wounds. Nurs Resid Care n.d.;13:518–21.

[19] Percival SL, McCarty SM, Lipsky B. Biofilms and Wounds: An Overview of the Evidence. Adv Wound Care 2015;4:373–81.

[20] Joo H-S, Otto M. Molecular basis of *in vivo* biofilm formation by bacterial pathogens. Chem Biol 2012;19:1503–13.

[21] Toyofuku M, Inaba T, Kiyokawa T, Obana N, Yawata Y, Nomura N. Environmental factors that shape biofilm formation. Biosci Biotechnol Biochem 2015;80:7–12.

[22] Kim PJ, Steinberg JS. Wound care: biofilm and its impact on the latest treatment modalities for ulcerations of the diabetic foot. Semin Vasc Surg 2012;25:70–4.

[23] Evans LR, Linker A. Production and characterization of the slime polysaccharide of *Pseudomonas aeruginosa.* J Bacteriol 1973;116:915–24.

[24] Juhas M, Eberl L, Tümmler B. Quorum sensing: the power of cooperation in the world of Pseudomonas. Environ Microbiol 2005;7:459–71.

[25] Boyd A, Chakrabarty AM. *Pseudomonas aeruginosa* biofilms: role of the alginate exopolysaccharide. J Ind Microbiol 1995;15:162–8.

[26] Percival SL, Hill KE, Malic S, Thomas DW, Williams DW. Antimicrobial tolerance and the significance of persister cells in recalcitrant chronic wound biofilms. Wound Repair Regen Off Publ Wound Heal Soc Eur Tissue Repair Soc 2011;19:1–9.

[27] Gilbert P, Das J, Foley I. Biofilm susceptibility to antimicrobials. Adv Dent Res 1997;11:160–7.

[28] Conlon BP, Rowe SE, Lewis K. Persister cells in biofilm associated infections. Adv Exp Med Biol 2015;831:1–9.

[29] Bjarnsholt T, Kirketerp-Møller K, Jensen PØ, Madsen KG, Phipps R, Krogfelt K, *et al.* Why chronic wounds will not heal: a novel hypothesis. Wound Repair Regen Off Publ Wound Heal Soc Eur Tissue Repair Soc 2008;16:2–10.

[30] Anon. Silver dressings - do they work? Drug Ther Bull 2010:38–42.

[31] Clinton A, Carter T. Chronic Wound Biofilms: Pathogenesis and Potential Therapies. Lab Med 2015;46:277–84.

[32] Harper, DR, Parracho, HMRT, Walker, J, Sharp, R, Hughes, G, Werthen, M, *et al.* Bacteriophages and Biofilms. Antibiotics 2014;3:270–84.

[33] Fey PD. Modality of bacterial growth presents unique targets: how do we treat biofilm-mediated infections? Curr Opin Microbiol 2010;13:610–5.

[34] McDonnell, G., Russell, A.D. Antiseptics and Disinfectants: Activity, Action, and Resistance. Clin Microbiol Rev 1999;12:147–79.

[35] Singh PK, Parsek MR, Greenberg EP, Welsh MJ. A component of innate immunity prevents bacterial biofilm development. Nature 2002;417:552–5.

[36] Li X-H, Lee J-H. Antibiofilm agents: A new perspective for antimicrobial strategy. J Microbiol Seoul Korea 2017;55:753–66.

[37] Dong Y-H, Gusti AR, Zhang Q, Xu J-L, Zhang L-H. Identification of quorum-quenching N-acyl homoserine lactonases from *Bacillus* species. Appl Environ Microbiol 2002;68:1754–9.

[38] Vuotto C, Longo F, Donelli G. Probiotics to counteract biofilm-associated infections: promising and conflicting data. Int J Oral Sci 2014;6:189–94.

[39] Hughes KA, Sutherland IW, Jones MV. Biofilm susceptibility to bacteriophage attack: the role of phage-borne polysaccharide depolymerase. Microbiol Read Engl 1998;144 (Pt 11):3039–47.

[40] Ammons MCB. Anti-biofilm strategies and the need for innovations in wound care. Recent Patents Anti-Infect Drug Disc 2010;5:10–7.

[41] Craigen B, Dashiff A, Kadouri DE. The Use of Commercially Available Alpha-Amylase Compounds to Inhibit and Remove *Staphylococcus aureus* Biofilms. Open Microbiol J 2011;5:21–31.

[42] Wolcott RD, Rumbaugh KP, James G, Schultz G, Phillips P, Yang Q, *et al.* Biofilm maturity studies indicate sharp debridement opens a time- dependent therapeutic window. J Wound Care 2010;19:320–8.

[43] Prescription Services. NHS Business Services Authority. Pers Commun 2010.

[44] Khundkar R, Malic C, Burge T. Use of Acticoat dressings in burns: what is the evidence? Burns J Int Soc Burn Inj 2010;36:751–8.

[45] Polak, F, Clift, M, Bower, L, Sprange, K. Buyer's Guide: Advanced Wound Dressings. London: Centre for Evidence-Based purchasing; 2008.

[46] Storm-Versloot MN, Vos CG, Ubbink DT, Vermeulen H. Topical silver for preventing wound infection. Cochrane Database Syst Rev 2010:CD006478.

[47] Cavanagh MH, Burrell RE, Nadworny PL. Evaluating antimicrobial efficacy of new commercially available silver dressings. Int Wound J 2010;7:394–405.

[48] Guggenheim M, Thurnheer T, Gmür R, Giovanoli P, Guggenheim B. Validation of the Zürich burn-biofilm model. Burns J Int Soc Burn Inj 2011;37:1125–33.

[49] Fraise AP, Wilkinson M a. C, Bradley CR, Oppenheim B, Moiemen N. The antibacterial activity and stability of acetic acid. J Hosp Infect 2013;84:329–31.

[50] BSI Standards Publication. BS EN 13727. Chemical disinfectants and antiseptics - quantitative suspension test for the evaluation of bactericial activity in the medical area - test methods and requirements (phase 2, step 1). 2012.

[51] European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. Clin Microbiol Infect 2003;9:1–7.

[52] Baugh S, Ekanayaka AS, Piddock LJV, Webber MA. Loss of or inhibition of all multidrug resistance efflux pumps of *Salmonella enterica* serovar Typhimurium results in impaired ability to form a biofilm. J Antimicrob Chemother 2012;67:2409–17.

[53] McGuiness W, Vella E, Harrison D. Influence of dressing changes on wound temperature. J Wound Care 2004;13:383–5.

[54] Ceri H, Olson M, Morck D, Storey D, Read R, Buret A, *et al.* The MBEC Assay System: multiple equivalent biofilms for antibiotic and biocide susceptibility testing. Methods Enzymol 2001;337:377–85.

[55] Ryssel H, Kloeters O, Germann G, Schäfer T, Wiedemann G, Oehlbauer M. The antimicrobial effect of acetic acid--an alternative to common local antiseptics? Burns J Int Soc Burn Inj 2009;35:695–700.

[56] Phillips I, Lobo AZ, Fernandes R, Gundara NS. Acetic acid in the treatment of superficial wounds infected by *Pseudomonas aeruginosa.* Lancet Lond Engl 1968;1:11–4.

[57] McManus AT, Mason AD, McManus WF, Pruitt BA. Twenty-five year review of *Pseudomonas aeruginosa* bacteremia in a burn center. Eur J Clin Microbiol 1985;4:219–23.

[58] Smith DT. Causes and treatments of otitis media; I. Observations on 205 cases occuring in 613 consecutive hospital admissions. JAMA Paediatr 1924;28.

[59] Currence WW. Acetic acid aerosol in treatment of purulent bronchiectasis due to *Pseudomonas aeruginosa*. AMA Am J Dis Child 1952;83:637–41.

[60] Kass EH, Sossen HS. Prevention of infection of urinary tract in presence of indwelling catheters; description of electromechanical valve to provide intermittent drainage of the bladder. J Am Med Assoc 1959;169:1181–3.

[61] Sloss JM, Cumberland N, Milner SM. Acetic acid used for the elimination of *Pseudomonas aeruginosa* from burn and soft tissue wounds. J R Army Med Corps 1993;139:49–51.

[62] Milner SM. Acetic acid to treat *Pseudomonas aeruginosa* in superficial wounds and burns. Lancet Lond Engl 1992;340:61.

[63] Madhusudhan VL. Efficacy of 1% acetic acid in the treatment of chronic wounds infected with *Pseudomonas aeruginosa*: prospective randomised controlled clinical trial. Int Wound J 2016;13:1129–36.

[64] Bjarnsholt T, Alhede M, Jensen PØ, Nielsen AK, Johansen HK, Homøe P, *et al.* Antibiofilm Properties of Acetic Acid. Adv Wound Care 2015;4:363–72.

[65] Anon. Chronic wounds: advanced wound dressings and antimicrobial dressings (ESMPB2). NICE Evid Summ 2016.

[66] British National Formulary. Appendix 5: Wound management products and elasticated garments. A5.3 Antimicrobbial Dressings. 2017.

[67] Stone HH. Review of pseudomonas sepsis in thermal burns: verdoglobin determination and gentamicin therapy. Ann Surg 1966;163:297–305.

[68] Miraftab M, Masood R, Edward-Jones V. A new carbohydrate-based wound dressing fibre with superior absorption and antimicrobial potency. Carbohydr Polym 2014;101:1184–90.

[69] Lara HH, Ayala-Nuñez NV, Ixtepan-Turrent L, Rodriguez-Padilla C. Mode of antiviral action of silver nanoparticles against HIV-1. J Nanobiotechnology 2010;8:1.

[70] Bowler PG, Jones SA, Walker M, Parsons D. Microbicidal properties of a silver-containing hydrofiber dressing against a variety of burn wound pathogens. J Burn Care Rehabil 2004;25:192–6.

[71] Atiyeh BS, Costagliola M, Hayek SN, Dibo SA. Effect of silver on burn wound infection control and healing: review of the literature. Burns J Int Soc Burn Inj 2007;33:139–48.

[72] Fong J. The use of silver products in the management of burn wounds: change in practice for the burn unit at Royal Perth Hospital. Prim Intent 2005;13:S16-22.

[73] Fong J, Wood F. Nanocrystalline silver dressings in wound management: a review. Int J Nanomedicine 2006;1:441–9.

[74] Collier M. Silver dressings: more evidence is needed to support their widespread clinical use. J Wound Care 2009;18:77–8.

[75] Anon. "Evidence-based prescribing of advanced wound dressings for chronic wounds in primary care." MeReC Bull 2010;21.

[76] Holm S. A simple sequentially rejective multiple test procedure. Scand J Stat 1979;6:65–70.

[77] Armstrong DG. Manuka honey improved wound healing in patients with sloughy venous leg ulcers. Evid Based Med 2009;14:148.

[78] Mizrahi, A, Lensky, Y. Honey as an antimicrobial agent. Bee Prod. Prop. Appl. Apitherapy, Plenum Press; 1996.

[79] Tan HT, Rahman RA, Gan SH, Halim AS, Hassan SA, Sulaiman SA, et al. The antibacterial properties of Malaysian tualang honey against wound and enteric microorganisms in comparison to manuka honey. BMC Complement Altern Med 2009;9:34.

[80] Cooper, R, Molan, P, White, R. Antimicrobial activity of honey. Honey Mod. Wound Manag., Wounds UK; 2009.

[81] Cooper, R. Honey as an effective antimicrobial treatment for chronic wounds: is there a place for it in modern medicine? Chronic Wound Care Manag Res 2014;1:15–22.

[82] Dryden M, Lockyer G, Saeed K, Cooke J. Engineered honey: *In vitro* antimicrobial activity of a novel topical wound care treatment. J Glob Antimicrob Resist 2014;2:168–72.

[83] Dryden M, Dickinson A, Brooks J, Hudgell L, Saeed K, Cutting KF. A multi-centre clinical evaluation of reactive oxygen topical wound gel in 114 wounds. J Wound Care 2016;25:140, 142–6.

[84] Maclean M, MacGregor SJ, Anderson JG, Woolsey G. Inactivation of bacterial pathogens following exposure to light from a 405-nanometer light-emitting diode array. Appl Environ Microbiol 2009;75:1932–7.

[85] Murdoch LE, McKenzie K, Maclean M, Macgregor SJ, Anderson JG. Lethal effects of high-intensity violet 405-nm light on *Saccharomyces cerevisiae, Candida albicans*, and on dormant and germinating spores of *Aspergillus niger*. Fungal Biol 2013;117:519–27.

[86] Maclean M, McKenzie K, Anderson JG, Gettinby G, MacGregor SJ. 405 nm light technology for the inactivation of pathogens and its potential role for environmental disinfection and infection control. J Hosp Infect 2014;88:1–11.

[87] Dai T, Gupta A, Murray CK, Vrahas MS, Tegos GP, Hamblin MR. Blue light for infectious diseases: *Propionibacterium acnes, Helicobacter pylori*, and beyond? Drug Resist Updat Rev Comment Antimicrob Anticancer Chemother 2012;15:223–36.

[88] Hamblin MR, Hasan T. Photodynamic therapy: a new antimicrobial approach to infectious disease? Photochem Photobiol Sci Off J Eur Photochem Assoc Eur Soc Photobiol 2004;3:436–50.

[89] Maclean M, Macgregor SJ, Anderson JG, Woolsey GA. The role of oxygen in the visible-light inactivation of *Staphylococcus aureus*. J Photochem Photobiol B 2008;92:180–4.

[90] Goldoni A. Porphyrins: fascinating molecules with biological significance. Sincrotrone TriesteSCpA, Trieste, Italy; 2002.

[91] Enwemeka CS, Williams D, Hollosi S, Yens D, Enwemeka SK. Visible 405 nm SLD light photo-destroys methicillin-resistant *Staphylococcus aureus* (MRSA) in vitro. Lasers Surg Med 2008;40:734–7.

[92] Murdoch LE, Maclean M, Endarko E, MacGregor SJ, Anderson JG. Bactericidal effects of 405 nm light exposure demonstrated by inactivation of Escherichia, Salmonella, Shigella, Listeria, and Mycobacterium species in liquid suspensions and on exposed surfaces. Scientific World Journal 2012; 137805.

[93] Maclean M, Murdoch LE, MacGregor SJ, Anderson JG. Sporicidal effects of high-intensity 405 nm visible light on endospore-forming bacteria. Photochem Photobiol 2013;89:120–6.

[94] Dai T, Gupta A, Huang Y-Y, Yin R, Murray CK, Vrahas MS, *et al.* Blue light rescues mice from potentially fatal *Pseudomonas aeruginosa* burn infection: efficacy, safety, and mechanism of action. Antimicrob Agents Chemother 2013;57:1238–45.

[95] Dai T, Gupta A, Huang Y-Y, Sherwood ME, Murray CK, Vrahas MS, *et al.* Blue light eliminates community-acquired methicillin-resistant *Staphylococcus aureus* in infected mouse skin abrasions. Photomed Laser Surg 2013;31:531–8.

[96] Zhang Y, Zhu Y, Gupta A, Huang Y, Murray CK, Vrahas MS, *et al*. Antimicrobial blue light therapy for multidrug-resistant *Acinetobacter baumannii* infection in a mouse burn model: implications for prophylaxis and treatment of combat-related wound infections. J Infect Dis 2014;209:1963–71.

[97] Noborio R, Nishida E, Kurokawa M, Morita A. A new targeted blue light phototherapy for the treatment of acne. Photodermatol Photoimmunol Photomed 2007;23:32–4.

[98] Ganz RA, Viveiros J, Ahmad A, Ahmadi A, Khalil A, Tolkoff MJ, *et al. Helicobacter pylori* in patients can be killed by visible light. Lasers Surg Med 2005;36:260–5.

[99] Bache SE, Maclean M, MacGregor SJ, Anderson JG, Gettinby G, Coia JE, *et al.* Clinical studies of the High-Intensity Narrow-Spectrum light Environmental Decontamination System (HINS-light EDS), for continuous disinfection in the burn unit inpatient and outpatient settings. Burns J Int Soc Burn Inj 2012;38:69–76.

[100] Maclean M, Macgregor SJ, Anderson JG, Woolsey GA, Coia JE, Hamilton K, *et al.* Environmental decontamination of a hospital isolation room using high-intensity narrow-spectrum light. J Hosp Infect 2010;76:247–51.

[101] R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria: 2016.

[102] Frank E Harrell Jr. rms: Regression Modelling Strategies. 2017.

[103] H Wickham. ggplot2: Elegant Graphics for Data Analysis. New York: Springer-Verlag; 2009.

[104] Fox, J. Effect Displays in R for Generalised Linear Models. J Stat Softw;8:1–27.

[105] Halstead FD, Rauf M, Bamford A, Wearn CM, Bishop JRB, Burt R, *et al.* Antimicrobial dressings: Comparison of the ability of a panel of dressings to prevent biofilm formation by key burn wound pathogens. Burns J Int Soc Burn Inj 2015;41:1683–94.

[106] Benskin LLL. Limitations of *in vitro* antimicrobial dressings study. Burns J Int Soc Burn Inj 2016;42:1147–8.

[107] Bauer J, Siala W, Tulkens PM, Van Bambeke F. A combined pharmacodynamic quantitative and qualitative model reveals the potent activity of daptomycin and delafloxacin against *Staphylococcus aureus* biofilms. Antimicrob Agents Chemother 2013;57:2726–37.

[108] Anon. GraphPad Prism version 7.00 for Windows. La Jolla California USA.

[109] Burmølle M, Ren D, Bjarnsholt T, Sørensen SJ. Interactions in multispecies biofilms: do they actually matter? Trends Microbiol 2014;22:84–91.

[110] Trzaska WJ, Correia JN, Villegas MT, May RC, Voelz K. pH manipulation as a novel strategy for treating mucormycosis. Antimicrob Agents Chemother 2015;59:6968–74.

[111] Guffey JS, Payne W, Jones T, Martin K. Evidence of resistance development by *Staphylococcus aureus* to an *in vitro*, multiple stage application of 405 nm light from a supraluminous diode array. Photomed Laser Surg 2013;31:179–82.

[112] Nussbaum EL, Lilge L, Mazzulli T. Effects of 630-, 660-, 810-, and 905-nm laser irradiation delivering radiant exposure of 1-50 J/cm2 on three species of bacteria *in vitro*. J Clin Laser Med Surg 2002;20:325–33.

[113] Fila G, Kawiak A, Grinholc MS. Blue light treatment of *Pseudomonas aeruginosa:* Strong bactericidal activity, synergism with antibiotics and inactivation of virulence factors. Virulence 2017;8:938–58.

[114] Roe AJ, McLaggan D, Davidson I, O'Byrne C, Booth IR. Perturbation of anion balance during inhibition of growth of *Escherichia coli* by weak acids. J Bacteriol 1998;180:767–72.

[115] Hirshfield IN, Terzulli S, O'Byrne C. Weak organic acids: a panoply of effects on bacteria. Sci Prog 2003;86:245–69.

[116] Hughes G, Webber MA. Novel approaches to the treatment of bacterial biofilm infections. Br J Pharmacol 2017; 174:2237-2246.

[117] Sullivan TP, Eaglstein WH, Davis SC, Mertz P. The pig as a model for human wound healing. Wound Repair Regen Off Publ Wound Heal Soc Eur Tissue Repair Soc 2001;9:66–76.

[118] Harrison F, Muruli A, Higgins S, Diggle SP. Development of an *ex vivo* porcine lung model for studying growth, virulence, and signaling of *Pseudomonas aeruginosa*. Infect Immun 2014;82:3312–23.

[119] Dalton T, Dowd SE, Wolcott RD, Sun Y, Watters C, Griswold JA, *et al*. An *in vivo* polymicrobial biofilm wound infection model to study interspecies interactions. PloS One 2011;6:e27317.

[120] Trøstrup H, Thomsen K, Christophersen LJ, Hougen HP, Bjarnsholt T, Jensen PØ, *et al. Pseudomonas aeruginosa* biofilm aggravates skin inflammatory response in BALB/c mice in a novel chronic wound model. Wound Repair Regen Off Publ Wound Heal Soc Eur Tissue Repair Soc 2013;21:292–9.

[121] Camplin AL, Maddocks SE. Manuka honey treatment of biofilms of *Pseudomonas aeruginosa* results in the emergence of isolates with increased honey resistance. Ann Clin Microbiol Antimicrob 2014;13:19.

[122] Givskov MC, Bjarnsholt T, Kirketerp-Moller K. Wound care products. WO 2011076216 A3.

[123] Hurlow J, Couch K, Laforet K, Bolton L, Metcalf D, Bowler P. Clinical Biofilms: A Challenging Frontier in Wound Care. Adv Wound Care 2015;4:295–301.

[124] Percival SL, Vuotto C, Donelli G, Lipsky BA. Biofilms and Wounds: An Identification Algorithm and Potential Treatment Options. Adv Wound Care 2015;4:389–97.

[125] Piddock LJV. Reflecting on the final report of the O'Neill Review on Antimicrobial Resistance. Lancet Infect Dis 2016;16:767–8.

[126] Lund P, Tramonti A, De Biase D. Coping with low pH: molecular strategies in neutralophilic bacteria. FEMS Microbiol Rev 2014;38:1091–125.