TOWARDS NEW TOPICAL TREATMENTS FOR BACTERIAL INFECTION

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The candidate confirms that the work submitted is her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

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Work from the published paper will be included in the following chapter:

Chapter 5: Antibacterial activity and mechanism of action of batumin

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Abstract

Soft skin and tissue infections represent the most common bacterial infections, however in some cases these infections fail to heal. The presence of surface-attached communities known as biofilms has been observed on >60% of wounds that fail to heal and adversely impact wound healing. However, many of the existing antibacterial agents used to treat non-healing wounds have limited activity against biofilms. This thesis sought to identify and characterise compounds with antibacterial and antibiofilm activity against significant wound pathogens.

Initial experimentation sought to determine whether combining antibacterial agents could improve their antibiofilm activity. Synergism studies identified the combination of chlorhexidine and cetrimide, which displayed improved biofilm eradication against mono-species *S. aureus* and *P. aeruginosa* biofilms. Unfortunately, this combination had limited activity against more robust mixed species biofilm. Although this combination did not display improved activity against this mixed species biofilms, the methodology used in this chapter could be used in future to identify and evaluate other combinations with regards to their antibiofilm activity.

The void in broad spectrum antibiofilm agents could be filled by repurposed agents. Bronidox (BX) and bronopol (BP) were identified as compounds that are able to eradicate mono- and mixed biofilms at concentrations that are regarded for safe use. Investigation into their antibiofilm mode of action (MOA) revealed that BP and BX are able to kill stationary phase bacteria, it was additionally shown that both agents target biofilm cells directly and that biofilm matrix disruption is a secondary MOA.

Batumin displays potent antistaphylococcal activity (MIC= 0.0625 µg/mL) and may prove a useful topical antibacterial agent for skin infections; however its MOA is under debate. Initial studies revealed that the batumin exerts its antistaphylococcal effect through the inhibition of fatty acid synthesis. Characterisation of batumin-resistant mutants revealed mutations which were hypothesised to increase FabI expression and reduce susceptibility to batumin. Indeed, the over expression of FabI in *S. aureus* resulted in reduced susceptibility to batumin. Finally the inhibition of purified *S. aureus* FabI by batumin was confirmed *in*

vitro. Batumin inhibited saFabI with similar potency to triclosan- another FabI inhibitor. The studies provide the basis to explore batumin further as a topical antistaphylococcal agent.

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Abbreviations

ACP	Acyl carrier protein
ADA	N-(2-Acetamido) iminodiacetic acid
ATc	Anhydrotetracycline
ATCC	American Type Culture Collection
BHA	Brain Heart Infusion agar
C	Celsius
CBD	Calgary biofilm device
CDB	Cellulose disk biofilm
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
CONS	Coagulase negative staphylococci
C-di-GMP	Bis-(3'-5')-cyclic dimeric guanosine monophosphate
DMSO	Dimethylsulphoxide
DNA	Deoxyribose nucleic acid
ENR	Enolyl-ACP reductase
EPS	Exopolysaccharide
FAS	Fatty acid biosynthesis
FBEC	Fractional biofilm eradication concentration
FIC	Fractional inhibitory concentration
G	Gravity

GRAS	Generally regarded as safe
IC_{50}	Concentration required to inhibit enzyme activity by 50%
MAC	Maximum authorised concentration
MBC	Minimum bactericidal concentration
MBEC	Minimum biofilm eradication concentration
MHA II	Cation adjusted Mueller-Hinton agar
MHB II	Cation adjusted Mueller-Hinton broth
MIC	Minimum inhibitory concentration
MMS	Macromolecular synthesis
MOA	Mode of action
MRSA	Methicillin- resistant *Staphylococcus aureus*
MSA	Mannitol salt agar
MSSA	Methicillin sensitive *S. aureus*
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced Nicotinamide adenine dinucleotide phosphate
NCTC	National Collection of Type Cultures
NRPS	Non-ribosomal peptide synthetase
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
PHMB	Polyhexanide

PIA	Polysaccharide intercellular adhesin
PKS	Polyketide synthase
PMBN	Polymyxin B nonapeptide
PNAG	Polymeric N-acetyl-glucosamine
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SDW	Sterile dilution water
SSTI	Soft skin and tissue infection
TCA	Trichloroacetic acid
v/v	Volume per volume
w/v	Weight per volume
WGS	Whole genome sequencing

Chapter 1 Introduction

1.1 Bacterial skin infections

The body's largest organ, the skin, is the first line of defence against microbial attack, and is home to a wide range of microorganisms including bacteria, viruses and fungi (Grice and Segre, 2011). These microorganisms are usually commensal causing no harm and some may even exist in a mutualistic relationship; however, if there is break in the skin, these microorganisms may act opportunistically resulting in infection (Cogen et al., 2008). Soft skin and tissue infections (SSTIs) can vary in severity; from impetigo, bite wounds and boils to more severe conditions such as ulcers, cellulitis, necrotising fasciitis, surgical site and intravenous infusion site infections (Gemmell et al., 2006). SSSTIs are amongst the most common bacterial infections (Ki and Rotstein, 2008). Given the various presentations of SSTIs, more precise estimations of their prevalence prove difficult; however it has been estimated that 24.6 in 1000 people per annum will develop an SSTI (Ki et al., 2008). This could be an underestimation, as for most uncomplicated infections the body is able to clear an infection on its own and therefore antimicrobial treatment (or visiting a healthcare professional) is not always necessary (Ki et al., 2008). In the clinic determining the aetiological pathogen of a SSTI is not always possible, therefore topical or oral antibiotics are usually prescribed empirically (Ki et al., 2008). The Gram-positive microorganisms *Staphylococcus aureus* and *Streptococcus pyogenes* are commonly associated with SSTIs, therefore antibiotics are empirically prescribed based on this (Fleming et al., 2007). Despite intervention, some SSTIs fail

to heal and chronic wounds may develop (Guo and DiPietro, 2010). The factors that cause chronic wounds to develop and their impact on society will be discussed in the following section.

1.2 The burden of chronic wounds

1.2.1 Factors influencing wound healing

Following a break in the skin, wound healing occurs in four overlapping and complex phases; coagulation, inflammation, proliferation and remodelling (Guo et al., 2010). Ordinarily acute wounds undergo these four stages in a timely manner and are healed just after a few days. However, in the case of chronic wounds these primary stages are delayed, most commonly at the inflammation stage (Percival, Steven. L. et al., 2012). Chronic wounds may last for weeks, months or even years and are usually in the form of pressure ulcers, venous ulcers and diabetic foot ulceration (James et al., 2008). There are many factors which may perturb wound healing and lead to chronic wounds; these include systemic factors such as, nutritional status and medication and disease including; diabetes, AIDS and cancers (Guo et al., 2010). Local factors include insufficient oxygenation, foreign bodies and bacterial infection (Guo et al., 2010); the importance of bacterial infection in chronic wounds is highlighted, as staphylococcal infection has been shown to significantly reduce the rate of wound healing in a mouse wound model (Schierle et al., 2009).

Biofilms are communities of sessile bacteria that differ phenotypically from free-floating planktonic cells due to their ability to remain attached to biological and

non-biological surfaces whilst encapsulated in an extracellular matrix comprised primarily of exopolysaccharide (EPS) produced by the bacteria (Costerton et al., 1999; Davies, D., 2003). Study of the chronic wound bed using electron microscopy has determined that bacterial biofilms are present within a high number of chronic wounds, with approximately 60% of chronic wounds containing biofilms in comparison with just 6% of acute wounds presenting biofilms (James et al., 2008). The presence of biofilms in chronic wounds has been extensively described and has been shown to be a factor that reduces healing rates (Schierle et al., 2009; Percival, Steven. L. et al., 2012; Rhoads, D.D. et al., 2012; Mancl et al., 2013).

Before the introduction of antibiotics, antiseptics and the knowledge that bacteria were able to cause infection; ancient civilisations used various topical treatments to heal chronic and acute wounds (Ovington, 2002). The ancient Egyptians regularly used a concoction of grease, lint and honey as part of their wound dressings and the ancient Greeks were the first civilisations to distinguish between acute and chronic wounds designating them as “fresh” and “non-healing” wounds, respectively (Ovington, 2002). Despite our advanced knowledge of the physiology of wounds, antimicrobial agents and the wound microbiome, many chronic wounds remain unhealed for many months or years. It is reported that in the UK there are approximately 650,000 people affected by chronic wounds and the burden of chronic wounds costs the NHS more than £4 billion a year (Thomas, S., 2006). Without appropriate and successful treatments, chronic wounds can be further complicated; in the US it is estimated that amputation will occur in 12% of patients with foot ulcers. Diabetes also increases the risk of amputation; of the people

receiving non-traumatic lower-extremity amputations, 67% were diabetics (Sen et al., 2009). In order to improve the outcome of chronic wounds, more must be done to control the biofilm communities that negatively impact wound healing.

1.2.2 Chronic wound microbiome

Knowledge surrounding the microbiome of chronic wounds may aid the development of new treatment regimens and antimicrobial agents designated to treat chronic wound infections. The human skin is home to a diverse range of bacteria, fungi and viruses, there is variation in colonising microorganisms at different sites across the body (Grice et al., 2011). The microorganisms present at a particular site are dependent on the microenvironment of the skin. This difference in commensal bacteria depends on whether the skin is dry, moist or sebaceous, which is influenced by host and environmental factors (Grice et al., 2011). On normal healthy skin *Propionibacterium, Staphylococcus, Streptococcus* and *Bacteroides* are most commonly isolated. Whereas, the wound microbiome demonstrates a very different population, anaerobes are more readily identified along with high numbers of Gram-negative bacteria and increased proportions of staphylococci and streptococci (Han et al., 2011).

When observing the wound bed there is often uncertainty over what constitutes an infection. The bacterial wound continuum is regularly used to establish the infection and replication status of the bacteria present in the wound bed (Siddiqui and Bernstein, 2010). Contamination can be described as the presence of bacteria within the wound that are not replicating, whilst colonisation occurs when the bacteria can freely replicate without causing any trauma to the host. In contrast,

critical colonisation/infection occurs when the bacteria are freely replicating and have invaded the tissue of the wound, eliciting the host's immune response. If not treated, critical colonisation can progress to an invasive infection and septicaemia (Siddiqui et al., 2010).

The number of bacteria present within a wound is often referred to as the "bioburden", however, there is still the question of how many bacteria are required to initiate an infection (Edwards and Harding, 2004; Salcido, 2007). Intact skin contains approximately 10^5 microorganisms per gram/cm^3; it is therefore thought that greater than 10^5 microorganisms is indicative of an infection. However this definition is restricted to the patients' clinical status as it may require less than 10^5 microorganisms to establish an infection in a patient who is immune compromised. Additionally, the virulence of the infecting microorganisms needs to be accounted for, for example β- haemolytic streptococci are able to infect at much lower numbers than 10^5 and their presence alone indicates the need for antimicrobial treatment (Bowler, Phillip. G., 2003; Edwards et al., 2004) .

Not only is the bacterial load noteworthy in chronic wound infection, the species present within the wound bed is important. Typical culture methods are biased to easily cultural microorganisms such as *S. aureus*. This bias may exclude other bacteria including anaerobic bacteria that may be present deeper within the wound. This has led to more molecular methods of screening the wound microbiota. For example, in the chronic wound microbiota explored by Melendez *et al.* samples were taken from diabetic foot ulcers, primary ulcers and venous ulcers and other chronic wounds to screen the microorganisms present using multiplex

real time PCR (RT-PCR) and qualitative and quantitative culture methods. Using qualitative culture methods it was found that at least one species was present in 97% of samples, whilst 60% of samples had three or more species. RT-PCR was able to detect organisms in 82% of samples, however the RT-PCR excluded organisms which were classified not clinically relevant including coagulase negative staphylococci (CONS) and *Corynebacterium. spp* (Melendez et al., 2010). CONS and *Corynebacterium. spp* are part of the commensal flora so are often excluded as contaminants, however for years *S. epidermidis* was disregarded from clinical samples but we now know that *S. epidermis* can cause intravascular catheter-related infections and surgical wound infections so should not be readily overlooked (Piette and Verschraegen, 2009). The most commonly identified organisms by culture and PCR in the study by Melendez were methicillin resistant *S. aureus* (MRSA), *Pseudomonas aeruginosa*, group B streptococci and methicillin sensitive *S. aureus* (MSSA). Furthermore, in 90% of samples that were surveyed by quantitative methods there were $\geq 10^5$ cfu/g of tissue, however information was not given regarding the status of the wounds (colonised or infected) so the significance of this finding is limited (Melendez et al., 2010).

As a wound infection progresses the species of bacteria present alters. During the earlier stages of wound infection aerobic bacteria which are present on the surface of the skin such as CONS and *Corynebacterium. spp,* are able to colonise the wound bed. Environmental bacteria such as Gram-negatives then start to invade the wound bed and compete with the other species. During the latter stages of wound infection anaerobic species become more prevalent (Daeschlein, 2013). In the

deeper layers of the wound strict anaerobes may be able to survive in the presence of oxygen by utilising a symbiotic relationship known as co-aggregation where aerobes (including facultative anaerobes) reduce oxygen, creating an anaerobic environment for anaerobes to persist (Daeschlein, 2013) . As chronic wounds also involve biofilms, it has been shown that oxygen is unable to substantially penetrate the substratum of the biofilm allowing anaerobes to thrive, supporting the hypothesis of co-aggregation (Rasmussen and Lewandowski, 1998).

The population of anaerobes and aerobes differs for each chronic wound type. For example, venous ulcers were found to have a greater population of facultative anaerobes, whilst pressure and diabetic ulcers were found to have greater numbers of strict anaerobes (Dowd, S. et al., 2008). Interestingly aerobic bacteria were not readily identified, highlighting that culture methods are only able to identify a snapshot of the wound microbiome (Dowd, S. et al., 2008). The complex and diverse nature of the chronic wound biofilm indicates that not only is recognising the species present within chronic wound biofilm important in infection management, but relative populations may provide vital information to aid treatment regimes.

Dowd and collegues set out to gain insight into to microbiome of diabetic foot ulcers without any culture bias. Using Bacterial Tag Encoded FLX Amplicon Pyro sequencing (bTEFAP) the species ubiquitous with diabetic foot ulcers were assessed (Dowd, S.E. et al., 2008). Corresponding with other studies, Corynebacterium was identified most frequently in 75% of samples along with anaerobic genera such as *Bacteroides. spp* (63%), *Peptoniphilus. spp* (63%) and *Fingoldia. spp*.(58%), despite

staphylococci (32%) and pseudomonads (20%) being not as commonly identified as in previous studies. Dowd and colleagues hypothesised that it may not be a single species alone which contributes to the pathogenesis of chronic wound biofilm infections but a selection of bacteria which work together enhance their survival and form Functional Equivalent Pathogroups (FEPs). Of the 8 FEPs proposed anaerobes were featured in all FEPs, supporting the co-aggregation relationship. Wound chronicity may be directly related to the presence of FEPs on the wound bed. Microorganisms such as *P. aeruginosa,* beta-haemolytic streptococci and *S. aureus* may produce sufficient virulence factors to ensure their survival in the biofilm, whilst the microorganisms present within the FEPs may each have their own role to contribute to virulence and survival (Dowd, S.E. et al., 2008).

1.2.3 Current antimicrobial wound management strategies

As described above, the chronic wound microbiome usually exists in a polymicrobial state (Rhoads, 2012, Melendez, 2010); this signifies the need for antibacterial agents that have broad spectrum activity and are effective against the diverse range of bacteria present within a wound. However, the use of antimicrobial therapy for chronic wounds is often cause for debate, as antibacterial agents which are often useful against planktonic cultures may have no or little effect on some biofilms (Costerton, 1999). Therefore, strategies are required to find effective broad spectrum antibiofilm agents to improve resolution of chronic wounds. Topical antibacterial agents are typically favoured over systemic antibiotics; when treating chronic wounds the efficacy of systemic antibiotics can be as low as 25% (Rhoads, D. et al., 2008). This reduced efficacy of systemic antibiotics is more problematic

when treating ischemic chronic wounds as the antibiotic is unable to reach the site of infection (Rhoads, D. et al., 2008). Additional challenges we face in finding effective wound treatments, are that treatments must be bactericidal to ensure that the wound is clear of bacteria which have the ability to recolonize the wound.

Concurrent debridement and antimicrobial therapy has been hailed the optimum strategy for reducing the wound bioburden (Phillips, 2012). Debridement is the physical removal of necrotic tissue and wound slough; in turn tissue contained within the biofilm will also be removed (Metcalf and Bowler, 2013). The use of debridement has been shown to be most effective on mature biofilms. Bacteria within mature biofilms have reduced metabolic activity and the process of debridement encourages the return to an increased metabolic rate to facilitate replication and biofilm regrowth, allowing antimicrobial agents to reach their target (Schultz et al., 2010). Despite the advantages of sharp debridement, it is not capable of complete clearance of biofilms and infected slough, biofilms have been known to reform within as little as 24 hours (Bowler, P., 2014). It is therefore important to combine the use of topical antimicrobial agents with debridement to ensure sufficient clearance.

There is a long standing history regarding the use of topical antiseptics for the management of chronic wounds infections (Lipsky and Hoey, 2009). Table 1.1 contains a list of antiseptic agents commonly used in the management of wound infections, along with information regarding their mechanism of action, spectrum of antibacterial activity and effect of biofilms.

Table 1. 1 Table of commonly used antibacterial wound agents, their spectrum of activity, mechanism of action, and effect on biofilms

Antibacterial agent	Spectrum of activity	Mechanism of action/Proposed mechanism of action	Effect on biofilms	Reference(s)
Acetic acid	Activity against Gram-positive and negative bacteria. Especially effective against *P. aeruginosa*	Mechanisms of actions unknown, however it is thought to act on the bacterial cell wall.	Limited antibiofilm activity	(Lipsky et al., 2009)
Cetrimide	Poor activity against *P.aeruginosa.* Has activity against other bacteria, fungi and yeast.	Targets the bacterial lipid bilayer. At bactericidal levels respiration, cell wall biosynthesis and solute transport are targeted.	Limited antibiofilm activity	(Gilbert and Moore, 2005; Group, 2012; Percival, Steven. L et al., 2014)
Chlorhexidine (CHX)	Active against Gram-negative and Gram positive bacteria	Chlorhexidine targets the bacterial cell wall and membrane, resulting in cytoplasmic leakage.	Little evidence of CHXs efficacy. Biofilm eradication ability of Chlorhexidine comparable to PHMB vs *S. aureus.*	(McDonnell and Russell, 1999)
Hydrogen Peroxide	Active against Gram-positive and Gram-negative bacteria	Affects DNA causing DNA strand breakage. Oxidising agent.	Hydrogen peroxide at 3% and 5% was able to rapidly eradicate *S. epidermidis* biofilms	(McDonnell et al., 1999; Presterl et al., 2007; Lipsky et al., 2009)

Table 1.1 (Continued) Table of commonly used antibacterial wound agents, their spectrum of activity, mechanism of action, and effect on biofilms

Antibacterial agent	Spectrum of activity	Mechanism of action/Proposed mechanism of action	Effect on biofilms	Reference(s)
Iodine	Active against Gram-positive and negative bacteria. Also active against fungi, viruses yeasts and protozoa	Mechanism of action not fully understood. Iodine is thought to oxidise membrane and cytoplasmic components.	Moderate antibiofilm activity. Can disrupt 3 and 7 day old polymicrobial biofilms.	(Lipsky et al., 2009; Percival, Steven. L et al., 2014; Fitzgerald et al., 2017)
Polyhexanide (PHMB)	Broad spectrum of activity against most bacteria, fungi and yeast	Targets bacterial cell membrane. Possible DNA damaging agent.	Good activity against *in vitro* biofilms of S*. aureus, E. coli* and *P. aeruginosa*	(Gilbert et al., 2005; Lipsky et al., 2009)
Silver	Bactericidal against Gram-positive and Gram-negative bacteria.	Membrane damage and interactions with proteins at thiol, carboxyl and sulfhydryl groups.	Limited biofilm prevention and eradication ability. Concentrations of 100 mg/L unable to disrupt or prevent biofilm formation. Nanoparticles and silver ions have been found to be most effective in reducing bacterial load.	(Lipsky et al., 2009; Wilkinson et al., 2011; Group, 2012; Randall et al., 2013; Percival, Steven. L et al., 2014)
Triclosan	Broad spectrum of activity, no activity against *P. aeruginosa*	Targets fatty acid synthesis, target, enoyl-acyl carrier protein reductase.	Limited antibiofilm activity	(Heath et al., 1999; Dann, 2011)

Silver and iodine represent antiseptic agents that have been used for many years, not only for the management of chronic wound but also for other SSTIs (Williamson et al., 2017); their use and antibiofilm activity is discussed below:

Silver ions are known to have bactericidal activity against both Gram-positives and negatives (Lipsky et al., 2009). Historically, solutions of silver nitrate have been used to cleanse wounds; however more recently new formulations of silver such as silver sulfadiazine (SSD) cream have been introduced (Group, 2012). The use of silver against biofilm infections is debated as *in vitro* studies have suggested that silver ions are unable to eradicate *S. aureus* biofilms (Randall et al., 2013), by contrast, Chaw and colleagues demonstrated that silver ions are able to partially destroy *S. epidermidis* biofilms (Chaw et al., 2005). In addition, silver toxicity to human cells is an issue; *in vitro* studies have demonstrated that silver may perturb wound healing due to cytotoxicity against keratinocytes and fibroblasts, although these findings are not reflected *in vivo* (Group, 2012).

Iodine has been used for its antimicrobial properties for in excess of 150 years (Lipsky et al., 2009). Povidone-iodine (PVP-I) and cadexomer iodine are the formulations most commonly used when treating chronic wound infections, however pain has been reported with use of both formulations (Lipsky et al., 2009; Ammons, 2010). Iodine has a broad spectrum of activity against both Gram-positives and negatives and fungal infections and PVP-I has been demonstrated to disrupt 3 and 7 day polymicrobial biofilms (Campbell, 2013). Cadexomer Iodine is now the formulation favoured and has been found useful when treating venous leg ulcers (Ammons, 2010; Fitzgerald et al., 2017), furthermore the disruption of *S.*

aureus biofilms was observed both *in vivo* and *in vitro* when challenged with cadexomer iodine (Akiyama et al., 2004). The broad spectrum of activity is thought to be attributed to iodine's ability affect multiple targets including proteins, nucleotides and cell membrane fatty acids (Campbell, 2013) .

Despite their long history of use, the antimicrobial agents mentioned here have varied efficacy against biofilm bacteria. With biofilms being observed in >60% of chronic wound samples (Malone et al., 2017a), it is advantageous to understand the underlying principles and mechanisms of biofilm formation and their structure, in order to optimise the management of biofilms related infections.

1.3 Introduction to bacterial biofilms

Biofilm communities are commonly documented and it is expected that over 99% of bacterial biomass on earth exist within a biofilm (Hall-Stoodley et al., 2004). Biofilms have been identified in water systems, on the surface of teeth, on early fossils and in biofouling of ships hulls (Hall-Stoodley et al., 2004; Kim, W. et al., 2013; Mancl et al., 2013). Antonie van Leeuwenhoek first observed bacteria living in communities under a primitive microscope; however at the time they were not known as biofilms. Since then bacteria in sessile communities have been identified, and in 1978 Costerton coined the term "biofilm" (Donlan and Costerton, 2002). Biofilms are implicated in approximately 80% of human infections; in spite of this, most methods of studying bacterial infections favour experimentation involving planktonic cells (Del Pozo and Patel, 2007). Bacterial biofilms are commonly associated with cystic fibrosis, periodontitis, chronic wounds, endocarditis,

indwelling catheters and other implanted medical devices (Zhao et al., 2013). As described in Section 1.2.2, the two most frequently isolated bacteria from chronic wound samples are *P. aeruginosa* and *S. aureus*. The following sections will mostly focus on the biofilms formed by these microorganisms and their structure.

1.3.2 Formation and structure of biofilms

The biofilm phenotype has been hypothesised as advantageous as it may protect bacteria against environmental stressors such as UV (ultra-violet light) exposure, metal toxicity and desiccation (Costerton et al., 1999; Hall-Stoodley et al., 2004). In addition to the protection provided, the biofilm phenotype is thought to be favoured due to the close proximity of other cells and the stable growth environment (Costerton et al., 1999). The phenotypic changes from planktonic to sessile may be a result of induction of RNA-polymerase-associated sigma factors (Davies, D., 2003). In *P. aeruginosa* the secondary messenger bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) plays a major role in the switch from a planktonic lifestyle to sessile (Ha and O'Toole, 2015). It is widely accepted that elevated concentrations of c-di-GMP increases biofilm formation in *P. aeruginosa* (Hengge, 2009; Ha et al., 2015). It has been demonstrated that elevated intracellular levels of c-di-GMP increases the expression exopolysaccharide (EPS) genes *pel* and *psl*, conversely flagellum and pilus genes are also down-regulated (Starkey et al., 2009), to promote biofilm formation.

Biofilm formation is a dynamic process which can be dependent on environmental factors and varies from species to species (Dufour et al., 2010). The formation of biofilms occurs in five principle stages; initial attachment, irreversible attachment,

maturation I (microcolony formation), maturation II (macrocolony formation) and dispersal, summarised in Figure. 1.1 (Monroe, 2007).

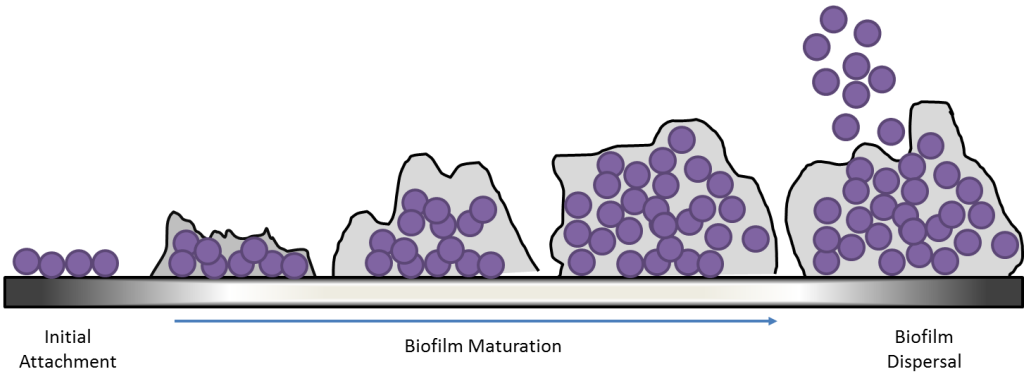


Figure 1. 1 The stages of biofilm development:
Initial attachment, planktonic cells attach to a surface. **Biofilm maturation,** initial colonisers divide and further cells become attached to the surface. Microcolonies form, which secrete EPS and the biofilm continues to grow. **Biofilm dispersal**, once the biofilm has reached a certain biomass, cells are released to go onto form other biofilms or return to planktonic growth. Adapted from (Monroe, 2007)

1.3.2.1 Attachment

In the initial stages of biofilm formation planktonic bacteria adhere to abiotic and biotic surfaces. Attachment is reliant on environmental factors such as pH, oxygen availability, nutrient availability and temperature and iron concentrations (Aparna and Yadav, 2008). To facilitate the attachment of bacteria, the surface is usually conditioned using organic molecules; these organic molecules create a conditioning film, which promotes adherence to the surface by creating sites for bacteria to become attached (Dufour et al., 2010). The initial stages of attachment are reversible and weak van der Waals interactions are made with the cell surface and the substratum, however attachment is reliant on close proximity between the surface and the bacterium (Dufour et al., 2010). Extracellular structures of bacteria,

such as type-V pili and flagella, along with LPS have also been shown to facilitate early attachment of bacterial cells to surfaces and may promote the recruitment of other bacteria and during the early stages of this biofilm development bacteria are exponentially dividing (O'Toole et al., 2000; Aparna et al., 2008). To irreversibly bind, weak van der Waals forces initiate stronger interactions such as hydrogen, ionic, dipole interactions between flagella, pili, LPS and exopolysaccharides and the surface (Dufour et al., 2010). On biotic surfaces such as the chronic wound bed *S. aureus* utilities surface-anchored proteins known as MSCRAMMs (Microbial surface components recognizing adhesive matrix molecules) to bind to host proteins (Otto, 2013).

1.3.2.2 Maturation and Dispersal

Following attachment of bacterial cells, the primary colonisers replicate to develop microcolonies, alternatively other bacteria may be recruited to enrich the microcolony (Aparna et al., 2008). The chronic wound biofilm environment is poorly understood, however the oral biofilms which have been studied for many years are often used as a model for biofilm formation. Bacteria in the oral biofilm utilise two mechanisms co-aggregation and co-adhesion to assist biofilm development (Mancl et al., 2013). Co-aggregation of the oral biofilm is described as the clumping of genetically distinct bacteria in suspension, whilst co-adhesion is the linking of bacteria attached to the substratum with planktonic bacteria in suspension (Mancl et al., 2013).

Once the microcolony has reached a thickness of approximately 10 µm, the biofilm develops to form a macrocolony and cell to cell interactions moderated by quorum

sensing increase (Aparna et al., 2008). Quorum sensing uses cell population density to regulate the expression of genes; once cell populations have reached a certain threshold autoinducers are released. Gram-negatives such as *P. aeruginosa* use acylated homoserine lactones (AHLs), a system first described in *V. fischeri*, to communicate; whilst Gram-positives use small peptides secreted by ATP-binding cassette transporters (Miller and Bassler, 2001). *P. aeruginosa* uses two quorum sensing systems, LasI/LasR and RhlI/RhlR; AHL autoinducers bind to LasR at high cell densities. The complex of autoinducer and LasR can then binds to promoter regions, stimulating the expression of desired genes. LasI/LasR quorum sensing is a positive feedback system; activation of LasR also actives LasI, which is responsible for the production of more AHLs (Miller et al., 2001). Quorum sensing is an astute mechanism of communication and bacteria may communicate with other species as well as their own (Miller et al., 2001; Lowery et al., 2008). In addition to its role in virulence and antimicrobial resistance, quorum sensing plays a role in biofilm formation and maturation. The role of quorum sensing and *P. aeruginosa* biofilm formation is relatively well studied; there are three biosynthetic genes in *P. aeruginosa* noted for the production of EPS; *alg*, *psl* and *pel*. In *P. aeruginosa* cells defective in the quorum sensing systems LasI and RhI, expression of *pel* was reduced, resulting in biofilms with poor structure and reduced pellicle formation at the liquid-air interface (De Kievit, 2009). In *S. aureus* the quorum sensing Agr (accessory gene regulator) system is associated with the formation of water channels to allow nutrients to flow through the biofilm (Otto, 2013).

Following the development of the biofilm to a macrocolony, the final stage of dispersal occurs and dispersal strategies vary from species to species. In non-mucoid *P. aeruginosa* biofilms "swarming dispersal" occurs where the cells in the inner layers of microcolonies are released following liquefaction of the inner regions of the biofilm (Hall-Stoodley et al., 2004). Alternatively, *S. aureus* may use clumping dispersal strategies where clumps or emboli of bacteria still encapsulated in EPS are released. These cells express a biofilm phenotype in contrast to the planktonic phenotype expressed by cells released by swarming dispersal (Hall-Stoodley et al., 2004). The Agr quorum sensing system is a key regulator of *S. aureus* biofilm maturation and is also primarily associated with dispersal and regrowth of the biofilm (Otto, 2013). The expression of proteases and phenol-soluble modulins (PSMs) and other toxin are upregulated as result of the Agr system, this leads to formation of water channels and biofilm dispersal (Le and Otto, 2015). Mutants deficient in the *agr* system have been demonstrated to produce sturdier biofilms (Vuong et al., 2000). Dispersal is a key element of biofilm development as the cells released may go on to other surfaces to form new biofilms.

1.3.2.3 Structure

As with biofilm formation, the structure of biofilms differs from species to species. One component present in all biofilms is the hydrated extracellular matrix encapsulating the cells (Del Pozo et al., 2007; Dufour et al., 2010). The extracellular matrix is primarily composed of protein and EPS, with extracellular DNA, RNA, lipids and nutrients also being present within the matrix and secreted by the bacteria (Del

Pozo et al., 2007; Mancl et al., 2013). EPS forms approximately 80-85% of biofilm structures with the remaining 15-20% containing bacterial cells (Dufour et al., 2010). In *S. aureus* biofilms, the major component of the matrix is polysaccharide intercellular adhesin (PIA) which is often referred to as polymeric N-acetyl-glucosamine (PNAG) (Lister and Horswill, 2014). PIA expression in *S. aureus* and *S. epidermidis* adherent cells is regulated by the *icaADBC* locus (Lister et al., 2014); the **i**ntra**c**elluar **a**dhesion proteins encoded by this operon are responsible for the excretion of PIA and are negatively regulated by IcaR (Cerca et al., 2008). There are some *S. aureus* biofilms that do not have PIA as their main matrix component (Archer et al., 2011); these PIA-independent biofilms form through the expression of cell wall associated proteins such as protein A (SpA), biofilm-associated proteins (Bap) and fibronectin binding proteins (FnBPs) (Archer et al., 2011). It has been noted that PIA-independent biofilm formation is strongly correlated with methicillin- resistant *S. aureus* (MRSA) isolates (O'Neill et al., 2007).

The EPS of *P. aeruginosa* constitutes three major exopolysaccharides Pel, Psl and alginate (De Kievit, 2009). Different *P. aeruginosa* isolates produce varying levels of each exopolysaccharide; the EPS of non-mucoid strains such as PAO1 and PA14 are primarily made up of Psl and Pel, respectively (Colvin et al., 2012). Mucoid strains of *P. aeruginosa* are typically isolated from patients with cystic fibrosis; these isolates overproduce alginate, however the Psl polysaccharide is still essential for structural stability of the biofilm (Ma et al., 2012; Wei and Ma, 2013).

The shape of biofilm structures is influenced by oxygen and nutrient availability; in *in vitro* flow cell systems where there is constant flow of fresh media, *P. aeruginosa*

microcolonies assume a mushroom-like structure (Bjarnsholt et al., 2013). The "mushroom shape" biofilm, formed by *P. aeruginosa,* is most commonly seen when glucose is used as a carbon source, however when an alternative carbon source is provided the biofilm aggregate is flat (Bjarnsholt et al., 2013). Throughout the biofilm structure there are open water channels which provide the biofilm with sufficient nutrients and the ability to remove waste products (Hall-Stoodley et al., 2004). The formation of water channels is aided by the presence of enzymes, such as alginate lyase in *P. aeruginosa* biofilms, which are able to degrade the EPS to give the biofilm its structure (Hall-Stoodley et al., 2004).

Throughout the biofilm matrix there is cell heterogeneity, in polymicrobial biofilms there may be phenotypic variation within and between species (Fig. 1.2) (Dufour et al., 2010). In the different regions of the mono-species biofilm genetically identical species may express different phenotypes, depending on the environmental signals such as oxygen and nutrient gradients (Dufour et al., 2010). Heterogeneity may also be present when genetically identical species in the same local environment express different phenotypes; this phenotypic variety arises from stochastic noise which is reliant on protein abundance in individual cells (Dufour et al., 2010). Finally heterogeneity may be observed when genetic mutations occur in populations under environmental stress (Dufour et al., 2010). The genetic mutations result in subpopulations of cells able to resist environmental stress.

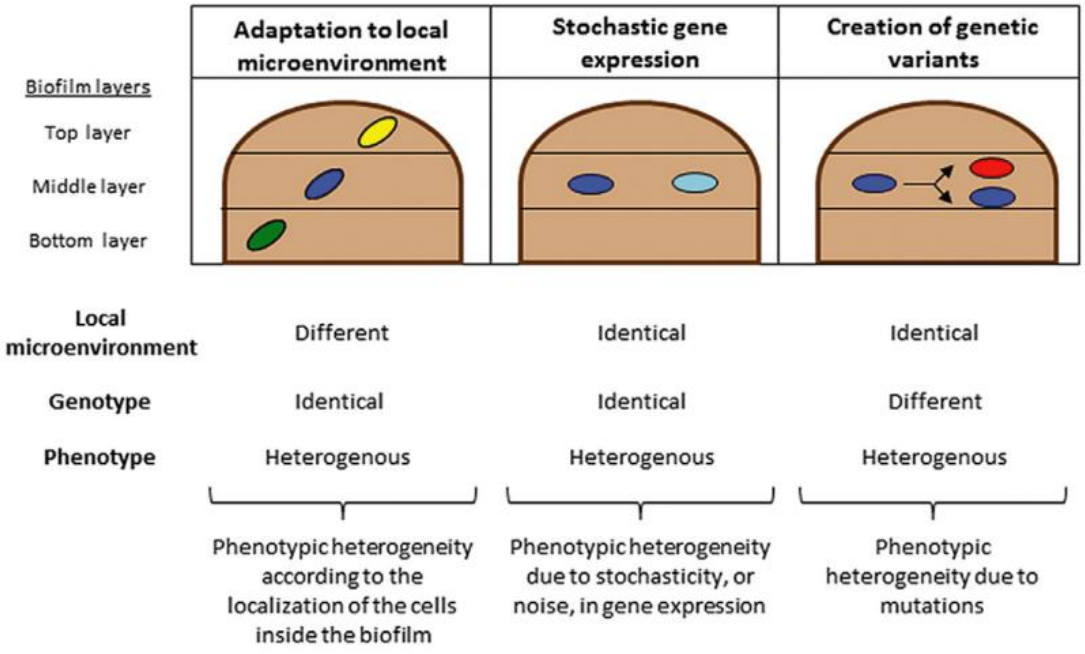


Figure 1. 2 The mechanisms of cell heterogenieity in mono-species biofilms. Phenotypic variation may be a result of adaption to the local microenviroment, cells in the inner layers of the biofilm are phenotypically different from cells in the outer layers of the biofilm. Heterogeneity can occur in the cells which share the same micro-enivronment, their phenotypic differences arise from stochasticity in gene expression. In environments where species are under stress, genetic mutations may occur to create phenotypic heterogeneity. Taken from (Dufour et al., 2010)

1.3.2.4 *Staphylococcus aureus* and *Pseudomonas aeruginosa*: interactions in mixed species wound biofilms

The presence of multiple species in the chronic wound bed, especially *S. aureus* and *P. aeruginosa*, has been linked to wound chronicity and reduced healing times (Dalton et al., 2011). Previous reports have demonstrated that, *in vivo* both microorganisms occupy different areas of the chronic wound biofilm; with *S. aureus* at the outer surface of the biofilm and *P. aeruginosa* in the deeper regions on biofilm closest to the wound bed (Fazli et al., 2009). Further study of wound

biofilms has demonstrated that the two microorganisms can closely associate, however distinct “pockets” of bacteria can be seen (Dalton et al., 2011). By contrast, in the same study and others, *in vitro* biofilms of both microorganisms are observed in close proximity (Dalton et al., 2011; DeLeon et al., 2014).

Drawing from the knowledge of *S. aureus* and *P. aeruginosa* co-culture in cystic fibrosis (CF) patients, it is often thought that the relationship between the two organisms is antagonistic (Hotterbeekx et al., 2017). In CF biofilms, *P. aeruginosa* outcompetes *S. aureus* during the latter stages of infection (Filkins et al., 2015). *P. aeruginosa* produces the compound 2-heptyl-4-hydroxyquinoline ***N***-oxide (HQNO) and various other siderophores, which inhibit the electron transport chain in *S. aureus* colonies (Filkins et al., 2015). In the chronic wound setting it has been demonstrated it *S. aureus* virulence factors such as Panton-Valentine leukocidin and α-haemolysin are upregulated in the presence of *P. aeruginosa*, it was suggested that the upregulation of such virulence factors contributes to reduced healing times (Pastar et al., 2013). Further study has revealed that the relationship between *S. aureus* and *P. aeruginosa* may actually be co-operative in the wound environment. The two microorganisms were described to interact directly, with *S. aureus* acting as early colonisers to facilitate the attachment of *P. aeruginosa* (Alves et al., 2018). Whilst more information regarding the nature of the relationship between *S. aureus* and *P. aeruginosa* is emerging; it is generally agreed that antimicrobial tolerance and resistance is enhanced in mixed species biofilms (Dalton et al., 2011; Beaudoin et al., 2017; Hotterbeekx et al., 2017).

1.4 Biofilms, antimicrobial tolerance and resistance: a recalcitrant mode of growth

As described in Section 1.2.3 the burden of chronic wounds is intensified by the limited efficacy of antimicrobials against biofilms. Studies have estimated that biofilms are up to 1500 times more tolerant to antibiotics in comparison with their planktonic counterparts, in addition biofilms are able to effectively evade the hosts' immune response (Mancl et al., 2013). Within the biofilm structure both antimicrobial resistance and tolerance can be observed. In order for antibacterial resistance to occur the bacteria must undergo genetic mutations or acquire resistance genes via horizontal gene transfer, however antibacterial tolerance is the survival of bacteria challenged by antimicrobials without genetic mutations (O'Neill, 2010). The tolerance of biofilms to antimicrobials is primarily due to alterations in phenotypic characteristics that separate biofilms from their planktonic counterparts (Dufour et al., 2010). (Figure 1.3)

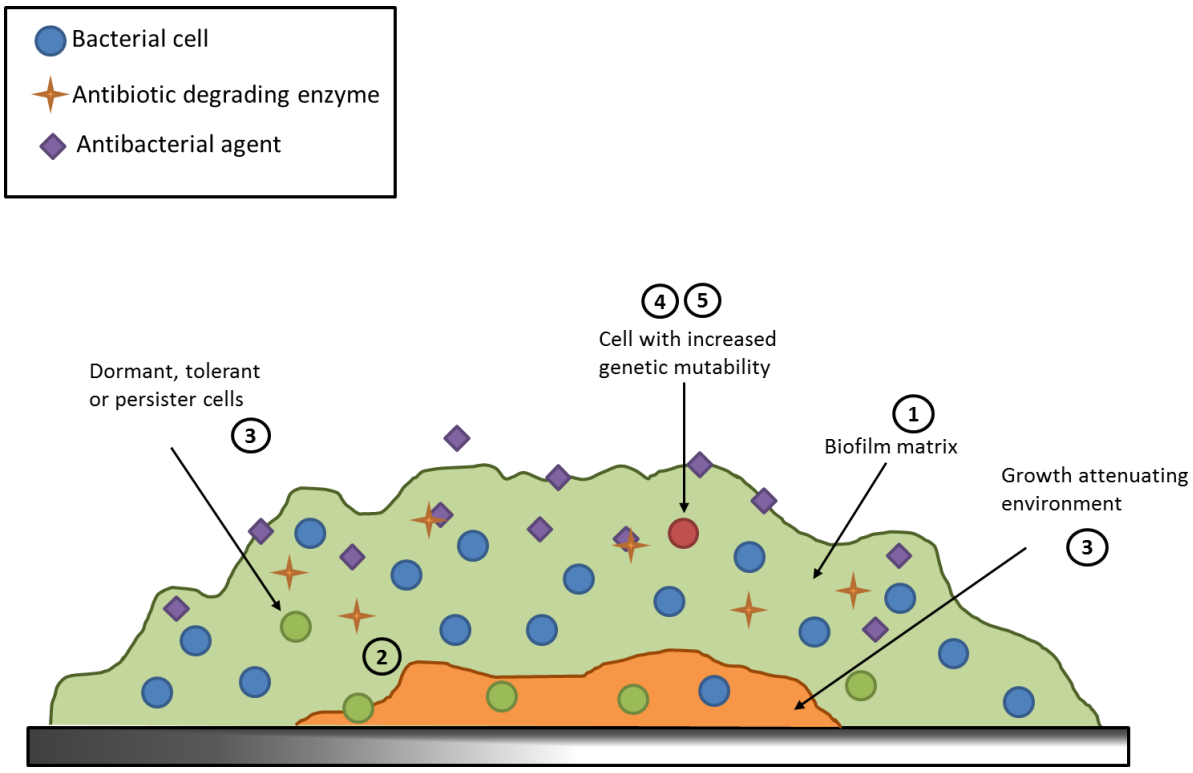


Figure 1. 3 Proposed mechanisms facilitating biofilm tolerance to antimicrobials

1| Antimicrobial activity is attenuated by poor diffusion across the matrix; however antimicrobials may be active against bacteria in the outer layers of the matrix or newly dispersed cells. **2|** Antibiotics may diffuse into the matrix but are neutralised and trapped by antibiotic chelator enzymes such as β-lactamases **3|** A subpopulation of slow- or non- growing cells are present within the biofilm, resulting in the target of antibiotics being absent or expressed at low levels**. 4|** Bacteria may express biofilm-specific genes that results in the expression of efflux pumps to remove the antimicrobial from the environment **5|**Antimicrobial degrading enzymes may be upregulated in response to environmental stressor (pH, oxygen concentrations and nutrient availability) Adapted from (Del Pozo et al., 2007)

1.4.1 Slow/non-growing cells

Within biofilm communities bacterial cells enter a quiescent state and are slow- or non-growing due to nutrient limitation and oxygen depletion within the matrix (Donlan, 2001). This poses a challenge as the majority of antimicrobial agents' mode of action involves the disruption of key biological processes such as synthesis

of DNA, protein, cell wall and essential metabolites. The existence of these slow-growing cells within the biofilm results in antibacterial indifference, as the target of antibiotic is either inactive or active at levels which are not sufficient to initiate killing (Ammons, 2010). Indeed, this recalcitrance to antibiotics is reversible and once the bacteria are released either by dispersal or physical removal they are susceptible to antibiotics again (Bjarnsholt et al., 2013).

1.4.2 Persistence

The phenomenon of bacterial persistence has been described in both planktonic and biofilm communities (Del Pozo et al., 2007; O'Neill, 2010). Persistence can occur when bacteria are challenged with a bactericidal antibiotic and a small population of these bacteria survive the antibacterial challenge (O'Neill, 2010). Like the bacteria deemed as “indifferent”, persister cells are in a quiescent state which provides them with the ability to circumvent antibacterial challenge (Del Pozo et al., 2007). However this non-growing state is not due to the restrictive environment of the biofilm but due to a phenotypic variation resulting in a “dormant” state (Dufour et al., 2010). The persistence model in biofilms suggests that metabolically active cells are killed following antibacterial exposure; however the persister cells in the biofilm remain intact. The persister cells protected by the biofilm matrix may then go on to repopulate the biofilm (Dufour et al., 2010). The persistence phenotype is thought to be distinct from the non-growing phenotype. This has been exemplified in *E. coli* where non-growing cells were susceptible to fluoroquinolones, however when *E. coli* persister cells were exposed to fluoroquinolones they remained unaffected (O'Neill, 2010).

1.4.3 Increased mutability

The physiological variations associated with the biofilm mode of growth are thought to be the main cause of reduced susceptibility to antimicrobial agents and genetic variability is often an overlooked as a source of this reduced susceptibility (Olsen, 2015). Oxidative stress has been demonstrated to increase the genetic mutability in *S. aureus* and *P. aeruginosa* biofilms. Selection of ciprofloxacin resistant *P. aeruginosa* mutants was shown to be around 105 fold higher for biofilm vs planktonic cultures (Driffield et al., 2008). Biofilm maturity can also be associated with the increased mutability of biofilm cells. For 6 day old *S. aureus* biofilms the selection of rifampicin resistant mutants increased ~60 fold, whilst the mutation frequency for 4 day old biofilms increased four-fold (Ryder et al., 2012). The close proximity of other cells creates the ideal environment for genetic transfer (Olsen, et al., 2013), it has been demonstrated in *S. aureus* biofilms that the transfer of plasmid DNA increases via conjugation (Savage et al., 2013).

1.4.4 The protective EPS matrix

In addition to providing biofilms with stability and nutrients, the extracellular matrix also plays a protective role against antimicrobials (Dufour et al., 2010). The EPS matrix may reduce penetration of the biofilm by antibacterial agents by either reducing diffusion or by binding to the antibiotic directly (Dufour et al., 2010). Diffusion of aminoglycosides is impaired by a negatively charged matrix, however the effect of reduced penetration is limited and only short term (Bjarnsholt et al., 2013). The short-term mechanism of reduced penetration of the EPS is exemplified by the ability of fluoroquinolones to penetrate the matrix of *P. aeruginosa* and

Klebsiella pneumoniae biofilms (Del Pozo et al., 2007). In *P. aeruginosa* biofilms antimicrobial peptides and aminoglycosides have been shown to be thwarted by extracellular DNA via chelation of cations creating a cation-limited environment (Bjarnsholt et al., 2013). In *Klebsiella pneumoniae* penetration of the biofilms EPS by β-lactams, such as ampicillin, is hindered by β-lactamase released from the bacteria as a stress response triggered by antibiotic exposure (Bjarnsholt et al., 2013). In biofilms deficient in β-lactamase, ampicillin was shown to penetrate the EPS. However, bacteria in these biofilms remained resistant to ampicillin treatment (Davies, D., 2003), highlighting that reduced penetration of the EPS alone is not culpable for reduced susceptibility to antimicrobials in biofilms.

1.5 Aims and objectives

The burden of topical infections including chronic wounds is increased by the lack of antibacterial agents able to successfully treat these infections; however, developing a new antibacterial agent requires an average of 17 years and billions of dollars (Zoë Slote et al., 2011). This body of work explores other avenues to shortcut antibacterial discovery and develop agents able to successfully treat topical infections, such as those observed in chronic wounds. Currently the treatment regimens for such infections are suboptimal and require substantial improvement (Han et al., 2011). The ideal antibacterial agent able to treat topical skin infections should fulfil all or most of the following criteria:

i. **Broad spectrum-** A vast array of microorganisms are associated with topical infections (Melendez et al., 2010), therefore effect treatments should be able to kill multiple bacterial species. Alternatively, antimicrobial treatments must be able to display potent activity against at least one of the essential infecting microorganisms e.g. *S. aureus* and *P. aeruginosa.*
ii. **Have a low propensity to developing resistance-** Antimicrobial resistance continues to be one of the major barriers for the effective resolution of topical infections (Davies, J. and Davies, 2010). Consequently, antimicrobial agents designated to treat topical infections should not be able to readily select for resistance in the associated pathogens.
iii. **Have bactericidal properties-** Resolution of topical infections requires antimicrobial agents able to not only inhibit bacterial growth (bacteriostatic) but display extensive killing (bactericidal). Individuals affected by topical infections often have weakened immune systems (Guo et al., 2010), therefore a bactericidal antimicrobial agents can compensate for the compromised immune system which may be unable to clear the infection sufficiently.

iv. **Eradicate biofilms-** The presence of biofilms in chronic topical infections presents a substantial challenge for their resolution (Hall-Stoodley et al., 2004; James et al., 2008). Biofilms are associated with reduced susceptibility to antimicrobial agents and protection from host mediated clearance. Effective eradication of bacteria encapsulated within the biofilm is essential to prevent the reformation of the biofilm and to ensure the resolution of topical infections.

This study present aimed to find effective ways to circumvent the lack of topical antibacterial agents using three strategies:

1. **Combination therapy-** The use of multiple agents in combination offers many advantages; and has been employed in many fields outside of antibacterial discovery (Sleire et al., 2017). Combination therapy has been demonstrated to reduce the risk of antimicrobial resistance, broaden the spectrum of bacterial targets and achieved increased killing (Ahmed et al., 2014). Chapter three aims to identify combinations of established antimicrobial agents used for the management of wounds that display improved kill against biofilm and planktonic cultures.
2. **Repurposing agents-** Using a drug or agent which has been previously designated for one application and using it for another is known as repurposing (Van den Driessche et al., 2017). Repurposing non-antibiotic agents which are currently licenced for use in topical applications may provide an accelerated route for their use as antibacterial agents. Chapter four aims to identify agents used in topical healthcare products, which display antibacterial and antibiofilm activity against microorganisms responsible for topical infections.
3. **Reviving unexploited antibiotics-** The rise of antimicrobial resistance has increased the need to find new antibacterial scaffolds. However this void has not been filled in the past 20 years. Revisiting abandoned scaffolds has proved successful in the case of antibiotics such as daptomycin, fidaxomicin and linezolid (Silver, 2011; McAlpine, 2017). With the preceding agents their

antimicrobial activity was known in the late 1970's to 80s, they were later introduced into the clinic after the turn of the 21st century after being previously removed from the antibiotic pipeline. The antibacterial activity and mode of action of an unexploited antibiotic will be discussed in Chapter five.

Chapter 2 Materials and Methods

2.1 Bacterial strains, plasmids and growth conditions

Bacterial strains used in this study are displayed in Table 2.1. Plasmids used in this study are listed in Table 2.2. Strains were propagated from -80 °C 20% (v/v) glycerol stocks in cation-adjusted Mueller-Hinton broth (MHB-II) (Sigma Aldrich, Poole, UK) and streaked onto cation-adjusted Mueller Hinton Agar (MHA-II) to obtain single colonies. Broth cultures were grown at 37 °C with aeration for 18-24 hours; agar plates were incubated for 24 hours at 37 °C. When necessary, antibiotic was added to both broth and agar.

Table 2. 1. Bacterial strains used in this study

Strain Microorganism	Comments, Source
***Enterococcus faecalis* ATCC29212**	Type Strain ATCC (Kim, E.B. et al., 2012)
***Pseudomonas aeruginosa* NCTC 13302**	Type Strain, NCTC
***Pseudomonas aeruginosa* PA14**	Highly virulent *P. aeruginosa* (Mikkelsen et al., 2011)
***Pseudomonas aeruginosa* PAO1**	(Holloway, 1955)
***Staphylococcus aureus* ATCC 25923**	Type Strain, ATCC
***Staphylococcus aureus* RN4220**	Restriction deficient *S. aureus* 8325-4 (Fairweather et al., 1983)
***Staphylococcus aureus* SH1000**	Derivative of *S. aureus* 8325-4 with functional *rsbU* (Horsburgh et al., 2002)
***Staphylococcus aureus* UAMS-1**	Proficient biofilm former (Gillaspy et al., 1995)
***Staphylococcus aureus* USA300**	Community acquired methicillin resistant *S. aureus*
***Staphylococcus epidermidis* RP62A**	Type Strain, Proficient biofilm former, (ATCC 35984)

Table2.1 (Continued) Bacterial Strains used in this study

Strain	Microorganism	Comments, Source
Acinetobacter baumannii **053736**		Clinical Isolate (Gift, Liverpool University)
Acinetobacter baumannii i**097373**		Clinical Isolate (Gift, Liverpool University)
Enterobacter cloacae **052026**		Clinical Isolate (Gift, Liverpool University)
Enterobacter cloacae**067255**		Clinical Isolate (Gift, Liverpool University)
Escherichia coli **32**		Clinical Isolate (Gift, Dublin Children's Hospital)
Escherichia coli **52**		Clinical Isolate (Gift, Dublin Children's Hospital)
Escherichia coli **BW25113**		Keio collection parental strain *E. coli* K-12 derivative (Baba et al., 2006)
Escherichia coli **ΔAcrA**		Keio Collection BW25113 ΔAcrA, Kanamycin @ 25 ug/mL (Baba et al., 2006)
Escherichia coli **ΔAcrB**		Keio Collection BW25113 ΔAcrB, Kanamycin @25 ug/mL (Baba et al., 2006)
Klebsiella pneumoniae **062046**		Clinical Isolate (Gift, Liverpool University)
Klebsiella pneumoniae **081360**		Clinical Isolate (Gift, Liverpool University)

Table 2. 2 Plasmids used in this study

Plasmid	Comments Source
pRAB11	*S. aureus* / *E. coli* shuttle vector. For expression of genes in *S. aureus* from *Pxyl/tet* anhydrotetracycline-inducible promoter *E. coli* : ampicillin^R at 100 µg/mL *S. aureus*: chloramphenicol^R at 10 µg/mL (Helle et al., 2011)
pOPIN-F	*V*ector for the expression of N-terminal His(6) tagged proteins in *E. coli* *E. coli:* ampicillin^R at 100 µg/mL (Berrow et al., 2007)

2.2 Antibacterial compounds, chemicals, reagents and kits

Antibiotics, antiseptics and chemicals used in this study were from Sigma Aldrich (Poole, UK) with the exception of povidone iodine (Santa Cruz Biotechnology, Dallas, TX, USA), triclosan (LG Life sciences, South Korea), anhydrotetracycline (Cayman Chemical, Ann Arbor, MI, USA), vancomycin (Duchefa Biochemie), ciprofloxacin (Bayer-Leverkusen, Germany), polyhexanide (PHMB) (Fluorochem LTD, Hadfield, UK), Dithiothreitol (DTT) (Fluorochem, UK), Sodium N-(2-Acetamido) iminodiacetic acid (ADA) (Insight Biotechnology, Wembley, UK) and ethanol (Fisher Scientific Ltd, Loughborough, UK). Batumin was initially from Enzo Life Sciences (Exeter, UK) and received as a gift from Dr. Joleen Masschelein (University of Warwick, UK). Antimicrobial agents and their solvents are displayed in Table 2.3.

The live/dead BacLight^{TM} kit was from Invitrogen along with Sypro® Ruby. Molecular biology reagents including T4 DNA ligase, restriction enzymes and Q5 DNA polymerase were from New England Biolabs. PurElute bacterial genomic extraction kit was from EdgeBio. E.Z.N.A. plasmid mini, gel extraction and cycle pure kits were from Omega Biotek (VWR). Human plasma was from Sera Laboratories (West Sussex, UK) and lithium heparin-treated whole equine blood was from Matrix Biologicals (Hull, UK). Antimicrobial wound dressings and irrigations solutions were from Williams Medical Supplies (Rhymney, UK).

Table 2. 3 Antibacterial agents and their solvents

Antibacterial Agent	Solvent
Acetic Acid	H_2O
Ampicillin	H_2O
Anhydrotetracycline	Ethanol
(AO2246) **2,2'-methylenebis-(4-methyl-6-tertiary-butylphenol)**	DMSO
Bakuchiol	DMSO
Batumin	Ethanol
Bromochlorophene	Ethanol
Bronidox	50% (v/v) Ethanol
Bronopol	H_2O
Cetrimide	H_2O
Chloramphenicol	Ethanol
Dimethyl Steramine	Ethanol
Erythromycin	50% (v/v)Ethanol
Hexitidine	Ethanol
(HTHQ) **1-O-hexyl-2,3,5-trimethylhydroquinone**	DMSO
Hydrogen peroxide	H_2O
Mupirocin	50% (v/v) Ethanol
Phytophingosine	Ethanol
Polymyxin B nonapeptide	H_2O
Povidone Iodine	50% (v/v) Ethanol
SDS	H_2O
Silver nitrate	H_2O
Tetracycline	H_2O
Triclosan	Ethanol
Vancomycin	H_2O
Zinc Pyrithione	DMSO

2.3 Antibacterial susceptibility testing

2.3.1 Minimum Inhibitory Concentration determinations

Broth MICs were determined by exposing *Staphylococcus aureus* SH1000 and *Pseudomonas aeruginosa* PAO1 to two-fold serial dilutions of common wound agents, control antibiotics and antioxidants (Table 2.3) according to the Clinical and Laboratory Standards Institute (CLSI) broth microdilution guidelines (CLSI, 2012). Saturated overnight cultures were diluted to 0.5 McFarland Standard using cation-adjusted Mueller Hinton Broth (MHB-II), and then further diluted to achieve approximately 5 $x10^4$ CFU/well. Cultures were incubated at 37°C +/- 2°C for 18-24 hours and the MIC was considered the lowest concentration of compound that completely inhibited all bacterial growth. To ensure reproducibility, experiments were conducted on at least three independent occasions.

2.3.2 Minimum Bactericidal Concentration determination

In parallel with MIC determinations, the minimum bactericidal concentration (MBC) of antimicrobial agents was determined by enumerating bacteria which survived at concentrations above the MIC (Barry et al., 1999). Bacterial cultures were plated onto MHA-II for 24 hours at 37 °C. The MBC is defined as the minimum concentration of antibacterial agent that caused 99.9% kill, compared to an untreated control.

2.3.3 Minimum Biofilm Eradication Concentration determination

The Calgary Biofilm Device (Ceri et al., 1999) was used to determine the minimum biofilm eradication concentration (MBEC) of antibacterial agents. Briefly, the Calgary biofilm device was fitted onto 96 well plates containing 200 µL of saturated overnight cultures diluted 1 in 100 in MHB-II and incubated for 24 hours at 37°C. After 24 hours, biofilms were washed in 200 µL saline twice and exposed to a two-fold serial dilution of antibacterial agents suspended in fresh MHB-II (200 µL) and incubated for a further 24 hours at 37°C. Pegs were washed twice in fresh saline and placed in 200 µL of MHB-II which did not contain any antibacterial agents, and were incubated for a further 24 hours at 37°C. The MBEC was considered as the lowest concentration of antibacterial agent required to completely eradicate biofilms and not allow re-seeding of the biofilm in fresh media. Biofilm growth or lack thereof was compared with an untreated control. To ensure reproducibility, experiments were conducted on at least three independent occasions.

2.3.4 Fractional Inhibitory Concentration Index Determination

To identify synergistic combinations of wound agents and antibiotics, the fractional inhibitory concentration (FIC) index was determined by exposing S*. aureus* and *P. aeruginosa* to two-fold serial dilutions of two antibacterial agents (from 2X the MIC to 1/32X MIC) in a checkerboard assay (Pillai, 2005). Saturated overnight cultures were diluted to 0.5 McFarland Standard using cation-adjusted Mueller Hinton Broth (MHB-II), and then further diluted to achieve approximately 5 x10^4 CFU/well. For agents that did not alone inhibit growth, a top concentration of 256 µg/mL was used. Following 24 hour incubation at 37°C, the FIC index was determined by

identifying the lowest concentration of both drugs required to inhibit the growth of planktonic cells.

The FIC Index was determined using the following calculation:

$$\text{FIC index} = \left(\frac{\text{lowest concentration A}}{\text{MIC A}}\right) + \left(\frac{\text{lowest concentration B}}{\text{MIC B}}\right)$$

An FIC index ≤0.5 indicated synergism, an FIC > 0.5 and ≤4 indicated an indifferent/ additive interaction and a FIC > 4 implied antagonism.

To identify synergistic combinations of wound agents and antibiotics against biofilms, the biofilm FIC index was determined by exposing *S. aureus* and *P. aeruginosa* biofilms to a combination of antimicrobial wound agents. Biofilms were propagated using the Calgary Biofilm Device, in which saturated overnight cultures were diluted 1 in 100 in MHB-II and incubated at 37 °C for 24 hours. Following 24 hour incubation, pegs were washed in sterile saline twice and exposed to two-fold serial dilutions of two antibacterial agents ranging from 2X the MBEC to 1/32X MBEC, in a checkerboard assay at 37 °C for 24 hours. Pegs were washed twice in fresh saline and placed in 200 µL of MHB-II which did not contain any antibacterial agents, and were incubated for a further 24 hours at 37°C. To ensure reproducibility, experiments were conducted on at least three independent occasions.

2.3.5 Time dependent killing studies

Early exponential phase cultures of *S. aureus* SH1000 were exposed to BX, BP and comparator agents (tetracycline and vancomycin) at 4X MIC (Ooi et al., 2015). Cell

viability was assessed by plating onto MHA-II hourly for the first 6 hours, and then at 24 hours. For time kill studies on stationary-phase cultures, the saturated overnight culture was pelleted at 5500 x *g* and resuspended to 0.2 OD 600nm in spent media prior to antibiotic challenge.

2.4 Cellulose Disk Biofilm Model

2.4.1 Biofilm culture and antimicrobial treatment

The previously established cellulose disk biofilm model (Ryder et al., 2012) was modified to assess the efficacy of antibacterial agent and wound dressings against mixed species biofilms grown on 25mm mixed cellulose disks (Millipore, Watford, UK).

Cellulose disks were incubated at 4 °C overnight in 4% (v/v) Human plasma (SeraLab, West Sussex, UK) in 0.05M carbonate buffer. Following incubation, disks were inoculated with saturated overnight cultures of *P. aeruginosa* and *S. aureus* in a ratio of 1:10, this ratio was selected because of the propensity of *P. aeruginosa* to outcompete *S. aureus* in co-culture (Biswas et al., 2009; Pastar et al., 2013). Disks were incubated on Brain Heart Infusion (BHI) agar for 48 hours at 37°C.

Following biofilm growth, biofilms were treated with antibacterial agent. To generate antimicrobial dressings, sterile gauze was saturated with 4 mL of antibacterial agent at the maximum authorised concentration for 5 minutes. Biofilms were transferred to fresh BHI agar and covered with the sterile gauze saturated with antimicrobial agent, commercial antimicrobial wound dressings and

an untreated control. Biofilms were incubated at 37°C for 24 hours. Alternatively biofilms were submerged in antibacterial agents at 37 °C with gentle shaking for 1 hour.

To remove planktonic cells and residual compound, disks were washed in sterile saline for 10 minutes. Adherent bacteria were detached from the cellulose disks by washing in buffered cellulase (1 mg/mL) in 0.05 M citrate buffer [0.5 M sodium citrate & 0.5M citric acid to pH 4.6] for 30 minutes at 37^{o}C with vigorous shaking and intermittent vortexing. Samples were centrifuged (10 minutes at 5,000 x *g*) to collect detached cells and re-suspended in sterile saline. Biofilm cultures were enumerated by dilution plating onto MHA-II and Mannitol Salt Agar (MSA).

2.5 Antibacterial mechanism of action (MOA) studies

2.5.1 Quantification of macromolecular biosynthesis

Inhibition of protein and fatty acid synthesis was monitored by exposing exponential phase cultures of *S. aureus* SH1000 containing radiolabelled precursors to antibacterial agent (Cotsonas King and Wu, 2001). *S. aureus* SH1000 cultures were grown in MHB-II at 37°C to early exponential phase (OD600nm = 0.2). Radiolabeled precursors of protein synthesis (glycine, [1-^{14}C]) and fatty acid biosynthesis (acetic acid, [1, 2-^{14}C],) were added to 1 µCi/mL and incubated for a further 10 minutes at 37°C. At time 0, 100 µL of radiolabelled culture was precipitated in 100 µL ice-cold TCA. The antibacterial agent was then added at 4X MIC to the radiolabelled culture and incubated for 10 minutes. Following incubation, 100 µL of treated culture was added to 10%(v/v) ice-cold TCA and

incubated on ice for 1 hour to facilitate the precipitation of radiolabelled macromolecules. Samples were processed by transferring 200 µL onto a Millipore Multiscreen Filter Plate under vacuum, a process that captured precipitated radiolabels on the filter. Filters were washed twice with 200 µL 5%(v/v) TCA, then twice with 200 µL acetic acid. Radioactivity was counted using the Plate CHAMELEON^{TM}V scintillation counter, following the addition of 25 µL MicroScint 20 Scintillation fluid to each filter. Results were expressed as percentage incorporation relative to incorporation at time 0.

2.5.2 Membrane perturbation assays

2.5.3 Haemolysis assay

The haemolysis assay was used to determine the effect of antibacterial agents on membrane integrity of horse red blood cells (Oliva et al., 2003). To prepare the erythrocytes, whole blood (lithium heparin treated) was centrifuged at 1000 x *g* for 10 minutes at 4°C. Erythrocytes were resuspended to 5% (v/v) in 10 mM Tris-HCl containing 0.9% (w/v) NaCl and stored at 4°C. For the assay the stock cell suspension was diluted 1 in 25 in 10 mM Tris-HCl containing 0.9% (w/v) NaCl. For each drug under investigation, 1 mL of erythrocytes was incubated for 15 minutes at 37°C in Eppendorf tubes. The antibacterial agent was added at 4X MIC and incubated without shaking at 37°C for 1 hour, inverting gently every 20 minutes to mix. Samples were centrifuged at 3000 x *g* for 5 mins and 200 µL of supernatant was transferred into a 96 well plate. Optical density was measured at 540 nm using a FLUOstar Omega plate reader. Haemolysis was expressed as a percentage relative to the positive control 5% (w/v) SDS.

2.5.4. BacLight™ Assay

The BacLight™ assay was used to determine the impact of antibacterial agents on bacterial cell membrane integrity (Hilliard et al., 1999). This assay utilises two dyes- Syto® 9 and propidium iodide, which stain cells with intact and compromised membranes, respectively. *S. aureus* SH1000 was grown to mid-exponential phase (OD600nm 0.5) in MHB-II at 37°C. Cells (500 µL) were collected by centrifugation at 16,000 x *g* for 3 minutes, washed in sterile dilution water and finally re-suspended in 900 µL SDW. Test compound was added to cells giving a final concentration of 4X MIC and incubated at 37°C for 10 minutes with shaking. The drug-cell mixture was centrifuged at 16,000 x *g* for 3 minutes, washed twice in sterile dilution water (SDW) and finally suspended in 1 mL SDW. A 50 µL aliquot of the sample was added to a clear bottom black microtitre plate (Nunc). In the dark, the BacLight™ reagent was made; Syto® 9 and Propidium Iodide were mixed in a 1:1 ratio, and then diluted 1 in 30 in SDW. The BacLight™ reagent (150 µL) was added to the wells of the microtitre plate and incubated for 15 minutes at RT. Fluorescence was read using a FLUOstar Omega plate reader; propidium iodide and Syto® 9 were excited at 485 nm with an emission of 630 nm and 545 nm, respectively. The green:red fluorescence ratio was determined and membrane integrity was expressed as a percentage relative to the positive control (5% (w/v) SDS).

2.5.5 Biofilm matrix quantification assay

The effect of antibacterial agents on staphylococcal biofilms was assessed using a method described by (Frank and Patel, 2007). Biofilm matrix and cells were stained with Syro® Ruby and Syto® 9, respectively, following exposure to antibacterial

agent at 4X MBEC. Microtitre plates were pre-conditioned with 20% (v/v) human plasma at 4°C overnight. *S. aureus* SH1000 biofilms were established by inoculating wells with a saturated overnight culture diluted in Tryptic Soy Broth (TSB) (1 in 100) and incubating for 24 hours at 37°C. Biofilms were washed with water to remove non-adherent material and exposed to antibacterial agent at 4X MBEC and 100 µg/mL Proteinase K in Tris-HCL (pH 7.0) for 1, 2, 4 and 24 hours at 37°C. Biofilms were washed with water and then stained with Sypro® Ruby containing 0.67 µM Syto® 9 for 30 minutes in the dark. Following removal of the stain, biofilms were washed with water and fluorescence was measured using the FLUROstar Omega plate reader. Sypro® Ruby and Syto® 9 were excited at 480 nm, with an emission wavelength at 620nm and 520nm respectively.

2.5.6 Assessment of biofilm viability

Biofilms grown on 96 well plates pre-conditioned with human plasma were exposed to antibacterial agent at 16X MBEC for 1, 2 and 6 hours (as in 2.5.5). Biofilms were washed with PBS and treated with Proteinase K (100 µg/mL in Tris-HCl pH 7.0) for one hour at 37°C. Cells were harvested by centrifugation and washed twice in PBS. Viable biofilm cells were observed by diluting cultures in PBS and plating onto MHA-II. Colonies were counted following incubation at 37°C for 18-24 hours.

2.5.7 Generation and analysis of antibacterial resistant mutants

To isolate spontaneous antibacterial resistant mutants, saturated overnight *S. aureus* cultures were spread onto Mueller-Hinton Agar containing antibiotic at 4X MIC. Plates were incubated at 37°C for 24 hours; if no colonies were observed,

plates were incubated for a further 24 hours. To confirm mutants were resistant, broth MICs were conducted using the standardised CSLI methodology (Section 2.3.1). Genomic DNA was amplified by PCR (Section 2.6.2) in regions where mutations were suspected and sent for sequence determination (Section 2.6.3). For isolates where polymorphisms were not observed by PCR, whole genome sequencing was used to identify polymorphisms (Section 2.6.3).

2.6 Molecular biology techniques

2.6.1 DNA purification

Bacterial genomic DNA was purified using the PurElute™ kit (EdgeBio) according to the manufacturer's instructions. To enhance the lysis of *S. aureus* cells, lysostaphin (100 µL) was added to 400 µL Spheroplast buffer and incubated at 37°C for 1 hour. To lyse *E. faecalis* cells, Spheroplast buffer was supplemented with lysozyme (100 µg/mL). Plasmid DNA was purified using the E.Z.N.A. ® Plasmid Mini Kit (VWR) according to the manufacturer's instructions. DNA preparations were stored at -20°C.

2.6.2 Polymerase chain reaction (PCR)

DNA was amplified using Q5 DNA polymerase (NEB), in reactions carried out according to the manufacturer's instructions. Oligonucleotide primers (Appendix A) were designed using OLIGO software and synthesised by Eurofins MWG Operon (Ebersburg, Germany). PCR products were visualised using agarose gel electrophoresis.

2.6.3 DNA Sequencing

Whole genome sequencing was performed at the Next Generation Sequencing facility at St James' University Hospital, Leeds, UK. Whole genome libraries were assembled using HiSeq 3000, 150 bp paired end sequencing and aligned with the reference genome sequences using CLC workbench (Qiagen Bioinformatics).

DNA sequence determination of PCR products were conducted by Beckman Coulter Genomics (Essex, UK).

2.6.3 Molecular cloning

Restriction enzymes and their respective buffers were purchased from NEB. Restriction digests were conducted according to the manufacturers' instructions in 50 µL reactions containing ≥ 1 µg of DNA. Digested DNA was purified using gel extraction or column purification (E.Z.N.A.® Cycle Pure Kit).

Vector DNA and PCR fragments were ligated using T4 DNA ligase from NEB. Ligations were conducted according to the manufacturers' instructions in 20 µL reactions; 50 ng of vector DNA was ligated with insert DNA in a 1:3 ratio (vector: insert) and incubated at 16 ºC overnight. Ligated DNA was transformed directly into chemically competent *E. coli* (Section2.6.4).

2.6.4 Transformation of chemically competent *E. coli*

For the transformation of DNA, chemically competent *E. coli* XL-Gold (Agilent Technologies, Cheshire) cells were thawed on ice, followed by the addition of DNA (1-10 ng) and incubated on ice for 1 hour. Cells were heat shocked at 42 ºC for 45

seconds and allowed to recover on ice for 2 minutes. Super Optimal Both containing 20 mM glucose (SOC), pre-warmed to 37 ºC was added to a final volume of 1 mL and incubated at 37 ºC for 1 hour. Following recovery, aliquots of culture was spread onto LB agar containing the appropriate selection antibiotic.

2.6.5 Electroporation of *S. aureus*

Electroporation of *S. aureus* was conducted using a method described by Monk and Foster (Monk et al., 2012). Competent *S. aureus* cells were thawed on ice and 2.5 µL of concentrated plasmid DNA was added to 50 µL of cells. The mixture was added to a 1 mm electroporation cuvette and pulsed at 21 kV/cm, 100 Ω and 25 µF. Immediately after pulsing, 950 µL of TSB-YE+ 50 mM sucrose was added to the cuvette. This mixture was incubated for 1-2 hours at 37°C. Following incubation, cells were spun at 16,000 x *g* for 1 minute and all but 50 µL of supernatant was removed. Cells were resuspended in the remaining supernatant, plated onto TSA containing the corresponding antibiotic, and incubated for 24 hours at 37° C.

2.7 Over-expression and purification of *Staphylococcus aureus* FabI (saFabI)

2.7.1 Preparation of buffers for protein purification

Buffers required for the purification of saFabI were prepared as in Table 2.4. The storage buffer was degassed and filter sterilised (0.22 µM filters, Merk Millipore) prior to use on the AKTA purification system. The composition of lysis, wash and elution buffers were from Scheibel et al. (Schiebel et al., 2012).

Buffer	Composition
Lysis	50 mM Tris-HCl pH 8.0, 500 mM NaCl
Wash	50 mM Tris-HCl pH 8.0, 1M NaCl
Elution	50 mM Tris-HCl pH 8.0, 500 mM NaCl, 250 mM imidazole
Storage	20 mM Tris-HCl pH 8.0, 280 mM NaCl, 1mM DTT

Table 2. 4 Composition of buffers used for the purification of saFabI

2.7.2 Purification of saFabI

The *fabI* gene from *S. aureus* SH1000 was amplified by PCR and inserted directly into the pOPINF (His-tagged) vector using InFusion cloning (Clontech), and transformed into *E. coli* BL21-Gold (DE3). A single colony was used to inoculate 400 mL 2ZY auto-induction media containing 100 µg/mL carbenicillin at 25°C for 48

hours. Cell pellets were harvested by centrifugation at 5000 x *g* and resuspended in 30 mL lysis buffer (Table 2.5) and stored at -80°C. For purification of FabI protein, cell pellets were thawed and disrupted via sonication and centrifuged at 16,000 x *g* for 30 minutes. The supernatant containing the soluble fraction was bound to 3 mL Protino Ni-TED suspension for 15 minutes at 4°C on a blood mixer. The suspension was subsequently loaded into a 25-mL free-flow gravity column (GeneFlow) and unbound protein was allowed to flow through. The column was washed with 5 column volumes of wash buffer (Table 2.5) and bound protein then eluted with 3 column volumes of elution buffer (Table 2.5). Eluted protein was concentrated to 5 ml and subjected to gel filtration on a Superdex-200 26/60 column (GE healthcare) pre-equilibrated with storage buffer (Table 2.5). Fractions containing saFabI according to the corresponding chromatograms were pooled and concentrated to 3.5 mg/mL (125 µM) using spin concentrators with a molecular cut off weight of 10,000 Da. Throughout; SDS PAGE was used to confirm the presence of saFabI.

2.8. FabI biochemical assay

The activity and inhibition of *Staphylococcus aureus* FabI (SaFabI) was measured by observing the consumption of NADPH at OD 340nm, previously described by Slater-Radosti et al (Slater-Radosti et al., 2001). Breifly, 100 µL assay mixtures contained 100 mM Sodium N-(2-Acetamido) iminodiacetic acid (ADA) pH 6.5, 4% (w/v) glycerol, 125 µM NAD(P)H, 250 µM Crotonyl-CoA and varying concentratons of SaFabI. In Ultra Violet (UV) plates (Greiner Bio-One) changes in NADPH concentration was monitored over 25 minutes at 30°C. The initial rate of this reaction over the first 3 minutes, was calculated in GraphPad Prism 7.

2.9 Over- expression of proteins involved in fatty acid biosynthesis in SH1000

Staphylococcus aureus SH1000 strains harbouring the pRAB11 plasmid were grown to mid- exponential phase (0.5 OD_{600nm}). Cultures were supplemented with 0.4 µM anhydrotetracycline (ATc) to induce the expression of *S. aureus* fatty acid biosynthesis genes under the control of the $P_{xyl/tet}$ promoter. Minimum inhibitory concentrations (Section 2.3.1) were determined for antibacterial agents against induced cultures.

Chapter 3 Can the antimicrobial properties of existing wound agents be enhanced by combining them?

3.1 Abstract

Efficacious antimicrobial treatments can positively impact the outcome of skin infections. However, most of the antimicrobial treatments in use to date for skin infections display limited efficacy against biofilms, which can lead to non-healing wounds and lower extremity amputations. Biofilms are involved in over 60% of chronic wound infections and are associated with increased tolerance and resistance to antimicrobial therapy. The work presented in the following chapter aimed to improve existing antimicrobial and antibiofilm therapies by identifying synergism between antimicrobial agents used in combination. Synergism studies were used to identify combinations of antimicrobial agents which displayed improved antibacterial activity and eradication of *S. aureus* SH1000 *and P. aeruginosa* PAO1 biofilms. Synergism studies identified the promising combination of chlorhexidine and cetrimide, which was able to disrupt single species biofilms of SH1000 and PAO1. The antibiofilm activity of chlorhexidine and cetrimide embedded into sterile dressings was evaluated alongside established antimicrobial wound dressings and solutions against mixed species biofilms grown on nitrocellulose disks. Unfortunately, chlorhexidine and cetrimide were not able to

disrupt or eradicate mixed species biofilms; this observation was also seen with all but one of the established antimicrobial agents.

3.2 Introduction

As the human population becomes older and more obese, the prevalence of wounds that fail to heal, such as pressure ulcer and diabetic foot ulcers, is steadily increasing (Mathus-Vliegen, 2004). In 2014, a UK study found that the prevalence of pressure ulcers was 4.6%, this data was collected from surveys across acute hospital wards, community and residential care and patients' home (Norman et al., 2016). Amongst the multiple factors that impact wound healing, bacterial colonisation and infection is thought to be detrimental to wound healing (Bowler, P. G. et al., 2001). To add to an already complex situation, the bacteria in non-healing wounds exist as biofilm communities; early studies suggested that 60% of chronic wounds involved a biofilm component (James et al., 2008), whilst more recent studies have raised that figure to 72.8% (Malone et al., 2017a). The presence of biofilms on chronic wounds presents a major challenge to treatment, since antibacterial agents which are often useful against bacteria growing in the planktonic phase often have no or little effect on some biofilms (Costerton et al., 1999).

The use of antimicrobials for non-healing wounds remains an area of contention. Whilst it was recommended in 2014, that antibiotics should not be used not for non- infected wounds (NICE, 2014). For infected wounds topical antimicrobials are often favoured over systemic antibiotics (Lipsky et al., 2009). Topical antimicrobials are typically split into two groups; antiseptics and antibiotics. The use of topical

antibiotics is restricted due to the rapid development of resistance (Siddiqui et al., 2010), nevertheless topical antibiotics like mupirocin and retapamulin are useful against staphylococcal (including MRSA) skin infections (Lipsky et al., 2009; Foster, 2017). Care must be taken when using mupirocin and retapamulin, which are designated for nasal decolonisation of MRSA. Antimicrobial resistance to both agents is emerging in the clinic and such repeated off-label use may be detrimental, limiting their usefulness for the treatment of biofilm related skin infections (McNeil et al., 2014).

By contrast, antiseptics are widely used for topical treatment of wound infections. These agents differ from antibiotics in that they often have multiple cellular targets, and consequently resistance to these agents is generally less common (Lipsky et al., 2009). Antiseptics such as chlorhexidine, povidone iodine, silver nitrate and triclosan have been utilised extensively in the prevention and treatment of both acute and non-healing wounds; however, evidence surrounding their efficacy is lacking (Williamson et al., 2017). In addition, many of these antiseptics have poor antibiofilm activity or their antibiofilm properties have not been widely studied (Percival, Steven. L et al., 2014).

Current antimicrobial strategies lack efficacy and many chronic wounds remain without resolution, leading to amputation and systemic infections (Siddiqui et al., 2010). In 2003 it was estimated that 2,600 lower extremity amputations in the UK, were performed on patients with ulcerative wounds (Posnett and Franks, 2008). An approach to improve the effectiveness of antimicrobial wound treatments is the use of combination therapy. By using antibacterial agents in combination improved

antibiofilm and bactericidal activity may be achieved (Ahmed et al., 2014). In addition, combination therapy may reduce the rate at which resistance to antimicrobials arises (Deresinski, 2009). Vancomycin is frequently used to treat bacteraemia caused by staphylococci; however the efficacy of vancomycin has been shown to be inferior to other systemic agents. In order to overcome this pitfall, vancomycin is often used in combination with rifampicin, β-lactams and aminoglycosides (Deresinski, 2009). Using topical antimicrobials in conjunction with each other or other adjuvants has the potential to enhance their antimicrobial and antibiofilm efficacy. Combination therapy could serve as one of the quickest routes to improve our antimicrobial therapies, without having to develop novel antibiotics.

3.3 Aims and objectives

The work in this chapter aimed to determine the activity of common antibacterial wound agents- alone and in combination- against the most frequently isolated bacteria in chronic wounds, *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Dowd, S. et al., 2008; Melendez et al., 2010). Synergism studies will be conducted to identify combinations of antibacterial agents that display improved eradication of single species biofilms. Combinations which display synergism against biofilm cultures will be evaluated against more complex mixed species biofilms.

3.4 Results

3.4.1 Antibacterial and antibiofilm activity of established wound care agents

Minimum inhibitory concentrations (MICs) (Section 2.3.1) were measured to determine the antimicrobial activity of common antibacterial wound agents (silver nitrate, chlorhexidine, povidone iodine, polyhexanide (PHMB), hydrogen peroxide, cetrimide, triclosan and acetic acid) against planktonic cultures of *S. aureus* SH1000 and *P. aeruginosa* PAO1. Of the wound agents tested, all displayed a degree of antimicrobial activity against *S. aureus* SH1000, with the MIC of these compounds ranging from 0.125 μg/mL to 2048 μg/mL (Table 3.1.). Of the wound agents tested against *P. aeruginosa* PAO1, most compounds had activity. The activity against PAO1 was comparable to SH1000; with an MIC range of 1 to 4096 μg/mL. Triclosan was unable to inhibit the growth of *P. aeruginosa* at 256 μg/mL- the highest concentration tested.

	SH1000 (µg/mL)			PAO1 (µg/mL)		
Compound	MIC	MBC	MBEC	MIC	MBC	MBEC
Acetic Acid	1024	2048	8192	1024	2048	2048
Cetrimide	2	64	32	128	256	256
Chlorhexidine	1	4	64	4	16	256
Hydrogen Peroxide	8	16	1024	128	128	8192
Polyhexanide (PHMB)	4	8	64	8	16	4096
Povidone Iodine	2048	4096	8192	4096	4096	16384
Silver Nitrate	16	32	1024	8	16	512
Triclosan	0.125	0.5	4	>256	n.d	n.d

Table 3. 1 Determination of MICs, MBCs and MBECs of common wound agents against *S. aureus* SH1000 and *P. aeruginosa* PAO1 Susceptibility studies were conducted on a minimum of three independent occasions to ensure reproducibility n.d- not determined

Antimicrobial agents employed to treat skin and wound infection should be able to kill (rather than inhibit the growth of) infecting microorganisms, in order to compensate for the often weakened immune system. Therefore, minimum bactericidal concentrations (MBCs)(Section 2.3.2) were defined to determine whether the wound agents were bactericidal or bacteriostatic (Table 3.1). Compounds were designated bactericidal if their MBC/MIC ratio was less than or

equal to 4 (Pankey and Sabath, 2004). Seven of the eight wound agents tested were bactericidal against *S. aureus* (Table 3.1); cetrimide is the only compound which did not exhibit bactericidal activity and was classified as bacteriostatic, with an MBC/MIC ratio of 32. Against *P. aeruginosa* all of the antimicrobial wound agents tested were bactericidal, with and MBC/MIC ratio range from 4-1.

Assessing antibacterial activity against planktonic cultures can guide selection of antimicrobial agents for use in treating skin infections where there is no biofilm component involved. However, in infections such as chronic wounds, biofilms play a significant role (James et al., 2008; Malone et al., 2017a) and therefore the antibiofilm activity of the agents in question requires evaluation. The effect of wound agents on *S. aureus* and *P. aeruginosa* biofilms was gauged by determining minimum biofilm eradication concentrations (MBECs) (Table 3.1). Of the compounds tested, all were able to eradicate *S. aureus* SH1000 biofilms using the Calgary Biofilm Device (Section 2.3.3), with MBECs in the range of 4- 8192 μg/mL. Chlorhexidine, PHMB, cetrimide and triclosan exhibited the most potent activity against *S. aureus* biofilms, with MBECs of ≤64 μg/mL. *P. aeruginosa* PAO1 biofilms were more resilient to treatment with common wound agents; only chlorhexidine and cetrimide were able to eradicate PAO1 biofilms at 256 μg/mL (Table 3.1). The remaining wound agents eradicated *P. aeruginosa* biofilm at concentrations greater than 256 μg/mL, but still within the range of in-use concentrations.

3.4.1.1 Antibiofilm activity against wound pathogens

The antibiofilm efficacy data presented thus far focused on one strain each of *S. aureus* (SH1000) and *P. aeruginosa* (PAO1), both of which are laboratory organisms widely used in bacterial research. In a setting such as the chronic wound biofilm multiple bacterial species may be present and there may be multiple strains of the same species (Dufour et al., 2010). To determine whether the antibiofilm activity observed with established wound care agents was also observed against other isolates of these species, MBECs were determined for the wound agents against a small panel of *S. aureus* and *P. aeruginosa* strains. The panel contained proficient biofilm former *S. aureus* UAMS-1 (Gillaspy et al., 1995), community-acquired methicillin resistant *S. aureus* (MRSA) USA300 (Pastar et al., 2013), a hyper virulent *P. aeruginosa* PA14 and type strain *P. aeruginosa* NCTC 10332. The antibiofilm activity observed with common wound agents was not specific to the laboratory strains *P. aeruginosa* PAO1 and *S. aureus* SH1000; in almost all cases the MBECs for all the agents tested was within 2 fold of that recorded against PAO1 and SH1000 biofilms (Table 3.2). However, hydrogen peroxide was unable to eradicate *P. aeruginosa* NCTC 10332 at the highest concentration tested (8192 μg/mL).

	S. aureus			*P. aeruginosa*		
	MBEC (μg/mL)					
	SH1000	UAMS-1	USA 300	PAO1	PA14	NCTC 10332
Acetic Acid	8192	4096	8192	4096	4096	4096
Cetrimide	64	32	32	256	64	256
Chlorhexidine	64	32	32	256	128	128
Hydrogen Peroxide	1024	512	512	8192	8192	>8192
Polyhexanide (PHMB)	256	256	1024	4096	8192	4096
Povidone Iodine	8192	8192	8192	16384	8192	8192
Silver Nitrate	512	512	512	512	256	256

Table 3. 2 Antibiofilm activity of established wound agents against a panel of *S. aureus* and *P. aeruginosa* strains Susceptibility studies were conducted on a minimum of three independent occasions to ensure reproducibility

3.4.2 Antibacterial and antibiofilm synergism between established wound agents

3.4.2.1 Antibacterial synergism

Initial synergism studies were conducted against planktonic cultures (Section 2.3.4), with a view to identify combinations which may also be effective against biofilm cultures. Of the 28 combinations tested against planktonic cultures of *S. aureus* SH1000, 1 synergistic interaction was identified (Table 3.3). Chlorhexidine and cetrimide displayed synergism against *S. aureus* SH1000 with an FIC index of 0.5 (Table 3.3). Chlorhexidine acted synergistically with silver nitrate and hydrogen peroxide against planktonic cultures of *P. aeruginosa* PAO1; with FIC indices of 0.2815 and 0.5 respectively (Table 3.1).

FIC Index		*S. aureus* SH1000							
		Silver Nitrate	**Chlorhexidine**	**Povidone Iodine**	**PHMB**	**Hydrogen Peroxide**	**Cetrimide**	**Triclosan**	**Acetic Acid**
***P. aeruginosa* PAO1**	**Silver Nitrate**		0.81	0.51	0.78	2.81	1.54	1.66	2
	Chlorhexidine	**0.28**		1.16	1.58	2	**0.5**	1.41	2
	Povidone Iodine	1	>0.5		1	2	0.63	1.5	0.75
	PHMB	0.53	>0.5	>0.5		0.53	0.75	1	2
	Hydrogen Peroxide	**0.5**	>0.5	>0.5	>0.5		>0.5	>0.5	>0.5
	Cetrimide	1	>0.5	>0.5	>0.5	>0.5		>0.5	>0.5
	Triclosan	n.d	n.d	n.d	n.d	n.d	n.d		>0.5
	Acetic Acid	1	>0.5	>0.5	>0.5	>0.5	>0.5	n.d	

Table 3. 3 FIC indices for commonly used wound agents against *S. aureus* (top) and *P. aeruginosa* (bottom) planktonic cultures. An FIC index ≤0.5 signifies a synergistic interaction. FIC studies were conducted on a minimum of three independent occasions and the mean result is given. Results highlighted in red represent synergistic combinations. FIC indices expressed as >0.5 were determined using a condensed FIC method. N.d- not determined

3.4.2.1 Antibiofilm synergism

One of the strategies to improve the efficacy of antibiofilm agents is the use of combination therapy (Hurdle et al., 2011). Synergistic interactions between silver nitrate and sodium hexametaphosphate have been observed when treating biofilm cultures of *S. aureu*s, *P. aeruginosa* and *Candida albicans* (Humphreys et al., 2011) . Another study involving a biofilm infection model in mice was able to demonstrate synergism between silver nitrate and gentamicin against *E.coli* biofilms (Morones-Ramirez et al., 2013). Combinations of common wound agents were exposed to biofilm cultures of *S. aureus* and *P. aeruginosa*, to identify potential synergistic interactions(Section 2.3.4).

The full-scale FIC determinations with planktonic cultures proved rather laborious and failed to yield a large number of synergistic interactions. Therefore subsequent studies initially involved a condensed biofilm FIC method, for more rapid identification of antibiofilm synergistic interactions. *S. aureus* and *P. aeruginosa* biofilms grown on the Calgary Biofilm Device were exposed to antibacterial agent alone at ¼X MBEC and in combination at ¼X MBEC. Chlorhexidine acted synergistically with PHMB, hydrogen peroxide and cetrimide against *S. aureus* biofilms (Table 3.4). Povidone iodine and acetic acid also displayed synergism against *S. aureus* biofilms. When combined against *P. aeruginosa* PAO1 biofilms synergism was observed between chlorhexidine and silver nitrate and cetrimide + silver nitrate (Table 3.4).

Biofilm FIC Index		*S. aureus* SH1000							
		Silver Nitrate	**Chlorhexidine**	**Povidone Iodine**	**PHMB**	**Hydrogen Peroxide**	**Cetrimide**	**Triclosan**	**Acetic Acid**
***P. aeruginosa* PAO1**	**Silver Nitrate**		>0.5	>0.5	>0.5	>0.5	>0.5	>0.5	>0.5
	Chlorhexidine	**≤0.5**		>0.5	**≤0.5**	**≤0.5**	**≤0.5**	>0.5	>0.5
	Povidone Iodine	>0.5	>0.5		>0.5	**≤0.5**	>0.5	>0.5	**≤0.5**
	PHMB	>0.5	>0.5	>0.5		>0.5	>0.5	>0.5	>0.5
	Hydrogen Peroxide	>0.5	>0.5	>0.5	>0.5		>0.5	>0.5	>0.5
	Cetrimide	**≤0.5**	>0.5	>0.5	>0.5	>0.5		>0.5	>0.5
	Triclosan	n.d	n.d	n.d	n.d	n.d	n.d		>0.5
	Acetic Acid	>0.5	>0.5	>0.5	>0.5	>0.5	>0.5	n.d	

Table 3. 4 Biofilm FIC indices for commonly used wound agents against *S. aureus* (top) and *P. aeruginosa* (bottom) biofilm cultures. Biofilm FIC indices were determined using a condensed biofilm FIC method. An FIC index ≤0.5 signifies a synergistic interaction. Results highlighted in bold represent synergistic combinations. Biofilm FIC studies were conducted on a minimum of three independent occasions and the mean result is given.

Whilst the condensed biofilm FIC method was initially useful for intitial indentification of potential synergyistic combinations, this method could possibly mis-identify synergyism or indifferent interactions. Full- scale biofilm FIC experiments were therefore conducted for all seven synergystic combinations identified in the condensed biofilm FIC experiments. These experiments revealed that four of the seven combinations acted synergystically against both *S. aureus* and *P. aeruginosa* biofilms with biofilm FIC indecies ≤ 0.5 (Table 3.5). The combination of chlorhexidine and hydrogen peroxide was particular potent against *P. aeruginosa* biofilms (biofilm FIC Index=0.188). As seen in the condensed biofilm FIC experiments, povidone iodone and hydrogen peroxide displayed synergyism against *S. aureus* biofilms but not *P.aeruginosa* biofilms; the same observation was seen with chlorhexidine and PHMB (Table 3.5). Conversely, silver nitrate and chlorhexidine did not display synergy against *S. aureus* biofilms, but was synergystic against *P. aeruginosa* biofilms (Table 3.5).

	Biofilm FIC Index	
	S. aureus SH1000	*P. aeruginosa* PAO1
Silver Nitrate + Cetrimide	0.125	≤0.5
Chlorhexidine + Cetrimide	0.5	0.5
Povidone iodine + Acetic acid	0.5	0.5
Chlorhexidine + Hydrogen peroxide	0.5	0.188
Chlorhexidine + PHMB	0.25	2
Povidone iodine + Hydrogen peroxide	0.5	2
Silver Nitrate + Chlorhexidine	0.813	0.281

Table 3. 5 Biofilm FICs indices for potential synergistic wound agent combinations previously identified using the condensed biofilm FIC method vs PAO1 and SH1000

An FIC index ≤0.5 signifies a synergistic interaction. Biofilm FIC studies were conducted on a minimum of three independent occasions and the mean result is given.

To establish whether this synergistic activity was also observed against other isolates of *S. aureus* and *P. aeruginosa*, FIC experiments were conducted with biofilms formed by several different strains. For the majority of combinations assayed, antibiofilm synergy was not strain specific (Table 3.6). For povidone iodine and acetic acid synergism was not identified against *P. aeruginosa* PA14 biofilms (FIC =0.625).

	Biofilm FIC index			
	Silver Nitrate + Cetrimide	Chlorhexidine + Cetrimide	Povidone Iodine + Acetic Acid	Chlorhexidine + Hydrogen Peroxide
***S. aureus* SH1000**	0.125	0.5	0.5	0.5
***S. aureus* UAMS-1**	0.375	0.094	0.375	0.188
***S. aureus* USA300**	0.094	0.281	0.5	0.188
***P. aeruginosa* PAO1**	≤0.5	0.5	0.5	0.188
***P. aeruginosa* PA14**	0.141	≤0.281	0.625	0.281
***P. aeruginosa* NCTC 10332**	≤0.5	0.5	0.5	≤0.531

Table 3. 6 Biofilm FIC indices for wound agent combinations against a panel of *S. aureus* and *P. aeruginosa* strains

An FIC index ≤0.5 signifies a synergistic interaction. Biofilm FIC studies were conducted on a minimum of three independent occasions and the mean result is given.

3.4.3 Evaluation of antibiofilm activity against mixed species biofilms

Improvement of chronic wound antibiofilm treatments relies on appropriate models able to replicate biofilm growth in the chronic wound bed *in vitro* and *in vivo* (Lebeaux et al., 2013). The Calgary Biofilm Device, whilst a useful tool for gauging the antibiofilm properties of wound care agents in a high-throughput setting; it may fail to successfully mimic the conditions of a chronic wound biofilm. Subsequent experiments therefore utilised the cellulose disk biofilm (CDB) model (Ryder et al., 2012), in an attempt to simulate the complex chronic wound biofilm

more closely and evaluate the antibiofilm efficacy of the synergistic combinations identified in section 3.4.2.1. The biofilms grown using this model were incubated for 48 hours producing a more mature biofilm with higher cell densities compared to those formed on the Calgary biofilm device. In addition, the substrate was primed with human plasma, which has been demonstrated to facilitate the development of a more robust biofilm (Chen et al., 2012). This model was adapted to propagate mixed species biofilm formed by *S. aureus* and *P. aeruginosa* (Section 2.4). Biofilms established using the CDB model were then subjected to treatment with antibacterial agent at ¼X MBEC alone and in combination for 1 hour; with a view to identify any combinations that displayed improved activity.

Treatment of established biofilms with hydrogen peroxide and chlorhexidine in combination did not yield a significant drop in biofilm viability compared to the untreated control (Figure 3.1). The combination of povidone iodine and acetic acid caused a significant drop in biofilm viability; however this combination was not superior to acetic acid alone (Figure 3.2).

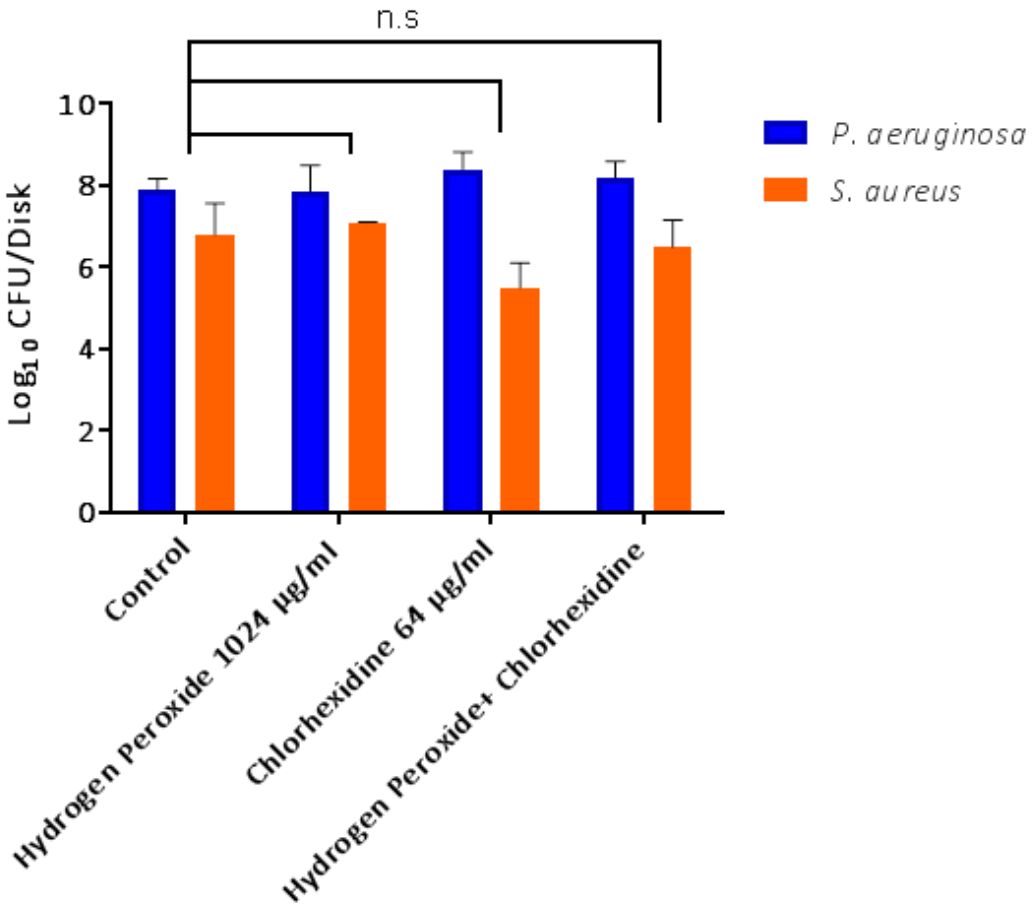


Figure 3. 1 The impact of hydrogen peroxide and chlorhexidine alone and in combination on bacterial viability of mixed species biofilms grown in the cellulose disk biofilm model (1 hour treatment). Mixed species biofilms formed by *S. aureus* SH1000 and *P. aeruginosa* PAO1 (mean of at least three independent replicates; error bars show standard deviations)
n.s- not significant <95% confidence, p value >0.05. Calculated using 2-Way ANOVA using GraphPad Prism 7.01

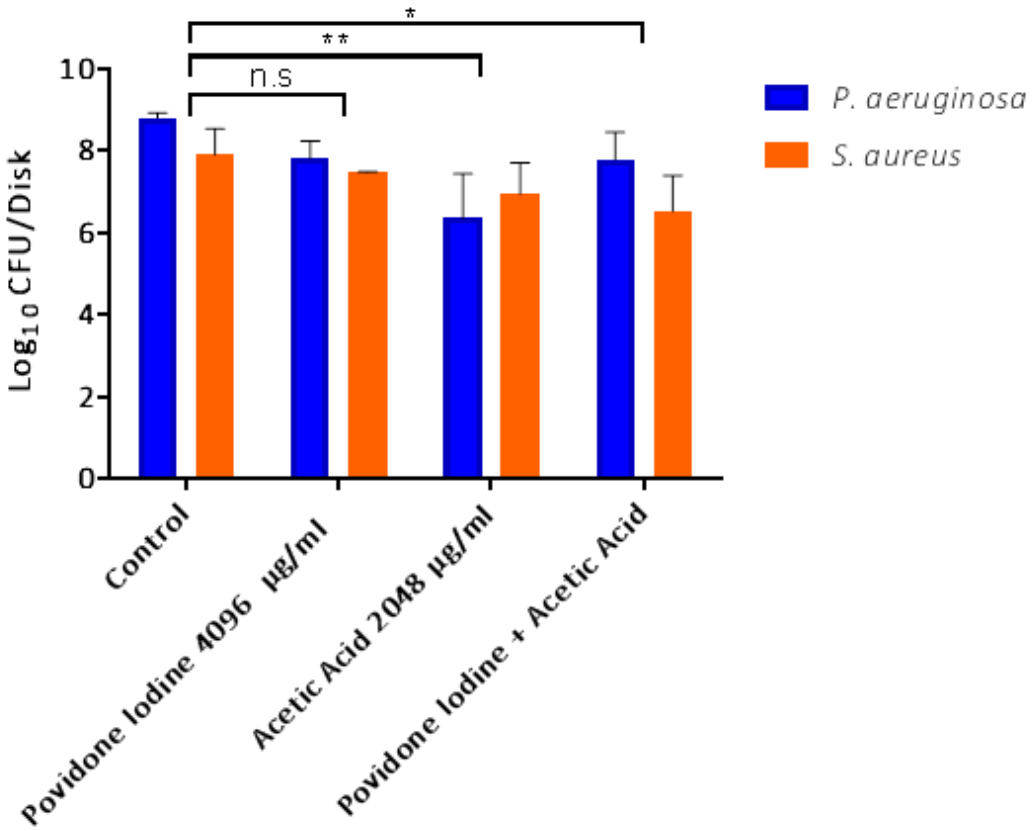


Figure 3. 2 The impact of povidone iodine and acetic acid alone and in combination on bacterial viability of mixed species biofilms grown in the cellulose disk biofilm model (1 hour treatment). Mixed species biofilms formed by *S. aureus* SH1000 and *P. aeruginosa* PAO1 (mean of at least three independent replicates; error bars show standard deviations)

n.s- not significant <95% confidence, p value >0.05. **-statistically significant >95% confidence, p value= 0.0012. *- p value= 0.0183. Calculated using 2-Way ANOVA using GraphPad Prism 7.01

A more promising result was observed with the combination of silver nitrate and cetrimide, with the *S. aureus* population being eradicated after 1 hour treatment. However, the *P. aeruginosa* population remained fully intact, with no loss of viability (Figure 3.3). The lack of killing observed is likely due to the relatively poor activity of cetrimide against *P. aeruginosa* compared to *S. aureus* (MBEC =256 µg/mL vs. 32 µg/mL).

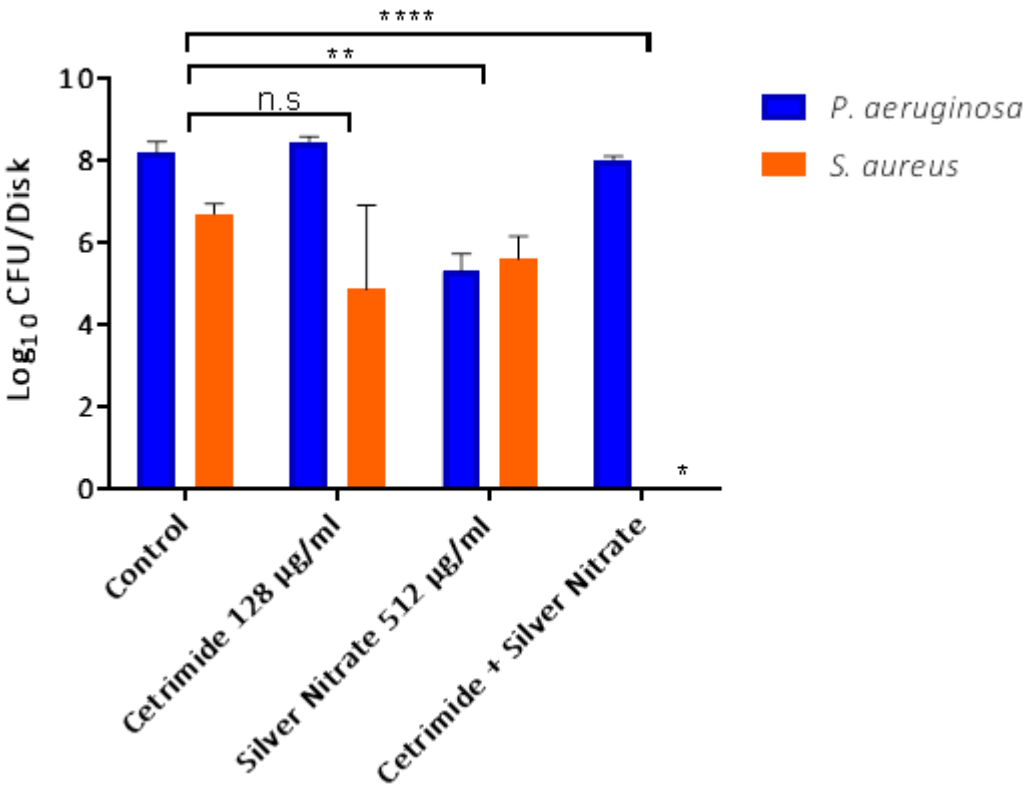


Figure 3. 3 The impact of cetrimide and silver nitrate alone and in combination on bacterial viability of mixed species biofilms grown in the cellulose disk biofilm model (1 hour treatment). Mixed species biofilms formed by *S. aureus* SH1000 and *P. aeruginosa* PAO1 (mean of at least three independent replicates; error bars show standard deviations) *- below the limit of detection (2 Log_{10} CFU/disk) n.s- not significant <95% confidence, p value >0.05. **-statistically significant >95% confidence, p value= 0.0012. ****- p value= 0.0001. Calculated using 2-Way ANOVA using GraphPad Prism 7.01

The combination of chlorhexidine and cetrimide was effective against both populations of the mixed species biofilm, with viability of *S. aureus* and *P. aeruginosa* both reduced ≥ 2.5 LogCFU/biofilm (Figure 3.4). In spite of the modest reduction observed, chlorhexidine and cetrimide were selected for further investigation with regards to their potential as an antibiofilm combination.

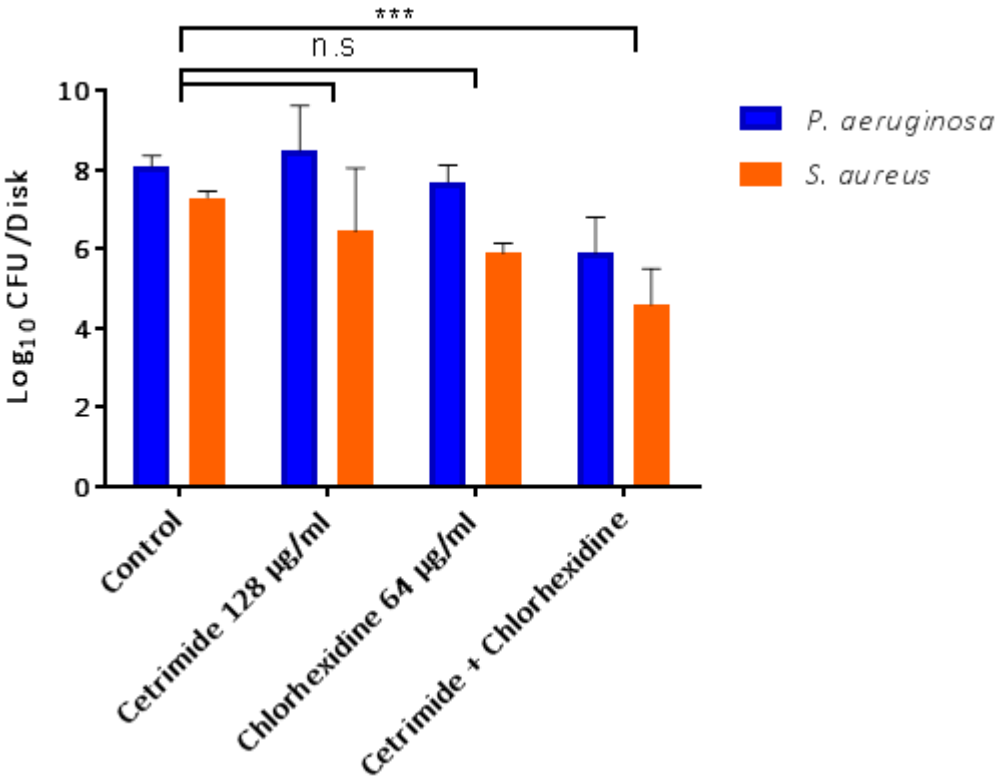


Figure 3. 4 The impact of cetrimide and chlorhexidine alone and in combination on bacterial viability mixed species biofilms grown in the cellulose disk biofilm model (1 hour treatment). Mixed species biofilms formed by *S. aureus* SH1000 and *P. aeruginosa* PAO1 (mean of at least three independent replicates; error bars show standard deviations)
n.s- not significant <95% confidence, p value >0.05. ***-statistically significant >95% confidence, p value= 0.0004. Calculated using 2-Way ANOVA using GraphPad Prism 7.01

The inability of the combinations to cause a substantial disruption in either population further highlights the need to use suitable models when evaluating antibiofilm activity. It remains that potency observed in one model is often not reflected in another; however the concentrations used in the previous experiments are much lower than those used in the clinic, which may account for the seemingly poor activity observed against more robust biofilms.

It is of note that single species biofilm of *S. aureus* and *P. aeruginosa* were not challenged with antimicrobial combinations. The absence of such experiments adds some reservations as to whether the lack of antibiofilm activity observed is a result of a more robust biofilm or the poor activity of the tested antimicrobial combinations.

3.4.4. Antibiofilm efficacy of chlorhexidine and cetrimide in an antibiofilm dressing

Biofilms propagated on the cellulose disk biofilm model were treated with a sterile adherent dressing impregnated with chlorhexidine and cetrimide at various concentrations (Section 2.4). Dressings and wound irrigation solutions which are currently employed for their antibacterial activity in the management of chronic wounds (Inadine, Iodoflex, Protosan and AqucelAg+) were also evaluated with regards to their antibiofilm efficacy. These experiments were conducted with a view to model an antimicrobial dressing being applied directly to a chronic wound biofilm *in vitro*. The efficacy of chlorhexidine and cetrimide against more complex biofilms can be compared alongside antibacterial dressings that claim to have antibiofilm properties (Thomas, M. et al., 2011; Fitzgerald et al., 2017).

Incorporated into a sterile dressing, chlorhexidine and cetrimide at 64 µg/mL and 128 µg/mL respectively were unable to eradicate *S. aureus* or *P. aeruginosa* in mixed species biofilms (Figure 3.5). Increasing the concentration of chlorhexidine and cetrimide (1024 and 64 µg/mL, respectively) resulted in eradication of the *S. aureus* population; however there was only limited reduction in viability of the *P. aeruginosa* population (Figure 3.5.).

Interestingly, in use wound dressings and irrigation solutions also had limited effect on the mixed species biofilms (Figure 3.5). Inadine, a povidone iodine based dressing, and the silver based dressing Aquacel Ag+ both had little effect on either population in the biofilm. The PHMB (0.1%) irrigation solution Prontosan had minimal effect on the *P. aeruginosa* within the biofilm, but was able to significantly reduce the *S. aureus* population. The only dressing able to cause eradication of the biofilm was Iodoflex. The cadexomer iodine based dressing was able to eradicate both populations within 24 hours (Figure 3.5); cadexomer iodine has previously been shown to act in a superior manner to other wound care agents against mature single species biofilms formed by *P. aeruginosa* and *S. aureus* in multiple models (Fitzgerald et al., 2017).

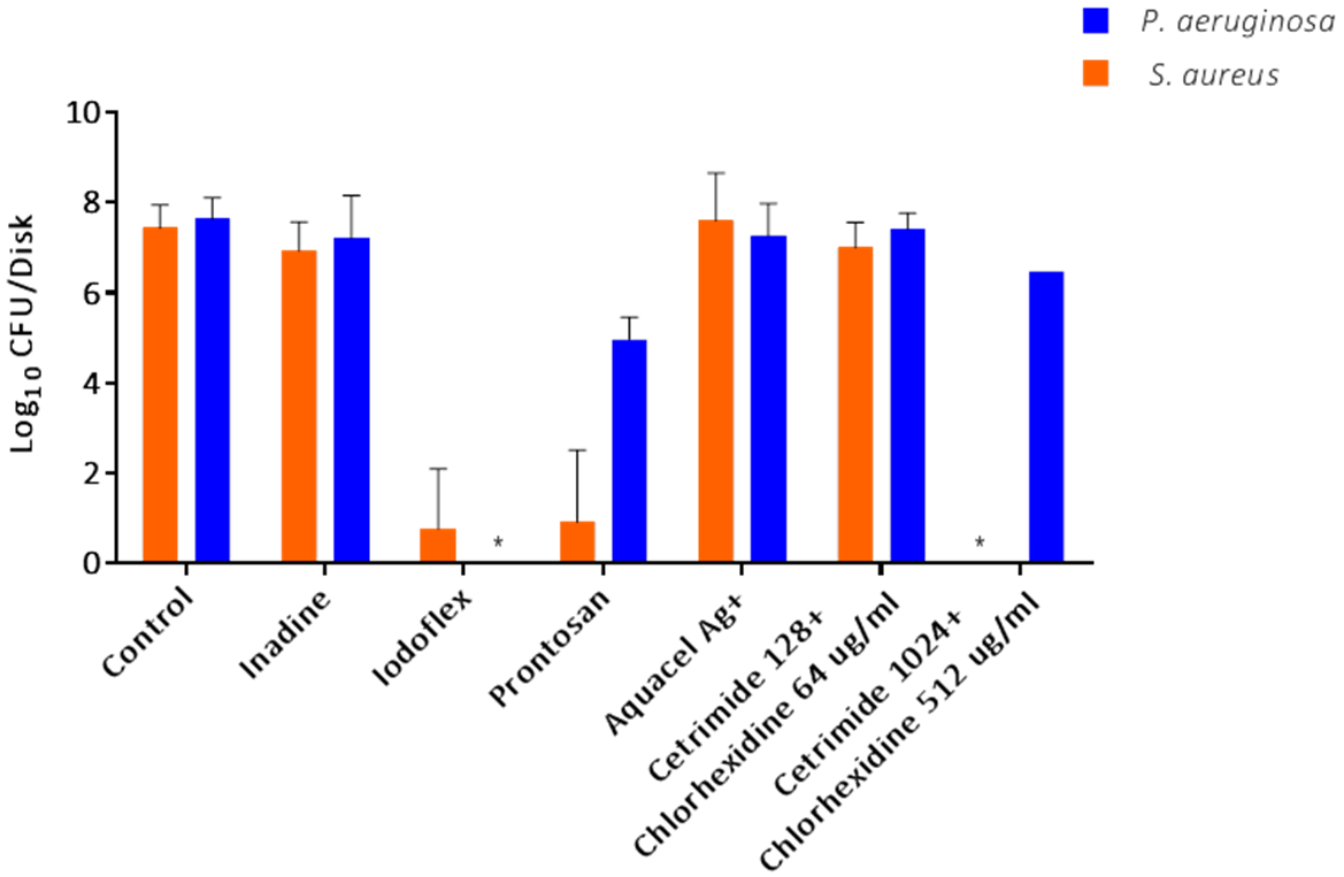


Figure 3. 5 The impact of chlorhexidine and cetrimide and established wound dressings on bacterial viability of mixed species biofilms. Mixed species biofilms formed by *S. aureus* SH1000 and *P. aeruginosa* PAO1 (mean of at least three independent replicates; error bars show standard deviations) * denotes below the limit of detection 2 Log_{10} CFU/disk

3.5 Discussion

There is currently limited and conflicting knowledge surrounding appropriate antimicrobial treatment for wounds; especially for chronic wounds (O'Meara et al., 2014). Many of the antimicrobial agents used currently, although active against planktonic bacteria, have demonstrated limited biofilm prevention and eradication abilities (Fitzgerald et al., 2017). When treating chronic wounds with systemic antibiotics efficacy can be reduced to as little as 25% (Rhoads, D. et al., 2008). The use of topical antimicrobials may be able to deliver higher and more sustained concentrations to the site of infection, reduce systemic toxicity and allow patients to treat their wounds at home due to ease of application (Lipsky et al., 2009). As the role of biofilms in chronic wounds becomes more apparent, the potentiation of antimicrobial agents to improve anti-biofilm activity has become a promising area of exploration. The work conducted in this chapter formed the basis of a model work flow, which can be used to identify and evaluate synergistic antibiofilm combinations.

The antibacterial wound agents in question displayed broad spectrum antibiofilm and antibacterial activity against *S. aureus* and *P. aeruginosa.* Nevertheless, the concentration of agent required to eradicate biofilms was in some cases 128-fold higher than those required to kill planktonic cells (Hydrogen peroxide vs *S. aureus).* This observation highlights the difficulty in treating biofilm infections. By contrast the concentrations tested in this study are much lower compared to the concentrations used to treat chronic wounds; however it is known that povidone

iodine, PHMB, cetrimide, triclosan and acetic acid have varied biofilm eradication properties in the clinic (Percival, Steven. L et al., 2014).

Chlorhexidine and cetrimide was identified as the combination able to cause the greatest disruption of both bacterial populations in mixed species biofilms formed by *S. aureus* and *P. aeruginosa*. Interestingly, chlorhexidine and cetrimide are used in combination in the antiseptic cream Savlon®, however, it is not known whether these active agents were selected based on previous synergism studies. Chlorhexidine acts on bacterial cell wall components, and like cetrimide, causes leakage of the cytoplasmic membrane (Percival, Steven. L et al., 2014). The combination of chlorhexidine and cetrimide enhances osmotic stress, resulting in increased potassium leakage and the precipitation and coagulation of the cytoplasm in bacterial cells (Gilbert et al., 2005; Percival, Steven. L et al., 2014). Unlike chlorhexidine and cetrimide, the other combinations identified in biofilm FIC studies did not cause significant reduction in biofilm viability for both populations after 1 hour.

Following the observation that chlorhexidine and cetrimide in combination show slightly improved antibiofilm activity against mixed species biofilms in 1 hour, the efficacy of the combination embedded into sterile dressings applied to mixed species biofilms was assessed. At higher concentrations the *S. aureus* population was impacted, however the *P. aeruginosa* population remained intact. The lack of activity against the *P. aeruginosa* population could possibly be due to relatively poor activity of cetrimide (Gilbert et al., 2005). In parallel experiments the antibiofilm efficacy of established wound dressings was also assessed;

chlorhexidine and cetrimide failed to disrupt both populations within the biofilm, as did three of the four established dressings tested. In contrast, Iodoflex, a cadexomer iodine dressing, was able to considerably reduce both bacterial populations after 24 hours. In this dressing iodine is slowly released from cadexomer beads resulting in sustained exposure of the wound biofilm to iodine (Fitzgerald et al., 2017; Malone et al., 2017b) the cadexomer vehicle has also been hypothesised to cause desiccation of biofilm structures (Phillips et al., 2015). It may be possible to improve the antibiofilm efficacy of chlorhexidine and cetrimide by binding the combination to a structure such as cadexomer.

The lack of antibiofilm efficacy observed with chlorhexidine and cetrimide and the established wound agents highlights the need to use appropriate *in vivo* and *in vitro* models of chronic wound biofilms when assessing antibiofilm efficacy. In this chapter antibiofilm efficacy of synergistic combinations were assessed against biofilms grown in the CDB model; which was selected for the ability to produce mixed species biofilms and the presence of host factors in the form of human plasma. The Lubbock chronic wound biofilm (LCWB) model is the earliest *in vitro* model for chronic wound biofilms, able to support mixed species cultures in the presence of multiple host factors (Sun et al., 2008). Since its introduction, the LWCB and many adaptions of the model have been used to assess antibiofilm efficacy of various wound treatments (Dowd et al., 2009; Brackman, G. et al., 2013; Kucera et al., 2014), however the LWCB was not selected due to difficulty in evaluating the efficacy of antimicrobial dressings. Although models such as the LCWB and CDB model have been utilised extensively, no model is perfect; as neither model uses a

flow system (Brackman, Gilles and Coenye, 2015). The drip flow reactor (DFR) biofilm model is able to form biofilms under low-shear flow along with a liquid-air interface which may mimic the chronic wound biofilm more closely (Strittmatter, 2014). The DRF has been used to asses antibiofilm efficacy of wound care products (Woods et al., 2012), nevertheless a disadvantage to using the DFR is the low throughput and laborious handling (Manner et al., 2017). All models have advantages and disadvantages; however in this study the use of the CDB model allowed for the evaluation of an earlier identified synergistic combination-chlorhexidine and cetrimide. Although chlorhexidine and cetrimide did not display improved antibiofilm activity in the CDB, the platform developed in this chapter could be used in future to evaluate other synergistic combinations.

3.6 Conclusions

Work described in the chapter aimed to identify combinations of antimicrobial agents which displayed increased potency against biofilm cultures, with the prospect to be used in antibiofilm wound treatments. Earlier work identified four combinations able to eradicate single species biofilms formed by *S. aureus* and *P. aeruginosa*. These combinations were found to be less effective when applied to a mixed species biofilm model.

Chlorhexidine and cetrimide were established as the most promising combination, due to the ability to reduce biofilm viability of both *S. aureus* and *P. aeruginosa* >2.5 LogCFU/biofilm in 1 hour (Figure 3.4). Unfortunately, this combination failed to cause disruption of both biofilm populations whilst embedded into sterile dressings.

Similarly, most of the established wound dressings and irrigation solutions were unable to significantly disrupt both bacterial populations within the biofilm- posing the question, whether the active ingredients in these products can actually reduce the chronic wound biofilms in a clinical setting? It is more likely that these established dressings prevent biofilm reformation, rather than biofilm eradication.

4 Potential for repurposing bronidox and bronopol as broad-spectrum antibiofilm agents

4.1 Abstract

There is an urgent need to identify agents able to disrupt the complex biofilm found on the surface of chronic wounds. This chapter investigated the potential for repurposing compounds with a history of safe use in topical healthcare products as antibiofilm agents. From a panel of healthcare agents, bronidox (BX) and bronopol (BP) were the only ones identified that displayed broad spectrum activity against established biofilms of the two major wound pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa* at ≤256 μg/mL. BX and BP substantially disrupted (>5 LogCFU/biofilm reduction) mixed-species biofilms formed by *S. aureus* and *P. aeruginosa* at concentrations permitted for safe use. Investigation into the antibiofilm mode action revealed that BX and BP were able to sterilise non-growing cultures of *S. aureus.* It was also observed that BX and BP can sterilise biofilm bacteria well before disruption of the biofilm matrix occurs. The potential for BX and BP as topical antibiofilm agents is further supported by the synergistic antibiofilm activity observed with established wound care agents.

4.2 Introduction

Sessile communities of bacteria known as biofilms are implicated in approximately 80% of chronic infections (Del Pozo et al., 2007). In the case of chronic wounds, biofilms have been observed in >70% of chronic wound samples (Malone et al., 2017a); by contrast, biofilms were detected in just 6% of acute wounds (James et al., 2008). The chronic wound biofilm is home to many bacterial species, including but not limited to *S. aureus*, *P. aerugionosa*, *E. faecalis*, *A. baumannii* and Group B streptococcus (Melendez et al., 2010). The presence of biofilm in the wound is considered a major barrier to wound healing and has been associated with persistent inflammation and reduced susceptibility to antimicrobial agents (Hall-Stoodley et al., 2004; Wolcott, 2015). The problem of biofilm-related infections is exacerbated by the lack of antibacterial agents that display potent, broad-spectrum antibiofilm activity (Percival et al., 2014). There is a lack of novel antibacterial agents in the discovery/ development pipeline (Silver, 2011); this is especially true for agents with antibiofilm activity. One approach that may have potential for rapidly delivering new antibiofilm compounds into the pipeline involves the repurposing of compounds.

4.2.1 Repurposing for the treatment of biofilm infections

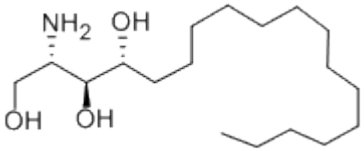
Repurposing is the process of taking an established agent from one therapeutic area and employing it for another. This approach has been employed successfully in many fields outside of antibacterial research, including cancer and HIV research (Strittmatter, 2014; Sleire et al., 2017). An advantage to repurposing compounds that have already been approved and licensed is the wealth of existing safety data

and knowledge surrounding pharmacokinetics and possible side effects (Van den Driessche et al., 2017). The access to such data may allow for repurposed therapeutic agents to quickly enter into clinical use, with fewer safety risks and significantly cheaper development costs. Screening compound libraries of mixed origins to be repurposed as antibiofilm agents has been fruitful (Ooi et al., 2015; Van den Driessche et al., 2017); however, the agents identified in these studies are narrow spectrum and may not prove useful against the multispecies chronic wound biofilm. Nevertheless, these studies highlight that repurposing compounds may provide a platform to discover new antibiofilm treatments.

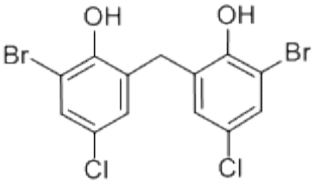
4.2.2 Where will newly repurposed antibiofilm agents come from?

The use of systemic antibiotics for the management of the chronic wound biofilm is considered controversial (Siddiqui et al., 2010). Previous recommendations have stated that systemic antibiotics should only be used when there are clinical signs of systemic infection (Powers et al., 2016). As described in section 1.2.3 topical antimicrobial agents are favoured over systemic antibiotics (Rhoads, D. et al., 2008; Lipsky et al., 2009). It therefore seemed sensible that the selection of appropriate candidates for repurposing as topical antibiofilm agents should start with compounds indicated for topical application. The agents considered in this study (Figure 4.1) came from a larger panel of 180 compounds that are licensed for use in healthcare products in the EU that have been shown to display a degree of antistaphylococcal activity (Personal Communications, A O'Neill); this list was further refined to exclude compounds from the same family, those with little or no antibacterial activity and those that had been evaluated in Chapter 3. The final list

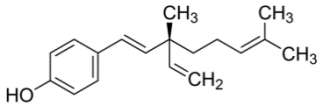
contained compounds that function as preservatives, skin conditioners, anti-static agents and antioxidants (EC, 2006), and all have established safety profiles.



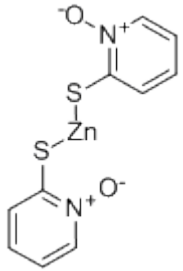
Phytosphingosine
$C_{18}H_{39}NO_3$



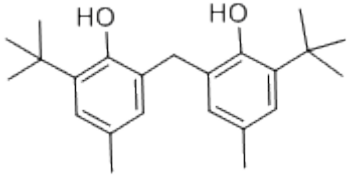
Bromochlorophene
$C_{13}H_8Br_2Cl_2O_2$



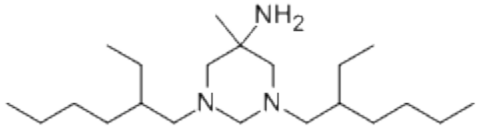
Bakuchiol
$C_{18}H_{24}O$



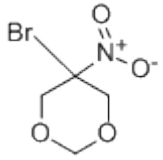
Zinc pyrithione
$C_{10}H_8N_2O_2S_2Zn$



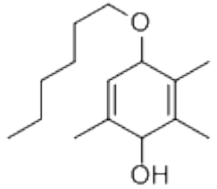
2,2'-Methylenebis(6-tert-butyl-4-methylphenol)
(AO2246)
$C_{23}H_{32}O_2$



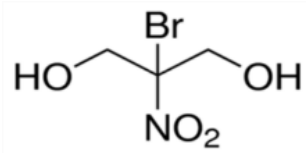
Hexetidine
$C_{21}H_{45}N_3$



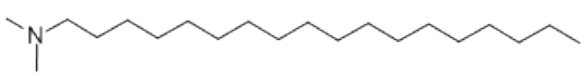
5-Bromo-5-nitro-1, 3-dioxane
(Bronidox)
$C_4H_6BrNO_4$



1-O-Hexyl-2, 3,5-trimethylhydroquinone
(HTHQ)
$C_{15}H_{26}O_2$



2-Bromo-2-nitro-1, 3-propanediol
(Bronopol)
$C_3H_6BrNO_4$



Dimethyl stearamine
$C_{20}H_{43}N$

Figure 4. 1 Structures and chemical formulas of a selection of agents licenced for use in topical agents. This list includes 10 agents that were considered in this study (structures and formulas were obtained from PubChem).

4.3 Aims and objectives

The work contained in this chapter explored the scope to repurpose agents which are licensed for use in topical healthcare products and evaluate their antibiofilm properties. The agents in question have a history of safe use, have defined maximum authorised concentrations (MACs) for permitted use and there is existing safety data available. The antibacterial and antibiofilm activity against common wound pathogens will be initially explored. Agents that display broad spectrum antibiofilm activity will be further investigated, with regards to their antibiofilm activity and their antibiofilm mechanism of action.

4.4 Results and discussion

4.4.1 Antibacterial activity of agents licensed for use in topical healthcare products

The panel of agents assayed have a variety of uses in topical healthcare products, including, but not limited to, preservatives (bronopol and bronidox), antioxidants (HTHQ, TBBQ, bakuchiol and AO2246), conditioning agents (phytosphingosine) and antimicrobials (zinc pyrithione and bromochlorophene). All have been previously reported to exhibit some antibacterial activity (Ghannoum et al., 1986; Shepherd et al., 1988; Ooi et al., 2015; Blanchard et al., 2016). However, most of the existing data did not use standardised susceptibility testing methodology, and information regarding antibiofilm activity is lacking. Antibacterial activity was initially evaluated for a larger panel (n=22) of antibacterial agents using the CSLI broth microdilution methodology (Section 2.3.1). Antibacterial activity against planktonic cultures of the laboratory strain *S. aureus* SH1000 was observed for 21 of the 22 agents tested, with MICs ranging from 0.25- 2048 µg/mL (Table 4.1). Ellagic acid did not exhibit any antibacterial activity at concentrations as high as 1024 µg/mL against SH1000.

Agent	MIC (µg/mL)	Agent	MIC (µg/mL)
1-O-Hexyl-2,3,5-trimethylhydroquinone (HTHQ)	16	**Hexamidine diisethionate**	0.25
2,2'-Methylenebis(6-tert-butyl-4-methylphenol) (AO2246)	2	**Hexetidine**	1
2-Bromo-2-nitro-1,3-propanediol (Bronopol)	16	**Menadione**	8
5-Bromo-5-nitro-1,3-dioxane (Bronidox)	16	**NDGA (Nordihydroguaiaretic acid)**	64
8-Hydroxyquinoline	2	**Octylisothiazolinone (OIT)**	16
Benzoyl peroxide	64	**Phytosphingosine**	12.5
Bromochlorophene	0.5	**Propyl gallate**	2048
Celastrol	0.5	**TBBQ**	8
Chiba (Bakuchiol)	4	**Thymohydroquinone**	16
Dimethyl stearamine	32	**Thymoquinone**	16
Ellagic acid	>1024	**Zinc pyrithione**	2

Table 4. 1 Antibacterial activity of agents licensed for use in healthcare products against *Staphylococcus aureus* SH1000. Minimum inhibitory concentration (MIC) determinations were conducted on a minimum of three independent occasions to ensure reproducibility.

Of the 22 compounds initially screened, 10 were selected for further investigation based on their potency against *S. aureus* (MIC ≤32 μg/mL) (Table 4.1). Some of the other agents (AO2246, TBBQ, thymohydroquinone, thymoquinone, bakuchiol, menadione and NDGA) were excluded from further study as their antibiofilm properties have recently been described (Ooi et al., 2015). Antibiofilm and antibacterial activity was assessed against the significant wound pathogens *S. aureus* and *P. aeruginosa* (Melendez et al., 2010). MIC determinations evaluated antibacterial activity, whilst antibiofilm activity was assessed using biofilms grown on the Calgary biofilm device. Eradication of *S. aureus* SH1000 biofilms was observed for all 10 agents at less than 256 μg/mL (Table 4.2).

By contrast, antibacterial and antibiofilm activity against *P. aeruginosa* PAO1 planktonic cultures was limited. Only zinc pyrithione, dimethyl stearamine, bronidox, and bronopol were active against PAO1 planktonic cultures, with MICs in the range of 8-32 μg/mL. Eradication of *P. aeruginosa* PAO1 biofilms at ≤256 μg/mL was observed for only two of the 10 GRAS compounds tested; bronidox (BX) and bronopol (BP) eradicated PAO1 biofilms at 128 and 64 μg/mL, respectively (Table 4.2). *P. aeruginosa* biofilms were more susceptible to BP than *S. aureus* biofilms (MBEC= 256 μg/mL) (Table 4.2). Early studies of the antibacterial activity of bronidox show a two-fold increase in the bronidox MIC against *P. aeruginosa* compared to *S. aureus* (Ghannoum et al., 1986), this observation was reflected in this study. BP and BX were therefore selected for further evaluation with regards to their antibacterial and antibiofilm properties and their potential to be repurposed as topical antibiofilm agents.

Compound	MIC	MBEC	MIC	MBEC
	µg/mL		µg/mL	
	Vs SH1000		Vs PAO1	
Bromochlorophene	0.5	16	>256	>256
Chiba (Bakuchiol)	4	32	>128	>128
Zinc pyrithione	2	256	16	>256
Hexetidine	1	4	>256	>256
AO2246	2	8	>256	>256
HTHQ	8	32	>256	>256
Phytosphingosine	16	128	>64	>256
Bronidox	16	128	32	128
Dimethyl stearamine	32	32	32	>256
Bronopol	16	256	8	64

Table 4. 2 Antibacterial and antibiofilm activity of compounds licensed for use in healthcare products against *Staphylococcus aureus* (SH1000) and *Pseudomonas aeruginosa* (PAO1) Susceptibility studies were conducted on a minimum of three independent occasions to ensure reproducibility. MIC- Minimum inhibitory concentration. MBEC- Minimum biofilm eradication concentration.

4.4.2 Spectrum of activity of bronidox and bronopol

The chronic wound biofilm consists of a diverse range of microorganisms, including aerobes, facultative anaerobes and obligate anaerobes (Dowd, S. et al., 2008). Standard culture techniques give us a snapshot of the diverse bacterial species present in the chronic wound microbiota, and DNA sequencing has revealed an

even greater level of diversity (Dowd, S. et al., 2008; Dowd, S.E. et al., 2008; Melendez et al., 2010). The antibacterial activity of BX and BP was assessed against a small panel of pathogens commonly isolated from the chronic wound biofilm, including *S. aureus, P. aeruginosa, E. faecalis* and *S. epidermidis* (Melendez et al., 2010). Activity against clinical isolates of Gram-negative bacteria associated with increased antibacterial resistance and nosocomial infections was also determined (Boucher et al., 2009); these latter microorganisms represent emerging wound pathogens, which are more commonly isolated from the deeper sections of the wound (Kirketerp-Møller et al., 2008).

BP was active against all the microorganisms tested with an MIC range of 4- 32 µg/ml (Table 4.3). BP was particularly active against the nosocomial pathogen *Acinetobacter. baumannii*, with an MIC of 4 µg/ml. *A. baumannii* is an emerging wound pathogen, and has been detected in over a quarter of wounds that failed to heal (Be et al., 2014).

BX inhibited growth of all the isolates screened at less than the highest concentration tested (256 µg/mL), with MICs ranging from 8-64 µg/mL. *P. aeruginosa* strains were the least susceptible to BX, with an MIC of 64 µg/mL.

Strain	Bronopol	Bronidox
	MIC	MIC
	µg/mL	
***S. aureus* SH1000**	16	8
***S. aureus* UAMS-1**	16	8
***S. aureus* USA300**	16	8
***P. aeruginosa* PAO1**	8	8
***P. aeruginosa* PA14**	16	64
***P. aeruginosa* NCTC 10332**	8	64
***E. faecalis* ATCC29212**	32	8
***S. epidermidis* RP62A**	8	8
***K. pneumoniae* 062**	16	16
***K. pneumoniae* 052**	16	16
***E.coli* 52**	8	16
***E. coli* 32**	8	16
***E. cloacae* 067**	8	16
***E. cloacae* 052**	16	16
***A. baumannii* 097**	4	8
***A. baumannii* 033**	4	8

Table 4. 3 Antibacterial activity of Bronopol and Bronidox against common wound and ESKAPE pathogens. Minimum inhibitory concentration (MIC) determination was conducted on a minimum of three independent occasions to ensure reproducibility

Another desirable property of a new antibacterial/antibiofilm agent is a low potential for the development of resistance (O'Neill and Chopra, 2004). To evaluate the resistance potential of BX and BP, *S. aureus* SH1000 cultures were exposed to sub-inhibitory concentrations of BP and BX over 15 passages to observe any decrease in susceptibility. Following 15 passages, the MICs of BP and BX did not increase greater than 2-fold, suggesting that both agents exhibit low resistance potential.

4.4.4 BP and BX antibiofilm activity against mixed-species biofilms

Biofilms present in chronic wounds are rarely comprised of a single microbial species, and indeed, most biofilms contain a diverse range of microorganisms (Dowd, S.E. et al., 2008). The Calgary biofilm device (CBD) provides a high throughput method of assaying antibiofilm activity; however, these biofilms are generally composed of a single species and the pegs of the CBD are only able to support small, immature biofilms of ~1×10^5 CFU (Ceri et al., 1999). Consequently, biofilms grown on the CBD do not necessarily represent those detected in chronic wounds. The impact of BP and BX on mature, mixed-species biofilms formed by the significant wound pathogens *S. aureus* and *P. aeruginosa* grown on nitrocellulose disks was examined (Chen et al., 2012; Ryder et al., 2012).

BX and BP may be used as active agents in antimicrobial dressings, therefore BP and BX were incorporated into sterile non-adherent dressings (Section 2.4.1), then added to the biofilms at their maximum authorised concentration (MAC) of 0.1% (w/v), the concentrations permitted for safe human use (Siegert, 2014).

Exposure of mixed species biofilms to BP and BX resulted in greater than 4 log CFU/disk reduction in both bacterial populations (*S. aureus and P. aeruginosa*) in 24 hours (Figure 4.2). Demonstrating eradication and disruption of biofilms at the MAC provides insight into how BP and BX may act if used in the clinic to treat biofilm infections.

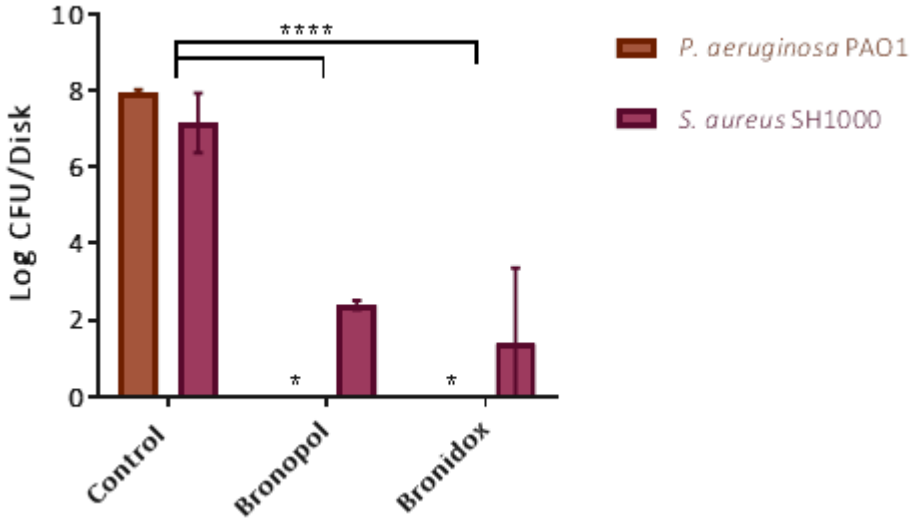


Figure 4. 2 Viability of *S. aureus* and *P. aeruginosa* biofilm cells following 24-hour exposure to BX and BP at their maximum authorised concentration 0.1% (w/v). (Mean of at least three independent replicates; error bars show standard deviations) *- below the limit of detection (2 Log_{10} CFU/disk) ****- Statically significant >95% confidence, p value= 0.0001. Calculated using 2-Way ANOVA using GraphPad Prism 7.01

4.4.3 Membrane damaging assays

Many biocides kill bacteria through the disruption of the bacterial cell membrane (Johnston et al., 2003; Lipsky et al., 2009). In order to determine whether membrane damage is the primary mode of action of BX and BP, the effect of the BX and BP on the bacterial membrane was determined using the BacLight assay (Section 2.5.4).

The BacLight assay utilises two fluorescent nucleic acid dyes, propidium iodide and Syto9 (Hilliard et al., 1999). Syto9 stains both cells with intact and compromised membranes; whist propidium iodide can only stain cells with disrupted membranes. Membrane integrity of bacterial cells exposed to antibacterial agents is assessed by observing the ratio of both dyes. Exposure of *S. aureus* cultures to BP for 10 minutes had minimal effect on cell membrane integrity (>85% bacterial membrane integrity), like the comparator antibacterial agent tetracycline, which is known not to perturb the bacterial membrane (Chopra and Roberts, 2001) (Figure 4.3). A more marked reduction (>35%) in cell membrane integrity was observed following treatment with BX. Due to toxicity issues BX is only allowed for rinse off formulations (Siegert, 2014), this may explain why a more marked effect on bacterial cell membranes was observed. However, the membrane-active lantibiotic, nisin, caused >90% membrane disruption.

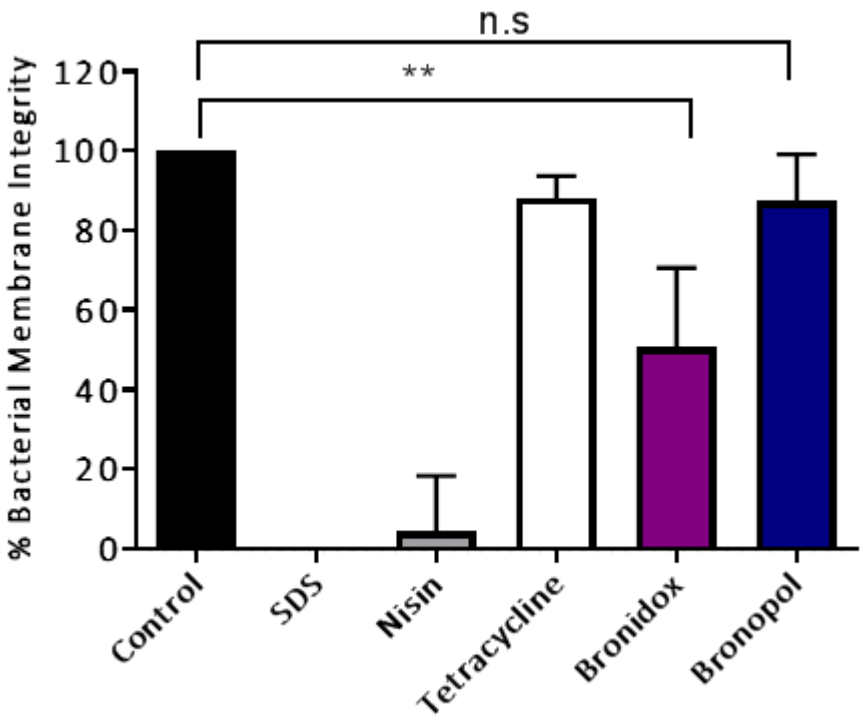


Figure 4. 3 The impact of BP, BX, and comparator agents at 4X MIC on *S. aureus* SH1000 membrane integrity. (mean of at least three independent replicates; error bars show standard deviations) n.s- not significant <95% confidence, p value >0.05. **- Statically significant >95% confidence, p value= 0.0026. Calculated using un-paired t-test using GraphPad Prism 7.01

BX and BP are generally recognised as safe to use, and possess defined concentrations permitted for safe human use (EC, 2006). However, BX was able to disrupt the bacterial membrane and antimicrobial agents that cause disruption of the bacterial cell membrane often have some effect on mammalian cells (Ooi et al., 2015). Therefore, the haemolysis assay (Section 2.5.3) was conducted in order to confirm bacterial selectivity. The haemolysis assay determines the influence of antibacterial agents at 4X MIC on the membrane integrity of equine red blood cells. Disruption of the membrane results in the release of haemoglobin, which can be

monitored by optical density at 540 nm. Equine erythrocyte integrity was marginally reduced following treatment with BX and BP (Figure 4.4). Membrane integrity was >80% for both compounds, unlike those treated with nisin which reduced erythrocyte membrane integrity over 40%. This finding corroborates that both agents should be safe for use on the skin.

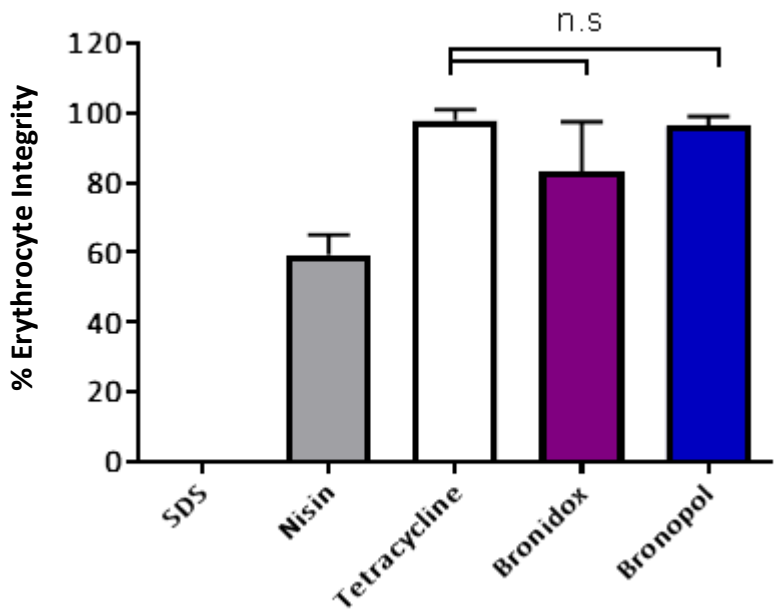


Figure 4. 4 The impact of BP, BX, and comparator agents at 4X MIC on equine erythrocyte membrane integrity. (mean of at least three independent replicates; error bars show standard deviations) n.s- not significant <95% confidence, p value >0.05. Calculated using un-paired t-test using GraphPad Prism 7.01

4.4.3 Antibiofilm mode of action of BP and BX

Within the biofilm, a large proportion of the bacterial cells are in a slow or non-growing (SONG) state (Donlan et al., 2002). SONG cells display a reduced susceptibility to antibacterial treatment and contribute to the difficulty in treating biofilm related infections (Donlan et al., 2002). With a view to understand whether the antibiofilm activity of BP and BX comes from the ability to act against SONG cells, the action of BP and BX on non-growing and exponential phase cultures of *S. aureus* was assessed (Section2.3.5).

Reduction in bacterial viability by 99.9% after 24 hours is indicative of bactericidal activity (Pankey et al., 2004). Within 24 hours, the viability of exponential phase *S. aureus* cultures exposed to BP and BX at 4X MIC was reduced by ≥5 logCFU/mL (Figure4. 5a). BX and BP have been demonstrated *in vitro* to oxidise essential thiol containing residues, this reaction results in the gradual generation of reactive oxygen species (ROS) including superoxide and peroxides (Ghannoum et al., 1986; Shepherd et al., 1988). The incremental release of ROS may explain why BX and BP are unable to rapidly kill growing cultures.

BP and BX sterilised stationary phase cultures after 24 hours, unlike the comparator agents vancomycin and tetracycline (Figure 4.5b). Vancomycin and tetracycline are both unable to eradicate *S. aureus* biofilms (Ooi et al., 2015).

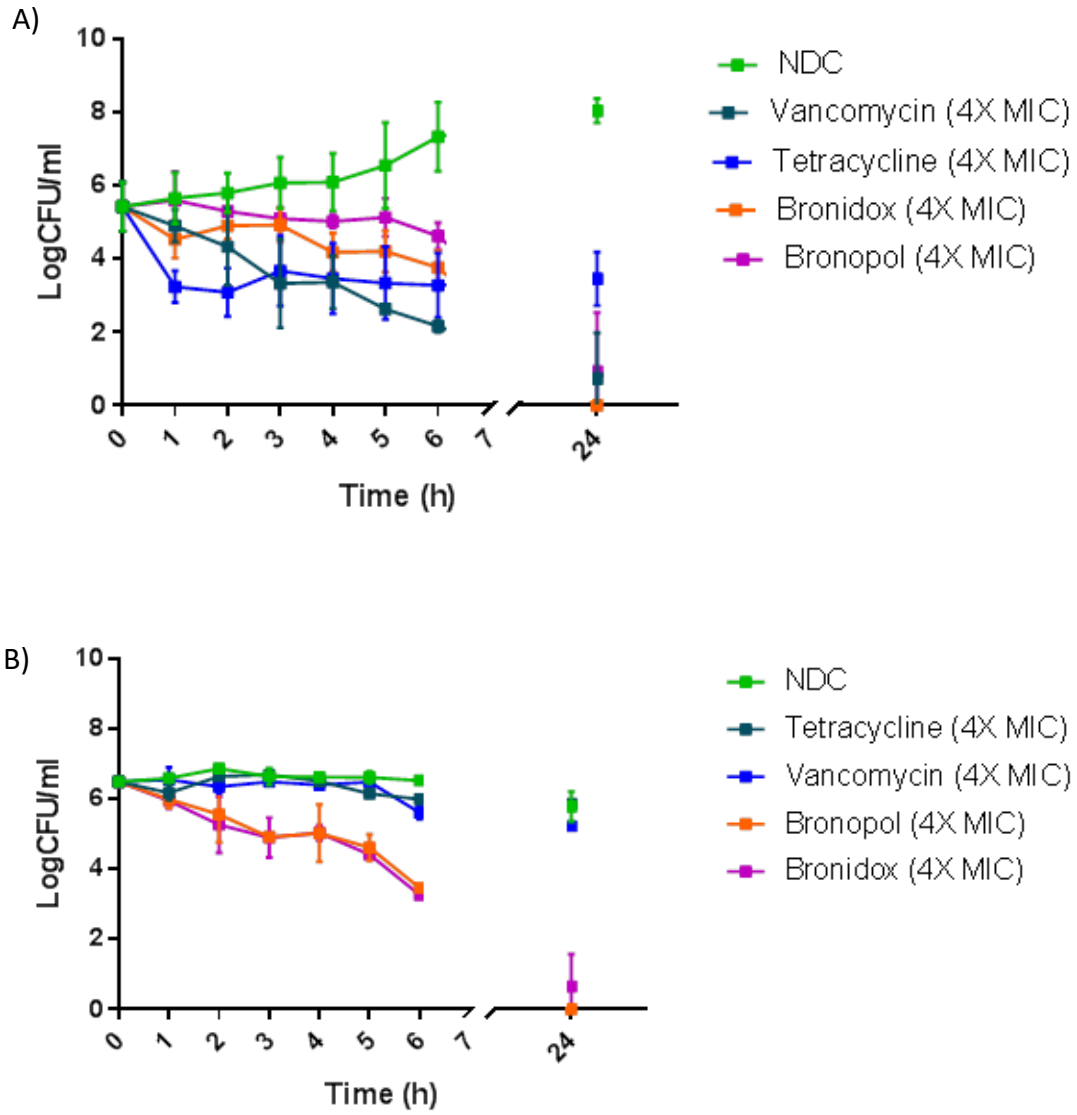


Figure 4. 5 Time kill studies BX and BP at 4X MIC against exponential (A.) and stationary phase (B.) cultures of *S. aureus* SH1000 (mean of at least three independent replicates; error bars show standard deviations)

The observation that BP and BX can sterilise slow/non-growing cultures may indicate why BX and BP effectively eradicate biofilms; this led to further investigations into of how BP and BX exert their antibiofilm action. Some antibiofilm

agents that eradicate biofilm have been demonstrated to do so through the disruption of the biofilm matrix (Ooi et al., 2015). To determine whether this is the case for BX and BP, *S. aureus* biofilms formed in the wells of pre-treated 96 well plates were exposed to BP and BX at 16X MBEC; and the effect on the biofilm structure was assessed by staining the biofilm matrix with SyproRuby and monitoring biofilm viability in parallel (Section 2.5.5 and Section 2.5.6). Testing at 16X MBEC allowed for monitoring alterations of the biofilm matrix and viability more readily as this concentration was unable to cause rapid eradication of the biofilm. The biofilm matrix is primarily composed of protein, EPS and extracellular DNA (Hall-Stoodley et al., 2004; Del Pozo et al., 2007). The matrix forms approximately 80-85% of biofilm structures with the remaining 15-20% containing bacterial cells (Dufour et al., 2010). The fluorescent dye Sypro Ruby stains the proteinaceous biofilm matrix; alterations of the biofilm matrix can therefore be monitored using the dye. In parallel biofilm viability experiments, proteinase-K was utilised for its ability to disrupt the biofilm matrix (Kumar Shukla and Rao, 2013).

The matrix of biofilms exposed to BX at 2048 µg/mL (16X MBEC) remained intact, whilst integrity of BP treated biofilms reduced approximately 40% relative to the untreated control at 6 hours (Figure 4.6). Treatment of *S. aureus* biofilms grown on 96 well plates with BP and BX at 16X MBEC eradicated viable cells in the structure within 6 hours (Figure 4.6). This suggests that BP and BX exert their antibiofilm action predominantly by killing growing and non-growing bacteria in the biofilm.

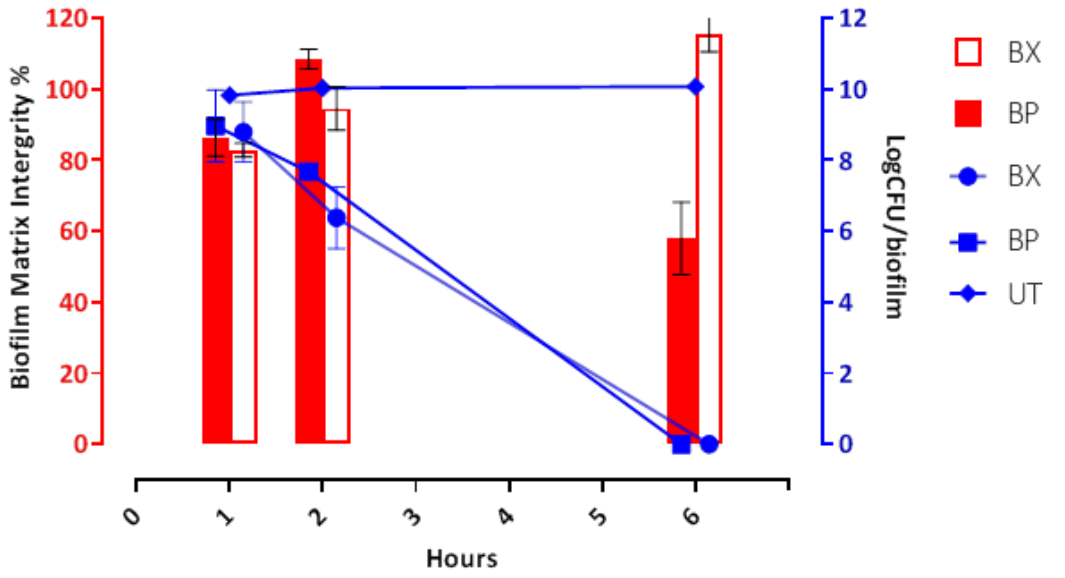


Figure 4. 6 Quantification of *S. aureus* SH1000 biofilm matrix and viable cells, following exposure to BX and BP at 16 XMBEC for 6 hours (mean of at least three independent replicates; error bars show standard deviations)

4.4.5 Synergy between BX and BP and established antimicrobial wound agents

The use of combination therapy has been suggested as a useful approach to combat biofilm infections (Hurdle et al., 2011). To investigate strategies to further enhance the antibiofilm activity of BX and BP, synergy between BX and BP and existing wound agents was examined.

In combination with silver nitrate, chlorhexidine, and cetrimide, BX and BP acted synergistically against *S. aureus* and *P. aeruginosa* single species biofilms (Table 4.4). However, the most synergistic combination was BP with chlorhexidine, which together showed the lowest FBEC index for both *S. aureus* (FBEC= 0.15) and *P. aeruginosa* (FBEC =0.25).

The antibiofilm synergy observed indicates that BP and BX may be used alongside established agents, to achieve improved biofilm disruption and eradication.

		FBEC Index		
		Silver Nitrate	Cetrimide	Chlorhexidine
BX	*S. aureus* SH1000	0.25	0.5	0.5
	P. aeruginosa PAO1	0.5	0.375	0.5
BP	*S. aureus* SH1000	0.5	0.25	0.15
	P. aeruginosa PAO1	0.25	0.375	0.25

Table 4. 4 Fractional Biofilm Eradication Concentration (FBEC) Index Determination; BP and BX in combination with wound agents against biofilm cultures of *S. aureus* and *P. aeruginosa*. An FBEC index less than or equal to 0.5 is indicative of a synergistic interaction. Biofilm FIC studies were conducted on a minimum of three independent occasions and the mean result is given.

4.5 Conclusion

To date, there are very few antibiofilm agents that are able to eradicate biofilms formed by both Gram-negative and Gram-positive species (Wolfmeier et al., 2017). This work aimed to identify and evaluate compounds that have a history of safe use in healthcare products, for their potential to be utilised as broad spectrum antibiofilm agents. An initial screen of 22 compounds identified bronidox and bronopol as compounds able to eradicate biofilms formed by *S. aureus* and *P. aeruginosa*. Both agents displayed broad spectrum antibacterial activity against known and emerging wound pathogens. Slow and non-growing cells are abundant in biofilm cultures, the ability to sterilised non-growing cultures of *S. aureus* pointed towards their possible antibiofilm mechanism of action. Monitoring their effect on the biofilm matrix and bacterial viability suggest that bronidox and bronopol target biofilm cells (including SONGs) directly and that the disruption of the biofilm matrix occurs secondary to the loss of bacterial viability. At concentrations permitted for safe topical use, bronidox and bronopol were able to cause a significant disruption of mixed species biofilms containing *S. aureus and P. aeruginosa*.

Bronidox and bronopol are both nitro-halo compounds functioning as preservatives used in cosmetic products. Both agents have been used for in excess of 40 years; however to date there is limited/no information pertaining to their antibiofilm activity. This work contains the first report of the antibiofilm activity of BX and BP, however, further work is warranted to assess the potential of BX and BP in the clinic.

A limitation to BX and BP is that they can release formaldehyde, in a pH and concentration dependent manner (Chandra Rastogi, 1992; Lv et al., 2015). This limitation may be avoided by using both agents at lower concentrations in synergistic combination with other active ingredients such as silver nitrate, cetrimide or chlorhexidine. In addition, BX is limited to use in rinse- off formulations (Siegert, 2014), therefore BX may serve better as the active ingredient of an irrigation solution rather than in a dressing.

The ability to diminish both prominent wound pathogens in a biofilm more similar to that which is observed in chronic wounds highlights their potential to be used as antibiofilm agents. Their potential as topical antibiofilm agents is further strengthened by the synergistic activity observed with antibacterial agents currently used in topical applications.

Overall, the findings of this study highlighted the utility of repurposing agents used in healthcare products as antibiofilm agents, this warrants a more comprehensive screen of such compounds.

Chapter 5 Antibacterial activity and mechanism of action of batumin

The work contained within this chapter has been published in part in:

LEE, V. E. & O'NEILL, A. J. 2017. Batumin does not exert its antistaphylococcal effect through inhibition of aminoacyl-tRNA synthetase enzymes. *International Journal of Antimicrobial Agents,* **49**(1)**,** 121-122.

5.1 Abstract

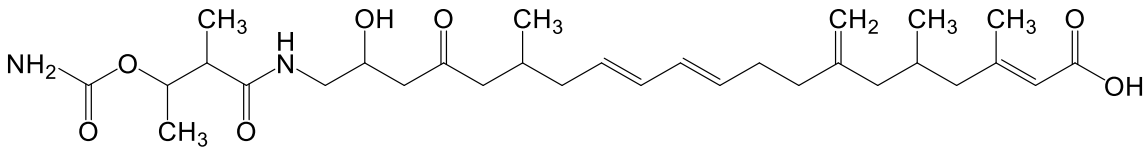
The antibiotic batumin displays antistaphylococcal activity. Since staphylococci represent the most common aetiological agents of skin infections, batumin may be valuable for treatment of such infections. Determining the mechanism of action (MOA) of an antibacterial agent is an important aspect of pre-clinical drug discovery, and therefore the MOA of batumin was investigated in this chapter. Previous work has suggested that batumin inhibits the growth of staphylococci by targeting the essential process of fatty acid biosynthesis (FAS). A macromolecular synthesis (MMS) assay revealed that batumin does indeed inhibit FAS. Laboratory-generated batumin-resistant mutants displayed cross resistance to the FAS inhibitor triclosan, which implied an overlapping target (FabI). DNA sequencing of batumin-resistant mutants revealed mutations in the coding and promoter regions of two FAS enzymes, FabI and FapR. The identified mutations were predicted to result in increased expression of FabI, and artificial overexpression of FabI in *S. aureus* resulted in decreased susceptibility to batumin, strengthening the case that FabI is

the molecular target of batumin in *S. aureus*. Finally, inhibition of purified *S. aureus* FabI by batumin was demonstrated *in vitro*. Collectively, these studies have established that batumin targets FAS by directly inhibiting the essential enoyl-ACP reductase, FabI.

5.2 Introduction

5.2.1 The antibiotic batumin

Batumin (Figure 5.1) is a polyketide antibiotic first purified from the bacterium *Pseudomonas batumici* UCM B-321, which was isolated from a soil specimen collected off the coast of Batumi, Georgia (Kiprianova et al., 2011). This antibiotic displays potent and selective antistaphylococcal activity (Churkina et al., 2015); indeed, its specificity for staphylococci is such that it has been used to create the diagnostic tool ("Diastaph") that has been patented in Ukraine (Churkina et al., 2015).



Batumin/Kalamanticin A

Chemical Formula: $C_{30}H_{48}N_2O_7$
Molecular Weight: 548.721

Figure 5. 1 Chemical structure of Batumin/Kalimantacin A (Adapted from Mattheus, 2010a)

The structure of the antibiotic kalimantacin A has been found to be identical to that of batumin, though it is produced by a different microorganism (Thistlethwaite et al., 2017). Genomic analysis of the kalimantacin A-producing organism (*Pseudomonas fluorescens* BCCM_ID9359) identified the *kal/bat* gene cluster that is responsible for the production of the antibiotic (Mattheus et al., 2010a). This *kal/bat* has also been identified in the batumin producing organism *P. batumici* UCM B-321 (Klochko et al., 2016). The *kal/bat* gene cluster is a 62 kb region containing 16 open reading frames (ORFs) that collectively encode hybrid polyketide synthase/ non-ribosomal peptide synthase (PKS-NRPS) machinery (*bat1-3*), trans-acting tailoring functions (*batA-M*) are used to extend this antibiotic (Mattheus et al., 2010a). It has been proposed that the protein encoded by *batG* serves no biosynthetic role in batumin production, but instead encodes a self-protection mechanism that spares the producer organism from the inhibitory effect of the antibiotic (Mattheus et al., 2010b). This gene encodes an isoform of enoyl-ACP reductase, FabV, that plays a role in FAS (Mattheus et al., 2010b); alignment of the *batG* coding sequence with *S. aureus* and *E. coli fabI* coding regions revealed moderate similarity, and strong similarity (76% similarity) was observed with the *fabV* gene from *P. aeruginosa* (Mattheus et al., 2010b). Heterologous expression of *batG* in *E. coli* and *S. aureus* was subsequently shown to reduce susceptibility to batumin >32 fold (Mattheus et al., 2010b). This finding appeared to implicate inhibition of FAS as the antibacterial mechanism of action of batumin.

Klochko and colleagues recently proposed an alternative hypothesis regarding the antibacterial target of batumin. On the basis of molecular docking studies and

analysis of the *P. batumici* UCM B-321 genome, they concluded that batumin acts in a similar fashion to the antibiotic mupirocin, binding to isoleucyl-tRNA synthetase (IleRS) to inhibit protein synthesis (Klochko et al., 2016). Their in *silico* docking studies demonstrated that batumin binds to IleRS from *S. aureus* with a similar binding affinity to mupirocin, whilst no affinity to FabI was observed (Klochko et al., 2016). They predicted that the primary mode of action of batumin is through the inhibition of aminoacyl-tRNA synthetase enzymes, and that inhibition of FAS is a secondary effect, related to the induction of the stringent response (Klochko et al., 2016). These two conflicting hypotheses demonstrate that the precise mechanism by which batumin inhibits the growth of staphylococci remains to be established.

5.2.2 Mechanism of action studies

Defining the mechanism of action (MOA) of a novel antibacterial agent is an essential part of preclinical drug discovery. Knowledge of an agent's MOA in conjunction with pharmacological (PK/PD) data provides useful insight into its potential clinical utility (O'Neill et al., 2004). Most antibiotics inhibit the biosynthesis of essential macromolecules, including protein, nucleic acid, folates and peptidoglycan (Wright, 2010). Following the identification of an agent which displays potent antibacterial activity, macromolecular synthesis (MMS) assays can be conducted to identify whether inhibition of one of these essential biological processes occurs (O'Neill et al., 2004). The MMS assay utilises radiolabelled precursors of these essential pathways and follows the incorporation of these precursors following challenge with test agents and positive controls (Cotsonas King et al., 2001). If an agent does not inhibit a particular pathway or there is disruption

of all pathways this can be indicative of membrane damage or non-specific effects (Ooi et al., 2009).

Genotypic analysis of laboratory mutants resistant to the antibacterial agent in question may also aid in the identification of its target (O'Neill et al., 2004). Selection of resistant mutants also anticipates the likelihood of resistance development in the clinic and can be used to identify cross resistance with existing antimicrobial agents. The generation of strains with altered expression of the proposed target can also aid the determination of MOA; overexpression of the target often results in reduced susceptibility to the antibacterial agent, whilst hyper-susceptibility occurs following down-regulation of the target (O'Neill et al., 2004). Additionally, biochemical studies using purified proteins can provide direct evidence of *in vitro* inhibition of the proposed target.

5.2.3 Aims and Objectives

The work in this chapter investigated the antibacterial mechanism of action of batumin in *S. aureus*. Initial experiments evaluated the antibacterial spectrum of activity of batumin and the inhibition of key biosynthetic pathways. To establish the target of batumin, spontaneous batumin-resistant mutants were genetically characterised. *S. aureus* strains with altered expression of the proposed target of batumin were generated to provide further confirmation of the target. Finally, biochemical studies were used to confirm batumin is able to inhibit the suggested *S. aureus* target.

5.3 Results and Discussion

5.3.1 Antibacterial activity of batumin

To better define the spectrum of activity of batumin, the antibacterial activity of the agent was assessed against various clinical and laboratory strains of Gram-positive bacteria including *S. aureus* (n=5), *S. epidermidis* (n=1) and *E. faecalis* (n=1), Batumin displayed potent antistaphylococcal activity, with staphylococcal MICs ranging from 0.0625-0.25 μg/mL (Table 5.1), and no substantial difference in susceptibility of MRSA (*S. aureus* USA 300) and MSSA strains was observed. As previously reported, antibacterial activity was not observed against *E. faecalis;* batumin was unable to inhibit this organism at 16 μg/mL, the highest concentration tested.

The antibacterial activity of batumin was also determined against the Gram-negative pathogens, *E. coli* and *P. aeruginosa* (Table 5.1). No activity was observed for either microorganism at 16 μg/mL, the maximum concentration tested. The Gram- negative outer membrane provides a major barrier for the diffusion of many antibacterial agents, and prevents these compounds from reaching their intracellular target (Arzanlou et al., 2017). Furthermore, if a compound is able to traverse the Gram- negative outer membrane, bacterial efflux pumps can act to reduce the amount of antibiotic accumulating within the cell (Arzanlou et al., 2017). To understand whether the lack of activity in *E. coli* was due to lack of ingress or active efflux of batumin out of the cell, MICs were conducted against (i) *E. coli* strains deleted for the major multidrug efflux transporter (AcrAB-TolC) and (ii) *E.*

coli with artificially compromised membranes. *E. coli* deletion strains of *acrA* and *acrB* were susceptible to batumin at 8 and 4 μg/mL, respectively (Table 5.1). Permeabilisation of the *E. coli* outer membrane with polymyxin B nonapeptide (PMBN) resulted in increased susceptibility to batumin (MIC= 0.5 μg/mL), a level of activity comparable to that observed against *S. aureus.* Thus, the lack of activity of batumin against *E. coli* is predominantly due to the inability of the antibiotic to traverse the outer membrane, though active efflux also appears to play a lesser role.

Biofilms play a role in many chronic skin infections, and therefore the antibiofilm activity of batumin was also assessed. Batumin was unable to eradicate biofilms formed by *S. aureus* SH1000 on the Calgary biofilm device at the highest concentration tested (32 μg/mL).

Strain	MIC (µg/mL)
***S. aureus* SH1000**	0.0625
***S. aureus* RN4220**	0.25
***S. aureus* USA300**	0.125
***S. aureus* UAMS-1**	0.25
***S. aureus* ATCC 25923**	0.25
***S. epidermidis* RP62A**	0.0625
***E. faecalis* ATCC29212**	>16
***P. aeruginosa* PAO1**	>16
***E. coli* BW25113**	>16
E. coli* Δ*acrA	8
E. coli* Δ*acrB	4
***E.coli* + PMBN @4 µg/mL**	0.5

Table 5. 1 Antibacterial activity of batumin against planktonic cultures. Minimum inhibitory concentration (MIC) determinations were conducted on a minimum of three independent occasions to ensure reproducibility. PMBN- Polymyxin B Nonapeptide

5.3.2 Effect of batumin on biosynthetic pathways

As described above, previous studies regarding the MOA of batumin have suggested the inhibition of fatty acid biosynthesis or protein synthesis (Mattheus et al., 2010b; Klochko et al., 2016). To investigate whether batumin inhibits fatty acid or protein synthesis in staphylococci, the incorporation of radiolabelled precursors into macromolecules was monitored (Section 2.5.1). Triclosan and mupirocin are known inhibitors of fatty acid biosynthesis and protein synthesis, respectively, and

were used as positive control agents. At 4X MIC, batumin caused a substantial reduction (>60%) of incorporation of the fatty acid biosynthesis precursor (^{14}C acetic acid) after 10 minutes, an effect comparable to that of triclosan (Figure 5.2). Batumin did not inhibit protein synthesis (~100 % incorporation), whilst the positive control inhibitor mupirocin reduced macromolecular incorporation by over 60% and had no impact on fatty acid biosynthesis at 10 minutes (Figure 5.2). Failure to observe any inhibition of protein synthesis in this experiment argues against the proposal of Klochko et al. that batumin exerts its antistaphylococcal activity primarily through inhibition of protein synthesis (Klochko et al., 2016). By contrast, the rapid and substantial inhibition of fatty acid synthesis achieved by batumin observed, strongly suggests that batumin exerts it antistaphylococcal activity through the inhibition of fatty acid biosynthesis. This observation led to the rejection of the hypothesis set out by Klochko et al., that inhibition of FAS by batumin is a secondary MOA.

Further studies were initiated to more precisely delineate the molecular target of batumin. There are multiple enzymes involved in FAS and to date various antibiotics have been demonstrated to inhibit FabI (Heath et al., 2000), FabH (Price et al., 2001), FabB and FabF (Slayden et al., 1996). It is possible that batumin inhibits one or more of these enzymes, or any other of the enzymes involved in the FAS pathway.

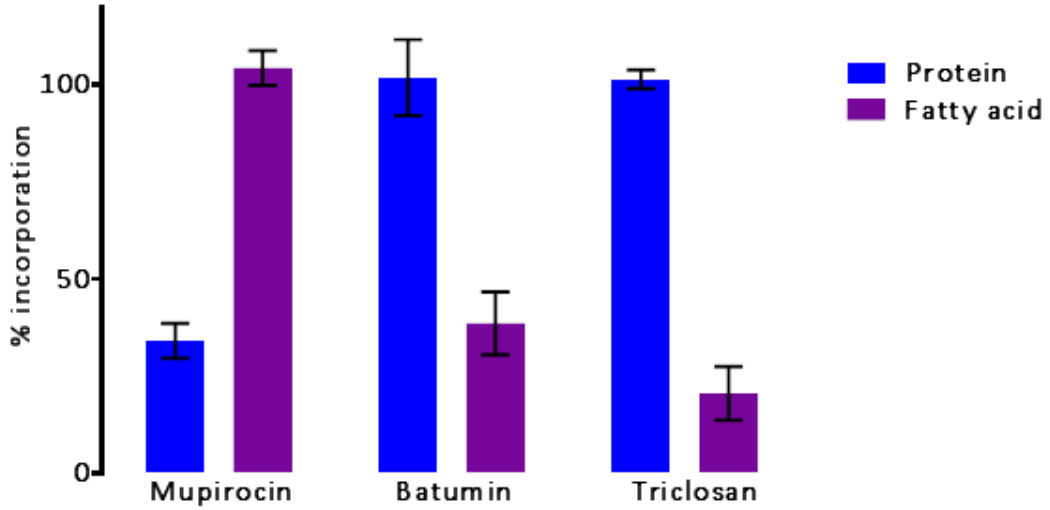


Figure 5. 2 Effects of mupirocin, batumin and triclosan on protein and fatty acid biosynthesis pathways in *S. aureus* SH1000. Percentage incorporation of ^{3}H glutamine and ^{14}C acetic acid into SH1000 protein and fatty acid, respectively. (mean of at least three independent replicates; error bars show standard deviations)

5.3.3 Generation and characterisation of batumin-resistant mutants

With a view to identifying the molecular target of batumin in *S. aureus*, spontaneous batumin resistant mutants were recovered (Section 2.5.7). Genomic analysis of resistant mutants can identify loci responsible for reduced susceptibility, and it is often the case that mutations will actually lie within the gene encoding the target (O'Neill et al., 2004).

Batumin-resistant mutants were selected by plating volumes (1 mL) of saturated overnight *S. aureus* SH1000 culture onto MHA-II plates containing batumin at 4X MIC. Following 24 hours' incubation, six batumin resistant mutants were recovered.

One isolate (designated BM 6) was initially chosen at random for further characterisation. The MIC of batumin was determined against the resistant mutant to confirm the reduced susceptibility; BM 6 was 32-fold less susceptible to batumin (MIC= 2 μg/mL) than the parental strain, *S. aureus* SH1000 (Table 5.2).

To understand the genetic basis of the altered susceptibility to batumin in BM 6, the whole genome sequence (WGS) was determined to identify mutations responsible for reduced susceptibility. Analysis of the BM 6 genome identified a mutation ($C_{88}T$) in SAOUHSC_01196, the gene encoding the fatty acid biosynthesis negative transcriptional regulator, FapR (Schujman et al., 2003); this missense substitution resulted in the amino acid substitution, $H_{30}Y$, in the encoded protein. FapR has been demonstrated to directly interact with the *fabI* promoter region thereby directly repressing expression (Jang et al., 2008). It seemed plausible that this amino acid substitution resulted in reduced FapR activity, thereby preventing repression of FabI; the reduced susceptibility to batumin in BM 6 could therefore potentially be attributable to overexpression of FabI. This idea was tested by examining whether BM 6 exhibited cross-resistance to the FabI inhibitor, triclosan, since overexpression of FabI is known to mediate reduced susceptibility to this agent (Grandgirard et al., 2015). BM 6 was less susceptible to triclosan with an MIC of 1 μg/mL, suggesting a potential overlap in the molecular target. In addition, a laboratory generated triclosan-resistant mutant was also resistant to batumin (MIC=2 μg/mL), strengthening the case of an overlapping target.

Batumin Resistant Mutant	MIC (µg/mL)	Mutation
SH1000	0.0625	N/A
BM 1	0.5	-106 FabI T→C
BM 2	1	-109 FabI T→G
BM 3	0.5	-72 FabI A→G
BM 4	1	-72 FabI A→G
BM 5	2	Unknown
BM 6	2	FapR $C_{88}T$
BM 7	0.5	-109 FabI T→G
BM 8	0.5	Unknown

Table 5. 2 Determination of minimum inhibitory concentrations for batumin against SH1000 and batumin- resistant derivatives. Minimum inhibitory concentration (MIC) determinations were conducted on a minimum of three independent occasions to ensure reproducibility

Additional batumin-resistant mutants were subsequently generated from seven independent *S. aureus* SH1000 cultures. The MIC of batumin for these mutants increased 8- 32 fold relative to SH1000, with MICs ranging from 0.5-2 µg/mL (Table 5.2). For each mutant, the coding and promoter regions of *fabI* and *fapR* were amplified by PCR and their DNA sequence determined to identify any mutations that may play a role in batumin resistance. No mutations were identified in the *fapR* coding or promoter region of these mutants. For isolates BM 3 and BM 4, a mutation ($A\text{-}_{72}G$) was identified in the promoter region of *fabI*; this mutation has previously been identified in laboratory-generated triclosan-resistant *S. aureus* strains (Grandgirard et al., 2015). A further mutation was identified in the *fabI* upstream regions of BM 2 and BM 7 ($T\text{-}_{109}G$); similarly, this mutation has previously

been identified in clinical isolates resistant to triclosan (Grandgirard et al., 2015). Position -109 lies within the putative FapR binding site, and the thymine ordinarily resident at this position has been shown to be central to FapR DNA recognition (Grandgirard et al., 2015). In BM1, another upstream mutation was identified in the putative FapR binding site ($T_{-106}C$); the effect of this latter mutation on FapR binding and/or triclosan resistance has not to date been described. No substitutions were identified in either *fabI* or *fapR* for BM5 and BM8, and consequently these isolates were sent for WGS for further analysis. Unfortunately, due to time constraints, I was unable analyse the genome of these mutants and further work will be required to do so. Mutations in the *fabI* upstream region of triclosan mutant identified by Grandgirard and colleagues were shown to result in the up-regulation of FabI and thereby reduced susceptibility to triclosan (Grandgirard et al., 2015). Some of the same mutations identified in these batumin-resistant mutants in this study have previously been found in triclosan-resistant *S. aureus* strains suggest that batumin and triclosan share the target (FabI).

5.3.4 Over-expression of proteins involved in fatty acid biosynthesis in *S. aureus* SH1000

To further corroborate the idea that reduced susceptibility to batumin can result from overexpression of FabI, and that FabI is therefore the likely target of batumin, the effect of overexpression of FabI on batumin susceptibility was determined (Section 2.9). The vector pRAB11 (Helle et al., 2011) was used for overexpression *in trans* of FabI in *S. aureus* SH1000. In parallel experiments, two other proteins were overexpressed using the same system to evaluate their effect on batumin

susceptibility. The fact that loss of function of the negative transcriptional regulator FapR mediates batumin resistance in *S. aureus* (Section 5.5.3) led me to ask the question: would overexpression of this regulatory protein actually serve to increase batumin susceptibility as a consequence of repressing FabI expression? To address this question, *fapR* from *S. aureus* SH1000 was also cloned in pRAB11. In *Enterococcus faecalis*, the Enoyl-ACP reductase FabK mediates triclosan resistance, with expression of *fabK* conferring between 10 to 100-fold decrease in susceptibility to triclosan in *E. coli* (Zhu et al., 2013). To determine whether *fabK* might also impact batumin susceptibility, this gene was also cloned in pRAB11.

To allow the expression of the fatty acid biosynthesis genes cloned into pRAB11, strains were induced with (anhydrotetracycline) ATc for 4 hours, and the MICs of batumin and triclosan were then determined. The MICs of batumin against the FabI overexpression strain *S. aureus* SH1000 (pRAB11: *fabI*) increased 16-fold (MIC =2 µg/mL) compared to the activity observed against the parental SH1000 (MIC=0.125 µg/mL). Overexpression of FabI also resulted in an elevated triclosan MIC; however only a four-fold increase was seen (Table 5.3). The discrepancies between the changes in susceptibility could be in part due to differences in the binding affinity of batumin and triclosan to *S. aureus* FabI; if batumin binds more weakly to FabI than triclosan, it is possible that more of the compound is required to inhibit increased levels of FabI. The decreased susceptibility of *S. aureus* SH1000 (pRab11:*fabI*) to batumin provides further evidence that the molecular target of batumin is FabI- as more batumin is required to overcome the elevated levels of FabI. It should be

considered that overexpression of FabI could result in the upregulation of the FAS pathway; it is therefore still possible that batumin targets another FAS enzyme.

In trans overexpression of the negative regulator FapR did not result in any change in batumin susceptibility. With increased expression of the negative regulator FapR, it was possible that FabI expression would be greatly reduced and thereby lead to increased susceptibility to batumin. However, Schujman and colleagues described in *Bacillus subtillis* that FapR activity and its activation is in response to changes in malonyl-CoA concentrations (Schujman et al., 2003). Malonyl-CoA plays a role in the initiating steps of FAS and has no impact of FabI activity (Fujita et al., 2007). The over expression of FapR, without a depletion of malonyl-CoA concentrations (which was not monitored in this assay), will not by itself lead to increased FabI repression.

The MIC of batumin was also determined against *S. aureus* expressing the *E. faecalis* ENR FabK *in trans*. *S. aureus* SH1000 (pRab11:*fabK*) exhibited a ≥32 fold reduction in batumin susceptibility, with an MIC of > 4 μg/mL, and the MIC of triclosan also increased 16-fold against this strain (MIC=2 μg/mL). This observation provides further support for the idea that batumin targets FabI, since introduction of FabK into *S. aureus* was sufficient to confer batumin resistance. Wild type *E. faecalis* contains both FabI and FabK, with FabK playing a lesser role in the modulation of FAS (Zhu et al., 2013). The over expression of FabK in *S. aureus* could "rescue" FAS, with more batumin being require to inhibit growth.

This observation provides some confirmation that batumin is unable to target the ENR FabK and can provide some explanation as to why batumin is inactive against *E. faecalis*. The intrinsic resistance of *E. faecalis* to batumin could be due to the

presence of FabK. This hypothesis could be tested by determining the activity of batumin against a *S. aureus* FabI knockout strain expressing FabK.

The results presented thus far suggest that the FabI enzyme is central to batumin's mode of action, since overexpression of FabI and other ENRs in *S. aureus* resulted in reduced susceptibility to batumin; further study is required to confirm this relationship. Confirmation of the inhibition of FabI by batumin can be assessed biochemically or through the generation of *S. aureus* FabI underexpression mutants (O'Neill et al., 2004); the former approach was explored in the subsequent section.

	Batumin MIC (µg/mL)		**Triclosan MIC (µg/mL)**	
	Uninduced	**Induced**	**Uninduced**	**Induced**
***S. aureus* SH1000**	0.125	0.125	0.125	0.125
***S. aureus* SH1000 (pRab11: *fabI*)**	0.25	2	0.125	0.5
***S. aureus* SH1000 (pRab11:*fapR*)**	0.25	0.25	0.125	0.06
***S. aureus* SH1000 (pRab11:*fabK*)**	0.25	>4	0.125	2

Table 5. 3 Determination of minimum inhibitory concentrations (MIC) for triclosan and batumin against *fabI, fapR* and *fabK* overexpression strains. Induced cultures were supplemented with anhydrotetracycline (0.4 µM) for 4 hours prior to MIC determination. MIC determinations were conducted on a minimum of three independent occasions to ensure reproducibility

5.3.5 Biochemical characterisation of the inhibition of *S. aureus* FabI by batumin

Finally, a biochemical approach was used to confirm FabI as the molecular target of batumin. *S. aureus* FabI (saFabI) was purified as described in Section 2.7. The activity of saFabI and its inhibition by batumin was monitored using a method devised by Slater-Radosti et al., (2001). FabI is an enoyl-ACP reductase required for the final stages of FAS elongation, and in this reaction enoyl-ACP is reduced to acyl-ACP and NAD(P)H is oxidised to form $NAD(P)^+$ (Lu and Tonge, 2008). Changes in NAD(P)H levels and FabI activity can be evaluated by monitoring absorbance at 340nm. The initial rate was measured over the first three minutes of the reaction (Section 2.8). Previous studies using this assay have used purified saFabI in nanomolar concentrations (~20 nM) (Slater-Radosti et al., 2001; Payne et al., 2002); however at these concentrations (10 nM-30 nM), no saFabI activity was observed in initial studies. The lack of activity observed at such concentrations may be a result of protein degradation or inactivation. Unfortunately, due to time constraints, the re-purification of saFabI and therefore determination of enzyme kinetics was not possible. Upon optimisation of the assay using the existing saFabI protein, the reduction of enoyl-ACP to acyl-ACP was observed using 8.5 µM saFabI.

The inhibitory activity of batumin on saFabI was compared to that of triclosan; mupirocin was also included as a control agent that is not able to inhibit FabI. Mupirocin binds directly to IleRS to cause the inhibition of protein synthesis, and as expected was unable to cause a substantial reduction in saFabI activity (Figure 5.3). It has been demonstrated previously that the IC_{50} of triclosan against saFabI is 3 µM

(Heath et al., 2000); in this study saFabI activity was reduced 35-40% at this concentration (Figure 5.3). Despite the low enzyme activity of purified saFabI, the inhibitory effect of triclosan at 3 µM is very similar to previously published research (Heath et al., 1999).

At 1.5 µM, batumin inhibited the activity of saFabI by ~20%, and approximately 40% inhibition was observed at 3 µM batumin (Figure 5.3). According to these results, the IC_{50} of batumin could be expected to lie between 3.5 and 4 µM; however, this remains to be confirmed.

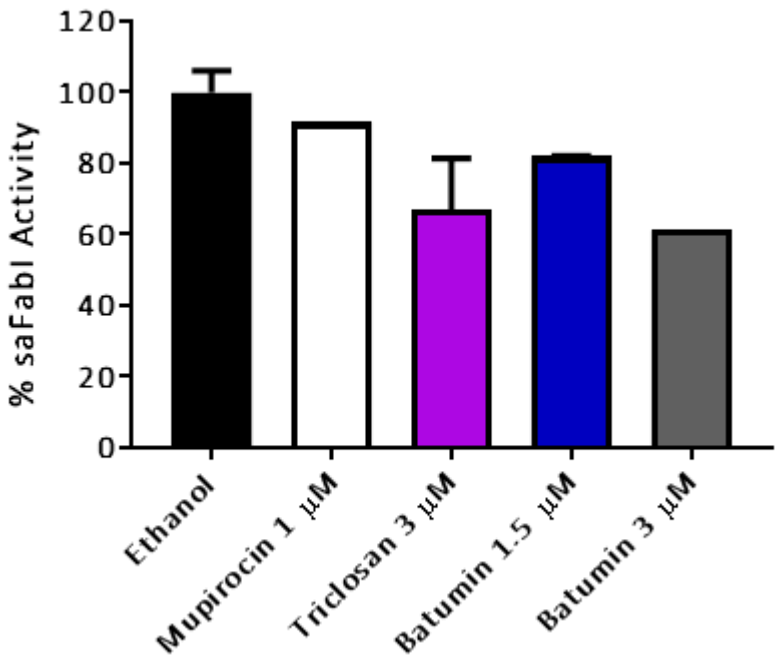


Figure 5. 3 The inhibitory effect of batumin, triclosan and mupirocin on SaFabI activity *in vitro* (mean of at least three independent replicates; error bars show standard deviations)

Batumin displayed similar potency to triclosan, with both agents causing a ~40% reduction in saFabI activity at 3 µM. This provides preliminary confirmation that batumin is able to inhibit saFabI *in vitro*.

5.4 Conclusion

The potent antistaphylococcal activity of batumin may provide a solution to the lack of agents able to successfully treat staphylococcal skin infections. This chapter aimed to define MOA of batumin, which forms an essential part of pre-clinical investigations. The MMS assay confirmed that the primary mode of action of batumin is through the inhibition of fatty acid biosyhthesis; which led to the rejection of the hypothesis described by Klochko and colleagues that batumin exerts its antistaphylococcal effects through the inhibition of protein synthesis (Klochko et al., 2016). Laboratory generated batumin-resistant *S. aureus* were resistant to the FAS inhibitor triclosan, suggesting an overlapping in target- the enoyl-ACP reductase FabI. Genetic characterisation of batumin-resistant mutants highlighted either FapR or FabI as the potential molecular target of batumin. The mutations observed in *fabI* promoter regions of batumin-resistant mutants have been previously demonstrated to result in increased FabI expression in triclosan-resistant *S. aureus*, providing further evidence that the two agents share the same target.

Batumin susceptibility decreased >16-fold when FabI and the ENR homolog FabK from *E. faecalis* were overexpressed *in trans*, further strengthening the case of FabI as the primary target of batumin.

Finally, the inhibition of *S. aureus* FabI by batumin was observed biochemically. Batumin was able to inhibit the activity of saFabI *in vitro* with similar potency to triclosan.

Resistance to batumin pre-existed in the clinic, in the form of triclosan resistance; therefore the use of batumin must be carefully considered. Triclosan resistance is a cause for concern in staphylococci, however only moderate levels of triclosan resistance are observed in strains where mutations result in increased FabI expression (Grandgirard et al., 2015). If batumin is to be used as a topical agent the risk of resistance development may be reduced, as topical agents are usually applied at very high concentrations. Further studies are required for the introduction of batumin as an antibiotic for the treatment topical infections, however the work described in this chapter provide a foundation for further studies.

Chapter 6 General conclusions and future work

6.1 General conclusions

The prevalence of skin infections such as chronic wounds is steadily rising, with this comes a high social and economic burden (Thomas, S., 2006). Part of the social and economic burden associated with skin infections comes from the lack of antibacterial agents able to effectively treat such infections (Williamson et al., 2017). The work described in this thesis aimed to accelerate the discovery and implementation of skin infection treatments, through the means of combination therapy, repurposing and exploiting underexplored scaffolds.

The ability of bacteria to form biofilms poses as a significant challenge in the effective treatment of skin infections. Work described in Chapter 3 investigated the potential to use antimicrobial agents with a longstanding history of use in topical treatments in combination to achieve an improved eradication of biofilms formed by the relevant pathogens *S. aureus* and *P. aeruginosa*. Initial studies identified four combinations which displayed synergism against biofilms formed by *S. aureus* and *P. aeruginosa* on the Calgary biofilm device. The synergistic combinations were subsequently evaluated against mixed-species biofilms of *S. aureus* and *P. aeruginosa* formed on nitrocellulose disks; the combination of chlorhexidine and cetrimide caused a >2.5 log reduction in biofilm viability in 1 hour, whilst the other combinations had limited impact on both populations. Chlorhexidine and cetrimide embedded into sterile dressings was unable to cause a substantial reduction in both bacterial populations in 24 hours; however, the majority of established

antimicrobial dressings and irrigation solutions were also unable to disrupt both populations. In this case, exploring combination therapy failed to identify combinations that displayed antibiofilm activity against more complex biofilms, however the approached used in this chapter could be used to evaluate other combinations.

Further studies investigated the prospect of repurposing agents licensed for use in topical healthcare products as antibiofilm agents for the management of skin infections. The agents in Chapter 4 all have a long history of safe use in topical healthcare products serving as preservatives, antioxidants and skin conditioners; and were investigated with the hope that they may have an accelerated route into clinical use. Initial studies identified two related compounds bronidox and bronopol which were able to eradicate *S. aureus* and *P. aeruginosa* biofilms at ≤256 μg/mL, a feature which the other agents investigated lacked. Further investigation of bronidox and bronopol revealed broad spectrum antibacterial activity against emerging wound pathogens. At concentrations permitted for safe use bronidox and bronopol were able to cause substantial disruption of mixed species biofilms formed by *S. aureus* and *P. aeruginosa*. Investigation into the antibiofilm action of bronidox and bronopol were both able to sterilise slow and non-growing *S. aureus* cultures, a feature implicated in the reduced susceptibility of biofilms to antimicrobial agents. Further investigation in *S. aureus* revealed that bronidox and bronopol exert their antibiofilm action by targeting the bacterial cells directly, leading to disorder of the biofilm matrix. Finally bronidox and bronopol displayed synergism with antimicrobial agents used in the management of skin infections; this

property demonstrates that if either agent cannot be used alone they may be a useful additive to other topical treatments.

Finally, the work conducted in Chapter 5 aimed to determine the antibacterial mechanism of action of batumin, an antistaphylococcal antibiotic not yet used in the clinic with the potential to be used as a topical agent. Initial studies into the MOA of batumin revealed preferential inhibition of fatty acid biosynthesis. Independent batumin resistant mutants isolated under selection displayed cross resistance with the FabI inhibitor triclosan suggesting an overlap in target. Analysis of the genome from batumin resistant mutants revealed mutations in the *fabI* promoter region and the *fapR* coding region, providing insight into the molecular target of batumin. Over expression of FabI and the enoyl-ACP reductase (ENR) from *E. faecalis* FabK in *S. aureus* resulted in a >16 fold reduction in batumin susceptibility, this observation contributed further evidence that FabI is central to the mechanism of action of batumin. Finally, inhibition of saFabI by batumin was characterised biochemically; batumin inhibited the activity of saFabI *in vitro* ~40%. This biochemical characterisation provided confirmation that the molecular target of batumin is the ENR FabI. Understanding the MOA is crucial for the pre-clinical development of a new antibacterial agent, the work presented here provides a solid foundation for further studies. This work also highlighted that other under-exploited antimicrobial substances that have been discarded due to toxicity and other issues can be revisited, to explore their potential as topical antibacterial agents.

The ideal topical antimicrobial agent described in Section 1.5 has a broad spectrum of activity and a low propensity for resistance development, is bactericidal and eradicates biofilms; this study identified agents such as bronidox and bronopol which fulfil these criteria. The approach of exploring combination therapy did not, unfortunately, prove as fruitful.

Overall this study revealed that strategies such as repurposing agents, and reviving underexploited antibiotics are a platform to discover new topical therapies for bacterial infections. The compounds identified through these platforms may provide some relief where our current treatment regimens for topical bacterial infections fall short.

6.2 Future work

In chapter 3 the combination of chlorhexidine and cetrimide embedded into sterile dressing failed to significantly disrupt mixed species biofilm formed on nitrocellulose disks. However, the lack of activity may be concentration dependent. To test this hypothesis cetrimide and chlorhexidine can be evaluated at further concentrations and in different ratios. Furthermore chlorhexidine and cetrimide are both used in combination in the antiseptic agent Savlon, their efficacy against mixed species biofilms could be tested at their in use concentrations.

Mutations were not observed in either FabI or FapR of *S. aureus* BM5 and BM8 (Chapter 5), WGS analysis of these mutants could identify further mutations which are responsible for batumin resistance. Transcriptional profiling can be used to complement the mode of action studies presented in this thesis (O'Neill et al.,

2004). The expression of genes in cells exposed to batumin can be compared to untreated cells; analysis of the genes which display altered expression could provide further information into the mode of action batumin.

The study demonstrated that batumin binds to FabI from *S. aureus*, further investigation could involve the co-crystallisation of saFabI and batumin. The crystal structure of saFabI in complex with batumin could identify moieties essential for inhibition; this could allow for the rational design of derivatives of batumin able to inhibit FabI more efficiently or increase the spectrum of activity.

An important step to facilitate the development of agents considered in this study is the use of relevant models; to allow the transition from *in vitro* and *in vivo* screening to clinical studies. The agents highlighted in this thesis were investigated with regards to their use as topical antimicrobial agents; therefore, selected models should ideally reflect the wound environment as closely as possible. In this study, the cellulose disk model was chosen in an attempt to mimic the chronic wound biofilm and antimicrobial treatment *in vitro*. As briefly described in Section 3.5, there are other *in vitro* models for wound biofilm infections. Future work could involve testing the efficacy of antimicrobial agents or combinations against biofilm formed using the drip flow reactor (DFR) model. The low shear-flow of the DFR can mimic the flow of wound exudate and the efficacy of antimicrobial preparations can be easily assessed (Woods et al., 2012; Kim, H. and Izadjoo, 2016; Fitzgerald et al., 2017). In addition, the DFR is able to support the growth of polymicrobial biofilms, previous studies using the DFR have cultivated biofilm containing 3 species (Woods et al., 2012). A disadvantage to the DFR is the use of an abiotic surface or coupon,

the absence of a biological surface prevents the study of any interactions between the biofilm and host. Skin-equivalents may serve as alternative *in vitro* model for wound infections. Previous work has demonstrated that biofilms are able to grow on skin equivalents (Charles et al., 2009), however, to date they are yet to be used to assess the antibiofilm activity of antimicrobial agent.

In addition to bacterial colonisation and infection; host factors also contribute to non-healing skin infections and should therefore be considered (Guo et al., 2010). *In vivo* models are a useful tool to examine the efficacy of antimicrobial agents, in a setting closer to human infections. Various animal models exist for acute and chronic wound infections; the uses of such models allow us to understand host responses and better define clinical end-points (e.g. wound closure and biofilm eradication)(Ganesh et al., 2015). Murine wound models have been developed to evaluate the activity of antimicrobial dressings (Fitzgerald et al., 2017). Porcine models of wound infections may be favoured over murine models, as the healing process of pig skin is very similar to that of humans (Ganesh et al., 2015). In humans, chronic wound may persist for months (James et al., 2008). Taking into account the persistence of chronic wounds, mixed species wound biofilms formed have been sustained in porcine models for up to 56 days (Sashwati et al., 2014); the use of this long -term model may reflect the human chronic wound more closely. Furthermore, this porcine model has also been used to assess the antibiofilm activity of established wound dressing (Sashwati et al., 2014). The evidence gathered from both *in vitro* and *in vivo* studies can be used to advance the agents described in this thesis to clinical trials and eventually clinical use.

Appendix

Oligonucleotide Primer	Description	Primer Sequence 5'-3'
FABIFwd	For amplification of *fabI* from *S. aureus*	GGATTAGATATTCTATCCGTTAAATTAATTATTATAAGGAG
FABIRev		CGTGAACAAAGCTGTTGAATGATA
FabIPOPFwd	For introduction of f*abI* into pOPINF	AAGTTCTGTTTCAGGGCCCGATGTTAAATCTTGAAAACAAAACATATG
FabIPOPRev		CTGGTCTAGAAAGCTTTATTTAATTGCGTGGAATCC
FabIpRABFwd	For introduction of *fabI* into pRAB11	CGATCGTCGGTACCTATAAGGAGTTATCTTACATGTTAA
FabIpRABRev		AGGTCGATGAGCTCATATTATTTAATTGCGTGGAATCC
FapRFwd	For amplification of *fapR* from *S. aureus*	GAGGAATGTTTAAGACTAGGT
FapRRev		CCCATCATATCAATTGCTAAT

Table A.1 Oligonucleotide Primers used in this study- Underling denotes restriction sites

Oligonucleotide Primer	Description	Primer Sequence 5'-3'
FapRpRABFwd	For introduction of *fapR* into pRAB11	cgatcgtcGGTACCGAGGAATGTTTAAGACTAGGT
FapRpRABRev		aggtcgatGAGCTCCCCATCATATCAATTGCTAAT
FabIPromFwd	For amplification of *fabI* promoter from *S.*	GTTTGATACAGAAAGGACTAAATCA
FabIPromRev	*aureus*	CCACCTTCTGGCATTAATTTTTTAG
FapRPromFwd	For amplification of *fapR* promoter from	GTGATATGACACTTGAACTATTTAA
FapRPromRev	*S. aureus*	TTCTGCTCTTACCGTATCATTTAAT
FabKpRABFwd	For introduction of *fapR* into pRAB11	CGATCGTCGGTACCGTACTTATCTTAGAACTAAAGGACG
FabKpRABRev		AGGTCGATGAGCTCTTTGTTAAAATTATTCACTTAGCCC
FabIpMUTINFwd	For introduction of *fabI* into pMUTIN4	TCTGAGTACGCGGCCGCCTATCCGTTAAATTAATTATTATAAGGAG
FabIpMUTINRev		GGATCGCAGGATCCACGTGAAGTTTCAGAAAAG

Table A.1 (continued) Oligonucleotide Primers used in this study - Underling denotes restriction sites

Compound	Function	MAC
1-O-Hexyl-2,3,5-trimethylhydroquinone (HTHQ)	Antioxidant	n.s
2,2'-Methylenebis(6-tert-butyl-4-methylphenol) (AO2246)	Antioxidant	n.s
2-Bromo-2-nitro-1,3-propanediol (Bronopol)	Preservative	0.1%
5-Bromo-5-nitro-1,3-dioxane (Bronidox)	Preservative	0.1%
8-Hydroxyquinoline	Chelator	0.3%
Benzoyl peroxide	Antimicrobial	0.7%
Bromochlorophene	Antimicrobial/ Anti-Plaque	0.1%
Celastrol	Antioxidant	n.s
Chiba (Bakuchiol)	Antimicrobial	1%
Dimethyl stearamine	Antimicrobial/ Anti-static	5%
Ellagic acid	Phytochemical	1%
Hexamidine diisethionate	Preservative	0.1%
Hexetidine	Preservative	0.1%
Menadione	Antioxidant	n.s
NDGA (Nordihydroguaiaretic acid)	Antioxidant	n.s
Octylisothiazolinone (OIT)	Antimicrobial	0.1%
Phytosphingosine	Phytochemical	0.01%
Propyl gallate	Antioxidant	n.s
TBBQ	Antioxidant	n.s
Thymohydroquinone	Antioxidant	n.s
Thymoquinone	Antioxidant	n.s
Zinc pyrithione	Antimicrobial	0.5%

Table A2- List of agents mentioned in the Cosmetic Ingredient (CosIng) database and their maximum authorised concentration (Siegert, 2014) . N.s = not specified.

Bibliography

Ahmed, A., Azim, A., Gurjar, M. and Baronia, A.K. 2014. Current concepts in combination antibiotic therapy for critically ill patients. *Indian Journal of Critical Care Medicine : Peer-reviewed, Official Publication of Indian Society of Critical Care Medicine.* **18**(5), pp.310-314.

Akiyama, H., Oono, T., Saito, M. and Iwatsuki, K. 2004. Assessment of cadexomer iodine against *Staphylococcus aureus* biofilm *in vivo* and *in vitro* using confocal laser scanning microscopy. *Journal of Dermatology.* **31**(7), pp.529-534.

Alves, P.M., Al-Badi, E., Withycombe, C., Jones, P.M., Purdy, K.J. and Maddocks, S.E. 2018. Interaction between *Staphylococcus aureus* and *Pseudomonas aeruginosa* is beneficial for colonisation and pathogenicity in a mixed biofilm. *Pathogens and Disease.* **76**(1), pp.2-10.

Ammons, M.C. 2010. Anti-biofilm strategies and the need for innovations in wound care. *Recent patents on anti-infective drug discovery.* **5**(1), pp.10-17.

Aparna, M.S. and Yadav, S. 2008. Biofilms: microbes and disease. *Brazilian Journal of Infectious Diseases.* **12**(6), pp.526-530.

Archer, N.K., Mazaitis, M.J., Costerton, J.W., Leid, J.G., Powers, M.E. and Shirtliff, M.E. 2011. *Staphylococcus aureus* biofilms: Properties, regulation and roles in human disease. *Virulence.* **2**(5), pp.445-459.

Arzanlou, M., Chai, Wern C. and Venter, H. 2017. Intrinsic, adaptive and acquired antimicrobial resistance in Gram-negative bacteria. *Essays In Biochemistry.* **61**(1), pp.49-59.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Tomita, M., Wanner, B.L. and Mori, H. 2006. Construction of *Escherichia coli* K - 12 in - frame, single - gene knockout mutants: the Keio collection. *Molecular Systems Biology.* **2**(1).

Barry, A.L., Craig, W.A., Nadler, H., Reller, L.B., Sanders, C.C. and Swenson, J.M. 1999. Methods for determining bactericidal activity of antimicrobial agents: approved guideline. *NCCLS document M26-A.* **19**(18).

Be, N.A., Allen, J.E., Brown, T.S., Gardner, S.N., McLoughlin, K.S., Forsberg, J.A., Kirkup, B.C., Chromy, B.A., Luciw, P.A., Elster, E.A. and Jaing, C.J. 2014. Microbial Profiling of Combat Wound Infection through Detection Microarray and Next-Generation Sequencing. *Journal of Clinical Microbiology.* **52**(7), pp.2583-2594.

Beaudoin, T., Yau, Y.C.W., Stapleton, P.J., Gong, Y., Wang, P.W., Guttman, D.S. and Waters, V. 2017. *Staphylococcus aureus* interaction with *Pseudomonas aeruginosa* biofilm enhances tobramycin resistance. *npj Biofilms and Microbiomes.* **3**(1), pp.25-34.

Berrow, N.S., Alderton, D., Sainsbury, S., Nettleship, J., Assenberg, R., Rahman, N., Stuart, D.I. and Owens, R.J. 2007. A versatile ligation-independent cloning method suitable for high-throughput expression screening applications. *Nucleic Acids Research.* **35**(6), pp.e45-e45.

Biswas, L., Biswas, R., Schlag, M., Bertram, R. and Götz, F. 2009. Small-Colony Variant Selection as a Survival Strategy for *Staphylococcus aureus* in the Presence of *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology.* **75**(21), pp.6910-6912.

Bjarnsholt, T., Ciofu, O., Molin, S., Givskov, M. and Høiby, N. 2013. Applying insights from biofilm biology to drug development -can a new approach be developed? *Nature Reviews Drug Discovery.* **12**(10), pp.791-808.

Blanchard, C., Brooks, L., Ebsworth-Mojica, K., Didione, L., Wucher, B., Dewhurst, S., Krysan, D., Dunman, P.M. and Wozniak, R.A.F. 2016. Zinc Pyrithione Improves the Antibacterial Activity of Silver Sulfadiazine Ointment. *mSphere.* **1**(5).

Boucher, H.W., Talbot, G.H., Bradley, J.S., Edwards, J.E., Gilbert, D., Rice, L.B., Scheld, M., Spellberg, B. and Bartlett, J. 2009. Bad Bugs, No Drugs: No ESKAPE! An Update from the Infectious Diseases Society of America. *Clinical Infectious Diseases.* **48**(1), pp.1-12.

Bowler, P. 2014. A clinical algorithm for wound biofilm identification. *Journal of Wound Care.* **23**(3).

Bowler, P.G. 2003. The 10^5 bacterial growth guideline: reassessing its clinical relevance in wound healing. *Ostomy/Wound Management.* **1**(49), pp.44-53.

Bowler, P.G., Duerden, B.I. and Armstrong, D.G. 2001. Wound Microbiology and Associated Approaches to Wound Management. *Clinical Microbiology Reviews.* **14**(2), pp.244-269.

Brackman, G. and Coenye, T. 2015. *In vitro* and *in vivo* biofilm wound models and their application. *Advances in Microbiology, Infectious Diseases and Public Health.* Springer, pp.15-32.

Brackman, G., De Meyer, L., Nelis, H.J. and Coenye, T. 2013. Biofilm inhibitory and eradicating activity of wound care products against *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms in an *in vitro* chronic wound model. *Journal of Applied Microbiology.* **114**(6), pp.1833- 1842.

Cerca, N., Brooks, J.L. and Jefferson, K.K. 2008. Regulation of the intercellular adhesin locus regulator (icaR) by SarA, σ(B), and IcaR in *Staphylococcus aureus*. *Journal of Bacteriology.* **190**(19), pp.6530-6533.

Ceri, H., Olson, M.E., Stremick, C., Read, R.R., Morck, D. and Buret, A. 1999. The Calgary Biofilm Device: New Technology for Rapid Determination of Antibiotic Susceptibilities of Bacterial Biofilms. *Journal of Clinical Microbiology.* **37**(6), pp.1771-1776.

Chandra Rastogi, S. 1992. A survey of formaldehyde in shampoos and skin creams on the Danish market. *Contact Dermatitis.* **27**(4), pp.235-240.

Charles, C.A., Ricotti, C.A., Davis, S.C., Mertz, P.M. and Kirsner, R.S. 2009. Use of tissue-engineered skin to study *in vitro* biofilm development. *Dermatologic Surgery.* **35**(9), pp.1334-1341.

Chaw, K.C., Manimaran, M. and Tay, F.E.H. 2005. Role of Silver Ions in Destabilization of Intermolecular Adhesion Forces Measured by Atomic Force

Microscopy in *Staphylococcus epidermidis* Biofilms. *Antimicrobial Agents and Chemotherapy.* **49**(12), pp.4853-4859.

Chen, P., Abercrombie, J.J., Jeffrey, N.R. and Leung, K.P. 2012. An improved medium for growing *Staphylococcus aureus* biofilm. *Journal of Microbiological Methods.* **90**(2), pp.115-118.

Chopra, I. and Roberts, M. 2001. Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance. *Microbiology and Molecular Biology Reviews.* **65**(2), pp.232-260.

Churkina, L., Vaneechoutte, M., Kiprianova, E., Perunova, N., Avdeeva, L. and Bukharin, O. 2015. Batumin—A Selective Inhibitor of Staphylococci—Reduces Biofilm Formation in Methicillin Resistant *Staphylococcus aureus*. *Open Journal of Medical Microbiology.* **5**(4), pp.193-201.

CLSI. 2012. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically (M07-A9).

Cogen, A.L., Nizet, V. and Gallo, R.L. 2008. Skin microbiota: a source of disease or defence? *The British Journal of Dermatology.* **158**(3), pp.442-455.

Colvin, K.M., Irie, Y., Tart, C.S., Urbano, R., Whitney, J.C., Ryder, C., Howell, P.L., Wozniak, D.J. and Parsek, M.R. 2012. The Pel and Psl polysaccharides provide *Pseudomonas aeruginosa* structural redundancy within the biofilm matrix. *Environmental microbiology.* **14**(8), pp.10.1111/j.1462-2920.2011.02657.x.

Costerton, J., Stewart, P.S. and Greenberg, E. 1999. Bacterial biofilms: a common cause of persistent infections. *Science.* **284**(5418), pp.1318-1322.

Cotsonas King, A. and Wu, L. 2001. Macromolecular Synthesis and Membrane Perturbation Assays for Mechanisms of Action Studies of Antimicrobial Agents. *Current Protocols in Pharmacology.* John Wiley & Sons, Inc., pp.1-23.

Daeschlein, G. 2013. Antimicrobial and antiseptic strategies in wound management. *International Wound Journal.* **10**(s1), pp.9-14.

Dalton, T., Dowd, S.E., Wolcott, R.D., Sun, Y., Watters, C., Griswold, J.A. and Rumbaugh, K.P. 2011. An *in vivo* polymicrobial biofilm wound infection model to study interspecies interactions. *PLOS ONE.* **6**(11), pe27317.

Dann, A.B., Hontela, A. 2011. Triclosan: environmental exposure, toxicity and mechanisms of action. *Journal of Applied Toxicology.* **31**(4), pp.285-311.

Davies, D. 2003. Understanding biofilm resistance to antibacterial agents. *Nature Reviews Drug Discovery.* **2**(2), pp.114-122.

Davies, J. and Davies, D. 2010. Origins and Evolution of Antibiotic Resistance. *Microbiology and Molecular Biology Reviews : MMBR.* **74**(3), pp.417-433.

De Kievit, T.R. 2009. Quorum sensing in *Pseudomonas aeruginosa* biofilms. *Environmental Microbiology.* **11**(2), pp.279-288.

Del Pozo, J. and Patel, R. 2007. The challenge of treating biofilm-associated bacterial infections. *Clinical Pharmacology & Therapeutics.* **82**(2), pp.204-209.

DeLeon, S., Clinton, A., Fowler, H., Everett, J., Horswill, A.R. and Rumbaugh, K.P. 2014. Synergistic interactions of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in an *in vitro* wound model. *Infection and Immunity.* **82**(11), pp.4718-4728.

Deresinski, S. 2009. Vancomycin in Combination with Other Antibiotics for the Treatment of Serious Methicillin-Resistant *Staphylococcus aureus* Infections. *Clinical Infectious Diseases.* **49**(7), pp.1072-1079.

Donlan, R.M. 2001. Biofilm Formation: A Clinically Relevant Microbiological Process. *Clinical Infectious Diseases.* **33**(8), pp.1387-1392.

Donlan, R.M. and Costerton, J.W. 2002. Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms. *Clinical Microbiology Reviews.* **15**(2), pp.167-193.

Dowd, S., Sun, Y., Secor, P., Rhoads, D., Wolcott, B., James, G. and Wolcott, R. 2008. Survey of bacterial diversity in chronic wounds using Pyrosequencing, DGGE, and full ribosome shotgun sequencing. *BMC Microbiology.* **8**(1), p43.

Dowd, S.E., Sun, Y., Smith, E., Kennedy, J.P., Jones, C.E. and Wolcott, R. 2009. Effects of biofilm treatments on the multi-species Lubbock chronic wound biofilm model. *Journal of Wound Care.* **18**(12), pp.508-512.

Dowd, S.E., Wolcott, R.D., Sun, Y., McKeehan, T., Smith, E. and Rhoads, D. 2008. Polymicrobial nature of chronic diabetic foot ulcer biofilm infections determined using bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP). *PloS one.* **3**(10), pe3326.

Driffield, K., Miller, K., Bostock, J.M., O'Neill, A.J. and Chopra, I. 2008. Increased mutability of Pseudomonas aeruginosa in biofilms. *Journal of Antimicrobial Chemotherapy.* **61**(5), pp.1053-1056.

Dufour, D., Leung, V. and Lévesque, C.M. 2010. Bacterial biofilm: structure, function, and antimicrobial resistance. *Endodontic Topics.* **22**(1), pp.2-16.

EC. 2006. *2006/257/EC: Commission Decision of 9 February 2006 amending Decision 96/335/EC establishing an inventory and a common nomenclature of ingredients employed in cosmetic products* [Online]. Available from: http://data.europa.eu/eli/dec/2006/257/oj

Edwards, R. and Harding, K.G. 2004. Bacteria and wound healing. *Current Opinion in Infectious Diseases.* **17**(2), pp.91-96.

Fairweather, N., Kennedy, S., Foster, T.J., Kehoe, M. and Dougan, G. 1983. Expression of a cloned *Staphylococcus aureus* alpha-hemolysin determinant in *Bacillus subtilis* and *Staphylococcus aureus*. *Infection and Immunity.* **41**(3), pp.1112-1117.

Fazli, M., Bjarnsholt, T., Kirketerp-Møller, K., Jørgensen, B., Andersen, A.S., Krogfelt, K.A., Givskov, M. and Tolker-Nielsen, T. 2009. Nonrandom distribution of *Pseudomonas aeruginosa* and *Staphylococcus aureu*s in chronic wounds. *Journal of Clinical Microbiology.* **47**(12), pp.4084-4089.

Filkins, L.M., Graber, J.A., Olson, D.G., Dolben, E.L., Lynd, L.R., Bhuju, S. and O'Toole, G.A. 2015. Coculture of *Staphylococcus aureus* with *Pseudomonas aeruginosa* drives *S. aureus* towards fermentative metabolism and reduced viability in a Cystic Fibrosis model. *Journal of Bacteriology.* **197**(14), pp.2252-2264.

Fitzgerald, D.J., Renick, P.J., Forrest, E.C., Tetens, S.P., Earnest, D.N., McMillan, J., Kiedaisch, B.M., Shi, L. and Roche, E.D. 2017. Cadexomer iodine provides superior

efficacy against bacterial wound biofilms *in vitro* and *in vivo*. *Wound Repair and Regeneration.* **25**(1), pp.13-24.

Fleming, D.M., Elliot, A.J. and Kendall, H. 2007. Skin infections and antibiotic prescribing: a comparison of surveillance and prescribing data. *The British Journal of General Practice.* **57**(540), pp.569-573.

Foster, T.J. 2017. Antibiotic resistance in *Staphylococcus aureus*. Current status and future prospects. *FEMS Microbiology Reviews.* **41**(3), pp.430-449.

Frank, K.L. and Patel, R. 2007. Poly-N-Acetylglucosamine Is Not a Major Component of the Extracellular Matrix in Biofilms Formed by icaADBC-Positive *Staphylococcus lugdunensis* Isolates. *Infection and Immunity.* **75**(10), pp.4728-4742.

Fujita, Y., Matsuoka, H. and Hirooka, K. 2007. Regulation of fatty acid metabolism in bacteria. *Molecular Microbiology.* **66**(4), pp.829-839.

Ganesh, K., Sinha, M., Mathew-Steiner, S.S., Das, A., Roy, S. and Sen, C.K. 2015. Chronic Wound Biofilm Model. *Advances in Wound Care.* **4**(7), pp.382-388.

Gemmell, C.G., Edwards, D.I., Fraise, A.P., Gould, F.K., Ridgway, G.L. and Warren, R.E. 2006. Guidelines for the prophylaxis and treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections in the UK. *Journal of Antimicrobial Chemotherapy.* **57**(4), pp.589-608.

Ghannoum, M., Thomson, M., Bowman, W. and Al-Khalil, S. 1986. Mode of action of the antimicrobial compound 5-Bromo-5-nitro-1, 3-dioxane (Bronidox). *Folia microbiologica.* **31**(1), pp.19-31.

Gilbert, P. and Moore, L.E. 2005. Cationic antiseptics: diversity of action under a common epithet. *Journal of Applied Microbiology.* **99**(4), pp.703-715.

Gillaspy, A.F., Hickmon, S.G., Skinner, R.A., Thomas, J.R., Nelson, C.L. and Smeltzer, M.S. 1995. Role of the accessory gene regulator (agr) in pathogenesis of staphylococcal osteomyelitis. *Infection and Immunity.* **63**(9), pp.3373-3380.

Grandgirard, D., Furi, L., Ciusa, M.L., Baldassarri, L., Knight, D.R., Morrissey, I., Largiadèr, C.R., Leib, S.L. and Oggioni, M.R. 2015. Mutations upstream of *fabI* in

triclosan resistant *Staphylococcus aureus* strains are associated with elevated fabI gene expression. *BMC Genomics.* **16**(1), p345.

Grice, E.A. and Segre, J.A. 2011. The skin microbiome. *Nature Reviews Microbiology.* **9**(4), pp.244-253.

Group, E.W. 2012. *Appropriate use of silver dressings in wounds: An expert working group consensus.* [Online]. Available from: www.woundsinternational.com

Guo, S. and DiPietro, L.A. 2010. Factors affecting wound healing. *Journal of Dental Research.* **89**(3), pp.219-229.

Ha, D.-G. and O'Toole, G.A. 2015. c-di-GMP and its effects on biofilm formation and dispersion: a Pseudomonas aeruginosa review. *Microbiology spectrum.* **3**(2), pp.10.1128/microbiolspec.MB-0003-2014.

Hall-Stoodley, L., Costerton, J.W. and Stoodley, P. 2004. Bacterial biofilms: from the natural environment to infectious diseases. *Nature Reviews Microbiology.* **2**(2), pp.95-108.

Han, A., Zenilman, J.M., Melendez, J.H., Shirtliff, M.E., Agostinho, A., James, G., Stewart, P.S., Mongodin, E.F., Rao, D. and Rickard, A.H. 2011. The importance of a multifaceted approach to characterizing the microbial flora of chronic wounds. *Wound Repair and Regeneration.* **19**(5), pp.532-541.

Heath, R.J., Li, J., Roland, G.E. and Rock, C.O. 2000. Inhibition of the *Staphylococcus aureus* NADPH-dependent enoyl-acyl carrier protein reductase by triclosan and hexachlorophene. *Journal of Biological Chemistry.* **275**(7), pp.4654-4659.

Heath, R.J., Rubin, J.R., Holland, D.R., Zhang, E., Snow, M.E. and Rock, C.O. 1999. Mechanism of triclosan inhibition of bacterial fatty acid synthesis. *Journal of Biological Chemistry.* **274**(16), pp.11110-11114.

Helle, L., Kull, M., Mayer, S., Marincola, G., Zelder, M.-E., Goerke, C., Wolz, C. and Bertram, R. 2011. Vectors for improved Tet repressor-dependent gradual gene induction or silencing in *Staphylococcus aureus*. *Microbiology.* **157**(12), pp.3314-3323.

Hengge, R. 2009. Principles of c-di-GMP signalling in bacteria. *Nature Reviews Microbiology.* **7**, p263.

Hilliard, J.J., Goldschmidt, R.M., Licata, L., Baum, E.Z. and Bush, K. 1999. Multiple Mechanisms of Action for Inhibitors of Histidine Protein Kinases from Bacterial Two-Component Systems. *Antimicrobial Agents and Chemotherapy.* **43**(7), pp.1693-1699.

Holloway, B.W. 1955. Genetic Recombination in *Pseudomonas aeruginosa*. *Journal of General Microbiology.* **13**(3), pp.572-581.

Horsburgh, M.J., Aish, J.L., White, I.J., Shaw, L., Lithgow, J.K. and Foster, S.J. 2002. σB modulates virulence determinant expression and stress resistance: Characterization of a functional rsbU strain derived from *Staphylococcus aureu*s 8325-4. *Journal of Bacteriology.* **184**(19), pp.5457-5467.

Hotterbeekx, A., Kumar-Singh, S., Goossens, H. and Malhotra-Kumar, S. 2017. *In vivo* and *in vitro* interactions between *Pseudomonas aeruginosa* and *Staphylococcus spp*. *Frontiers in Cellular and Infection Microbiology.* **7**(106), pp.1-13.

Humphreys, G., Lee, G.L., Percival, S.L. and McBain, A.J. 2011. Combinatorial activities of ionic silver and sodium hexametaphosphate against microorganisms associated with chronic wounds. *Journal of Antimicrobial Chemotherapy.* **66**(11), pp.2556-2561.

Hurdle, J.G., O'Neill, A.J., Chopra, I. and Lee, R.E. 2011. Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. *Nature Reviews. Microbiology.* **9**(1), pp.62-75.

James, G.A., Swogger, E., Wolcott, R., Pulcini, E.d., Secor, P., Sestrich, J., Costerton, J.W. and Stewart, P.S. 2008. Biofilms in chronic wounds. *Wound Repair and Regeneration.* **16**(1), pp.37-44.

Jang, H.-J., Chang, M.W., Toghrol, F. and Bentley, W.E. 2008. Microarray analysis of toxicogenomic effects of triclosan on *Staphylococcus aureus*. *Applied Microbiology and Biotechnology.* **78**(4), pp.695-707.

Johnston, M.D., Hanlon, G.W., Denyer, S.P. and Lambert, R.J.W. 2003. Membrane damage to bacteria caused by single and combined biocides. *Journal of Applied Microbiology.* **94**(6), pp.1015-1023.

Ki, V. and Rotstein, C. 2008. Bacterial skin and soft tissue infections in adults: A review of their epidemiology, pathogenesis, diagnosis, treatment and site of care. *The Canadian Journal of Infectious Diseases & Medical Microbiology.* **19**(2), pp.173-184.

Kim, E.B., Kopit, L.M., Harris, L.J. and Marco, M.L. 2012. Draft Genome Sequence of the Quality Control Strain *Enterococcus faecalis* ATCC 29212. *Journal of Bacteriology.* **194**(21), pp.6006-6007.

Kim, H. and Izadjoo, M. 2016. Antimicrobial activity of a bioelectric dressing using an *in vitro* wound pathogen colony drip-flow reactor biofilm model. *Journal of Wound Care.* **25**(Sup7), pp.S47-S52.

Kim, W., Tengra, F.K., Young, Z., Shong, J., Marchand, N., Chan, H.K., Pangule, R.C., Parra, M., Dordick, J.S. and Plawsky, J.L. 2013. Spaceflight promotes biofilm formation by *Pseudomonas aeruginosa*. *PloS one.* **8**(4), pe62437.

Kiprianova, E., Klochko, V., Zelena, L., Churkina, L. and Avdeeva, L. 2011. *Pseudomonas batumici sp. nov*., the antibiotic-producing bacteria isolated from soil of the Caucasus Black Sea coast. *Mikrobiolohichnyi zhurnal.* (73,№ 5), pp.3-8.

Kirketerp-Møller, K., Jensen, P.Ø., Fazli, M., Madsen, K.G., Pedersen, J., Moser, C., Tolker-Nielsen, T., Høiby, N., Givskov, M. and Bjarnsholt, T. 2008. Distribution, Organization, and Ecology of Bacteria in Chronic Wounds. *Journal of Clinical Microbiology.* **46**(8), pp.2717-2722.

Klochko, V.V., Zelena, L.B., Kim, J.Y., Avdeeva, L.V. and Reva, O.N. 2016. Prospects of a new antistaphylococcal drug batumin revealed by molecular docking and analysis of the complete genome sequence of the batumin-producer *Pseudomonas batumici* UCM B-321. *International Journal of Antimicrobial Agents.* **47**(1), pp.56-61.

Kucera, J., Sojka, M., Pavlik, V., Szuszkiewicz, K., Velebny, V. and Klein, P. 2014. Multispecies biofilm in an artificial wound bed—A novel model for *in vitro*

assessment of solid antimicrobial dressings. *Journal of Microbiological Methods.* **103**(Supplement C), pp.18-24.

Kumar Shukla, S. and Rao, T.S. 2013. Dispersal of Bap-mediated Staphylococcus aureus biofilm by proteinase K. *The Journal of Antibiotics.* **66**(2), pp.55-60.

Le, K.Y. and Otto, M. 2015. Quorum-sensing regulation in staphylococci—an overview. *Frontiers in Microbiology.* **6**, p1174.

Lebeaux, D., Chauhan, A., Rendueles, O. and Beloin, C. 2013. From *in vitro* to *in vivo* Models of Bacterial Biofilm-Related Infections. *Pathogens.* **2**(2), p288.

Lipsky, B.A. and Hoey, C. 2009. Topical antimicrobial therapy for treating chronic wounds. *Clinical Infectious Diseases.* **49**(10), pp.1541-1549.

Lister, J.L. and Horswill, A.R. 2014. *Staphylococcus aureus* biofilms: recent developments in biofilm dispersal. *Frontiers in Cellular and Infection Microbiology.* **4**(178).

Lowery, C.A., Dickerson, T.J. and Janda, K.D. 2008. Interspecies and interkingdom communication mediated by bacterial quorum sensing. *Chemical Society Reviews.* **37**(7), pp.1337-1346.

Lu, H. and Tonge, P.J. 2008. Inhibitors of FabI, an Enzyme Drug Target in the Bacterial Fatty Acid Biosynthesis Pathway. *Accounts of Chemical Research.* **41**(1), pp.11-20.

Lv, C., Hou, J., Xie, W. and Cheng, H. 2015. Investigation on formaldehyde release from preservatives in cosmetics. *International Journal of Cosmetic Science.* **37**(5), pp.474-478.

Ma, L., Wang, S., Wang, D., Parsek, M.R. and Wozniak, D.J. 2012. The roles of biofilm matrix polysaccharide Psl in mucoid *Pseudomonas aeruginosa* biofilms. *FEMS Immunology & Medical Microbiology.* **65**(2), pp.377-380.

Malone, M., Bjarnsholt, T., McBain, A.J., James, G.A., Stoodley, P., Leaper, D., Tachi, M., Schultz, G., Swanson, T. and Wolcott, R.D. 2017a. The prevalence of biofilms in chronic wounds: a systematic review and meta-analysis of published data. *Journal of Wound Care.* **26**(1), pp.20-25.

Malone, M., Johani, K., Jensen, S.O., Gosbell, I.B., Dickson, H.G., McLennan, S., Hu, H. and Vickery, K. 2017b. Effect of cadexomer iodine on the microbial load and diversity of chronic non-healing diabetic foot ulcers complicated by biofilm in vivo. *Journal of Antimicrobial Chemotherapy.* **72**(7), pp.2093-2101.

Mancl, K.A., Kirsner, R.S. and Ajdic, D. 2013. Wound biofilms: Lessons learned from oral biofilms. *Wound Repair and Regeneration.* **21**(3), pp.352-362.

Manner, S., Goeres, D.M., Skogman, M., Vuorela, P. and Fallarero, A. 2017. Prevention of *Staphylococcus aureus* biofilm formation by antibiotics in 96-Microtiter Well Plates and Drip Flow Reactors: critical factors influencing outcomes. *Scientific Reports.* **7**, p43854.

Mathus-Vliegen, E.M.H. 2004. Old Age, Malnutrition, and Pressure Sores: An Ill-Fated Alliance. *The Journals of Gerontology: Series A.* **59**(4), pp.M355-M360.

Mattheus, W., Gao, L.-J., Herdewijn, P., Landuyt, B., Verhaegen, J., Masschelein, J., Volckaert, G. and Lavigne, R. 2010a. Isolation and Purification of a New Kalimantacin/Batumin-Related Polyketide Antibiotic and Elucidation of Its Biosynthesis Gene Cluster. *Chemistry & Biology.* **17**(2), pp.149-159.

Mattheus, W., Masschelein, J., Gao, L.-J., Herdewijn, P., Landuyt, B., Volckaert, G. and Lavigne, R. 2010b. The Kalimantacin/Batumin Biosynthesis Operon Encodes a Self-Resistance Isoform of the FabI Bacterial Target. *Chemistry & Biology.* **17**(10), pp.1067-1071.

McAlpine, J.B. 2017. The ups and downs of drug discovery: the early history of Fidaxomicin. *The Journal Of Antibiotics.* **70**, p492.

McDonnell, G. and Russell, A.D. 1999. Antiseptics and disinfectants: activity, action, and resistance. *Clinical Microbiology Reviews.* **12**(1), pp.147-179.

McNeil, J.C., Hulten, K.G., Kaplan, S.L. and Mason, E.O. 2014. Decreased susceptibilities to retapamulin, mupirocin, and chlorhexidine among *Staphylococcus aureus* isolates causing skin and soft tissue infections in otherwise healthy children. *Antimicrobial Agents and Chemotherapy.* **58**(5), pp.2878-2883.

Melendez, J.H., Frankel, Y.M., An, A.T., Williams, L., Price, L.B., Wang, N.Y., Lazarus, G.S. and Zenilman, J.M. 2010. Real-time PCR assays compared to culture-based approaches for identification of aerobic bacteria in chronic wounds. *Clinical Microbiology and Infection.* **16**(12), pp.1762-1769.

Metcalf, D.G. and Bowler, P.G. 2013. Biofilm delays wound healing: A review of the evidence. *Burns and Trauma.* **1**(1), p5.

Mikkelsen, H., McMullan, R. and Filloux, A. 2011. The *Pseudomonas aeruginosa* reference strain PA14 displays increased virulence due to a mutation in ladS. *PLOS ONE.* **6**(12), pe29113.

Miller, M.B. and Bassler, B.L. 2001. Quorum sensing in bacteria. *Annual Reviews in Microbiology.* **55**(1), pp.165-199.

Monk, I.R., Shah, I.M., Xu, M., Tan, M.-W. and Foster, T.J. 2012. Transforming the Untransformable: Application of Direct Transformation To Manipulate Genetically *Staphylococcus aureu*s and *Staphylococcus epidermidis*. *mBio.* **3**(2).

Monroe, D. 2007. Looking for chinks in the armor of bacterial biofilms. *PLoS Biology.* **5**(11), pe307.

Morones-Ramirez, J.R., Winkler, J.A., Spina, C.S. and Collins, J.J. 2013. Silver enhances antibiotic activity against Gram-negative bacteria. *Science Translational Medicine.* **5**(190), p190ra181.

N Campbell, D.C. 2013. Evaluation of a non-adherent, povidone–iodine dressing in a case series of chronic wounds. *Journal of Wound Care.* **22**(8), p401.

NICE. 2014. *Pressure ulcers: prevention and management.* [Online]. Available from: https://www.nice.org.uk/guidance/cg179

Norman, G., Dumville, J.C., Moore, Z.E.H., Tanner, J., Christie, J. and Goto, S. 2016. Antibiotics and antiseptics for pressure ulcers. *Cochrane Database of Systematic Reviews.* **4**(4), pCD011586.

O'Meara, S., Al-Kurdi, D., Ologun, Y., Ovington, L.G., Martyn-St James, M. and Richardson, R. 2014. Antibiotics and antiseptics for venous leg ulcers. *Cochrane Database of Systematic Reviews.* **1**(1), pCD003557.

O'Neill, A.J. 2010. Bacterial phenotypes refractory to antibiotic-mediated killing: mechanisms and mitigation. In: Miller, A.A. ed. *Emerging trends in antibacterial discovery: answering a call to arms.* Norfolk, United Kingdom: Caister Academic Press, pp.195-210.

O'Neill, E., Pozzi, C., Houston, P., Smyth, D., Humphreys, H., Robinson, D.A. and O'Gara, J.P. 2007. Association between methicillin susceptibility and biofilm regulation in Staphylococcus aureus isolates from device-related infections. *Journal of Clinical Microbiology.* **45**(5), pp.1379-1388.

O'Toole, G., Kaplan, H.B. and Kolter, R. 2000. Biofilm Formation as Microbial Development. *Annual Review of Microbiology.* **54**(1), pp.49-79.

O'Neill, A.J. and Chopra, I. 2004. Preclinical evaluation of novel antibacterial agents by microbiological and molecular techniques. *Expert Opinion on Investigational Drugs.* **13**(8), pp.1045-1063.

Oliva, B., Miller, K., Caggiano, N., O'Neill, A.J., Cuny, G.D., Hoemann, M.Z., Hauske, J.R. and Chopra, I. 2003. Biological Properties of Novel Antistaphylococcal Quinoline-Indole Agents. *Antimicrobial Agents and Chemotherapy.* **47**(2), pp.458-466.

Olsen, I. 2015. Biofilm-specific antibiotic tolerance and resistance. *European Journal of Clinical Microbiology and Infectious Diseases.* **34**(5), pp.877-886.

Olsen, I., Tribble, G.D., Fiehn, N.-E. and Wang, B.-Y. 2013. Bacterial sex in dental plaque. *Journal of Oral Microbiology.* **5**(1), p20736.

Ooi, N., Eady, E.A., Cove, J.H. and O'Neill, A.J. 2015. Redox-active compounds with a history of human use: antistaphylococcal action and potential for repurposing as topical antibiofilm agents. *Journal of Antimicrobial Chemotherapy.* **70**(2), pp.479-488.

Ooi, N., Miller, K., Hobbs, J., Rhys-Williams, W., Love, W. and Chopra, I. 2009. XF-73, a novel antistaphylococcal membrane-active agent with rapid bactericidal activity. *Journal of Antimicrobial Chemotherapy.* **64**(4), pp.735-740.

Otto, M. 2013. Staphylococcal Infections: Mechanisms of Biofilm Maturation and Detachment as Critical Determinants of Pathogenicity*. *Annual Review of Medicine.* **64**(1), pp.175-188.

Ovington, L.G. 2002. The Evolution of Wound Management: Ancient Origins and Advances of the Past 20 Years. *Home Healthcare Nurse: The Journal for the Home Care and Hospice Professional.* **20**(10), pp.652-656.

Pankey, G.A. and Sabath, L.D. 2004. Clinical Relevance of Bacteriostatic versus Bactericidal Mechanisms of Action in the Treatment of Gram-Positive Bacterial Infections. *Clinical Infectious Diseases.* **38**(6), pp.864-870.

Pastar, I., Nusbaum, A.G., Gil, J., Patel, S.B., Chen, J., Valdes, J., Stojadinovic, O., Plano, L.R., Tomic-Canic, M. and Davis, S.C. 2013. Interactions of methicillin resistant *Staphylococcus aureus* USA300 and *Pseudomonas aeruginosa* in polymicrobial wound Infection. *PLOS One.* **8**(2), pe56846.

Payne, D.J., Miller, W.H., Berry, V., Brosky, J., Burgess, W.J., Chen, E., DeWolf, J.W.E., Fosberry, A.P., Greenwood, R., Head, M.S., Heerding, D.A., Janson, C.A., Jaworski, D.D., Keller, P.M., Manley, P.J., Moore, T.D., Newlander, K.A., Pearson, S., Polizzi, B.J., Qiu, X., Rittenhouse, S.F., Slater-Radosti, C., Salyers, K.L., Seefeld, M.A., Smyth, M.G., Takata, D.T., Uzinskas, I.N., Vaidya, K., Wallis, N.G., Winram, S.B., Yuan, C.C.K. and Huffman, W.F. 2002. Discovery of a Novel and Potent Class of FabI-Directed Antibacterial Agents. *Antimicrobial Agents and Chemotherapy.* **46**(10), pp.3118-3124.

Percival, S.L., Finnegan, S., Donelli, G., Vuotto, C., Rimmer, S. and Lipsky, B.A. 2014. Antiseptics for treating infected wounds: Efficacy on biofilms and effect of pH. *Critical Reviews in Microbiology.* **42**(2), pp.293-309.

Percival, S.L., Hill, K.E., Williams, D.W., Hooper, S.J., Thomas, D.W. and Costerton, J.W. 2012. A review of the scientific evidence for biofilms in wounds. *Wound Repair and Regeneration.* **20**(5), pp.647-657.

Phillips, P.L., Yang, Q., Davis, S., Sampson, E.M., Azeke, J.I., Hamad, A. and Schultz, G.S. 2015. Antimicrobial dressing efficacy against mature *Pseudomonas aeruginosa* biofilm on porcine skin explants. *International Wound Journal.* **12**(4), pp.469-483.

Piette, A. and Verschraegen, G. 2009. Role of coagulase-negative staphylococci in human disease. *Veterinary Microbiology.* **134**(1–2), pp.45-54.

Pillai SK, M.R. 2005. Antimicrobial combinations. *Antibiotics in laboratory medicine.* . New York: Lippincott Williams & Wilkins, pp.365-440.

Posnett, J. and Franks, P. 2008. The burden of chronic wounds in the UK. *Diabetic Medicine.* **14**(5), pp.S7-S85.

Powers, J.G., Higham, C., Broussard, K. and Phillips, T.J. 2016. Wound healing and treating wounds: Chronic wound care and management. *Journal of the American Academy of Dermatology.* **74**(4), pp.607-625.

Presterl, E., Suchomel, M., Eder, M., Reichmann, S., Lassnigg, A., Graninger, W. and Rotter, M. 2007. Effects of alcohols, povidone-iodine and hydrogen peroxide on biofilms of Staphylococcus epidermidis. *Journal of Antimicrobial Chemotherapy.* **60**(2), pp.417-420.

Price, A.C., Choi, K.-H., Heath, R.J., Li, Z., White, S.W. and Rock, C.O. 2001. Inhibition of β-ketoacyl-acyl carrier protein synthases by thiolactomycin and cerulenin: structure and mechanism. *Journal of Biological Chemistry.* **276**(9), pp.6551-6559.

Randall, C.P., Oyama, L.B., Bostock, J.M., Chopra, I. and O'Neill, A.J. 2013. The silver cation (Ag+): antistaphylococcal activity, mode of action and resistance studies. *Journal of Antimicrobial Chemotherapy.* **68**(1), pp.131-138.

Rasmussen, K. and Lewandowski, Z. 1998. Microelectrode measurements of local mass transport rates in heterogeneous biofilms. *Biotechnology and Bioengineering.* **59**(3), pp.302-309.

Rhoads, D., Wolcott, R. and Percival, S. 2008. Biofilms in wounds: management strategies. *Journal of Wound Care.* **17**(11), p502.

Rhoads, D.D., Wolcott, R.D., Sun, Y. and Dowd, S.E. 2012. Comparison of culture and molecular identification of bacteria in chronic wounds. *International Journal of Molecular Sciences.* **13**(3), pp.2535-2550.

Ryder, V.J., Chopra, I. and O'Neill, A.J. 2012. Increased Mutability of Staphylococci in Biofilms as a Consequence of Oxidative Stress. *PLOS One.* **7**(10), pe47695.

Salcido, R. 2007. What is Bioburden?: The Link to Chronic Wounds. *Advances in Skin and Wound Care.* **20**(7), pp.368-369.

Sashwati, R., Haytham, E., Mithun, S., Kasturi, G., Sarah, C., Ethan, M., Christina, M., Savita, K., K, B.V., M, P.H., H, C.C., M, G.G., J, W.D. and K, S.C. 2014. Mixed - species biofilm compromises wound healing by disrupting epidermal barrier function. *The Journal of Pathology.* **233**(4), pp.331-343.

Savage, V.J., Chopra, I. and O'Neill, A.J. 2013. *Staphylococcus aureus* biofilms promote horizontal transfer of antibiotic resistance. *Antimicrobial Agents and Chemotherapy.* **57**(4), pp.1968-1970.

Schiebel, J., Chang, A., Lu, H., Baxter, Michael V., Tonge, Peter J. and Kisker, C. 2012. *Staphylococcus aureus* FabI: inhibition, substrate recognition, and potential implications for *In Vivo* essentiality. *Structure.* **20**(5), pp.802-813.

Schierle, C.F., De la Garza, M., Mustoe, T.A. and Galiano, R.D. 2009. Staphylococcal biofilms impair wound healing by delaying reepithelialization in a murine cutaneous wound model. *Wound Repair and Regeneration.* **17**(3), pp.354-359.

Schujman, G.E., Paoletti, L., Grossman, A.D. and de Mendoza, D. 2003. FapR, a bacterial transcription factor involved in global regulation of membrane lipid biosynthesis. *Developmental Cell.* **4**(5), pp.663-672.

Schultz, G., Phillips, P., Yang, Q. and Stewart, P. 2010. Biofilm maturity studies indicate sharp debridement opens a time-dependent therapeutic window. *Journal of Wound Care.* **19**(8), p320.

Sen, C.K., Gordillo, G.M., Roy, S., Kirsner, R., Lambert, L., Hunt, T.K., Gottrup, F., Gurtner, G.C. and Longaker, M.T. 2009. Human skin wounds: A major and snowballing threat to public health and the economy. *Wound Repair and Regeneration.* **17**(6), pp.763-771.

Shepherd, J.A., Waigh, R.D. and Gilbert, P. 1988. Antibacterial action of 2-bromo-2-nitropropane-1, 3-diol (bronopol). *Antimicrobial Agents and Chemotherapy.* **32**(11), pp.1693-1698.

Siddiqui, A.R. and Bernstein, J.M. 2010. Chronic wound infection: Facts and controversies. *Clinics in Dermatology.* **28**(5), pp.519-526.

Siegert, W. 2014. Approved Preservatives for Cosmetics A Review of Actives Listed in Regulation (EC) No 1223/2009 on cosmetic products - Annex V. [Online]. Available from: https://ec.europa.eu/health/sites/health/files/endocrine_disruptors/docs/cosmetic_1223_2009_regulation_en.pdf

Silver, L.L. 2011. Challenges of antibacterial discovery. *Clinical Microbiology Reviews.* **24**(1), pp.71-109.

Slater-Radosti, C., Van Aller, G., Greenwood, R., Nicholas, R., Keller, P.M., DeWolf, J.W.E., Fan, F., Payne, D.J. and Jaworski, D.D. 2001. Biochemical and genetic characterization of the action of triclosan on *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy.* **48**(1), pp.1-6.

Slayden, R.A., Lee, R.E., Armour, J.W., Cooper, A.M., Orme, I.M., Brennan, P.J. and Besra, G.S. 1996. Antimycobacterial action of thiolactomycin: an inhibitor of fatty acid and mycolic acid synthesis. *Antimicrobial Agents and Chemotherapy.* **40**(12), pp.2813-2819.

Sleire, L., Førde-Tislevoll, H.E., Netland, I.A., Leiss, L., Skeie, B.S. and Enger, P.Ø. 2017. Drug repurposing in cancer. *Pharmacological Research.* **124**(Supplement C), pp.74-91.

Starkey, M., Hickman, J.H., Ma, L., Zhang, N., De Long, S., Hinz, A., Palacios, S., Manoil, C., Kirisits, M.J., Starner, T.D., Wozniak, D.J., Harwood, C.S. and Parsek, M.R. 2009. Pseudomonas aeruginosa Rugose Small-Colony Variants Have Adaptations That Likely Promote Persistence in the Cystic Fibrosis Lung. *Journal of Bacteriology.* **191**(11), pp.3492-3503.

Strittmatter, S.M. 2014. Overcoming drug development bottlenecks with repurposing: Old drugs learn new tricks. *Nature Medicine.* **20**(6), pp.590-591.

Sun, Y., Dowd, S.E., Smith, E., Rhoads, D.D. and Wolcott, R.D. 2008. *In vitro* multispecies Lubbock chronic wound biofilm model. *Wound Repair and Regeneration.* **16**(6), pp.805-813.

Thistlethwaite, I.R.G., Bull, Freya M., Cui, C., Walker, P.D., Gao, S.-S., Wang, L., Song, Z., Masschelein, J., Lavigne, R., Crump, M.P., Race, P.R., Simpson, T.J. and Willis, C.L. 2017. Elucidation of the relative and absolute stereochemistry of the kalimantacin/batumin antibiotics *Chemical Science.* **8**(9), pp.6196-6201.

Thomas, M., Hamdan, M., Hailes, S. and Walker, M. 2011. Evaluation of low-adherent antimicrobial dressings. *Wounds UK.* **7**(2), pp.32-45.

Thomas, S. 2006. Cost of managing chronic wounds in the UK, with particular emphasis on maggot debridement therapy. *Journal of Wound Care.* **15**(10), p465.

Van den Driessche, F., Brackman, G., Swimberghe, R., Rigole, P. and Coenye, T. 2017. Screening a repurposing library for potentiators of antibiotics against *Staphylococcus aureus* biofilms. *International Journal of Antimicrobial Agents.* **49**(3), pp.315-320.

Vuong, C., Saenz, H.L., Götz, F. and Otto, M. 2000. Impact of the *agr* quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*. *The Journal of Infectious Diseases.* **182**(6), pp.1688-1693.

Wei, Q. and Ma, L.Z. 2013. Biofilm matrix and its regulation in *Pseudomonas aeruginosa*. *International Journal of Molecular Sciences.* **14**(10), pp.20983-21005.

Wilkinson, L.J., White, R.J. and Chipman, J.K. 2011. Silver and nanoparticles of silver in wound dressings: a review of efficacy and safety. *Journal of Wound Care.* **20**(11), pp.543-549.

Williamson, D.A., Carter, G.P. and Howden, B.P. 2017. Current and Emerging Topical Antibacterials and Antiseptics: Agents, Action, and Resistance Patterns. *Clinical Microbiology Reviews.* **30**(3), pp.827-860.

Wolcott, R. 2015. Disrupting the biofilm matrix improves wound healing outcomes. *Journal of Wound Care.* **24**(8), pp.366-371.

Wolfmeier, H., Pletzer, D., Mansour, S.C. and Hancock, R.E.W. 2017. New Perspectives in Biofilm Eradication. *ACS Infectious Diseases.*

Woods, J., Boegli, L., Kirker, K.R., Agostinho, A.M., Durch, A.M., deLancey Pulcini, E., Stewart, P.S. and James, G.A. 2012. Development and application of a polymicrobial, *in vitro,* wound biofilm model. *Journal of Applied Microbiology.* **112**(5), pp.998-1006.

Wright, G.D. 2010. Q&A: Antibiotic resistance: where does it come from and what can we do about it? *BMC Biology.* **8**, pp.123-123.

Zhao, G., Usui, M.L., Lippman, S.I., James, G.A., Stewart, P.S., Fleckman, P. and Olerud, J.E. 2013. Biofilms and Inflammation in Chronic Wounds. *Advances in Wound Care.* **2**(7), pp.389-399.

Zhu, L., Bi, H., Ma, J., Hu, Z., Zhang, W., Cronan, J.E. and Wang, H. 2013. The two functional enoyl-acyl carrier protein reductases of *Enterococcus faecalis* do not mediate triclosan resistance. *mBio.* **4**(5), pp.e00613-00613.

Zoë Slote, M., Steven, W. and Jonathan, G. 2011. The answer is 17 years, what is the question: understanding time lags in translational research. *Journal of the Royal Society of Medicine.* **104**(12), pp.510-520.