An Investigation into the Impact of Culture Method on Biofilm Recalcitrance from the Perspective of Chronic Ischemic Wounds

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This thesis is dedicated to those I have lost along this journey.

For: Susan, Doreen, Darlene, Lee, Keith, Frank, and Jayne.

The world is a little less bright without you in it.

But it is mostly for my beloved Grandma J (Irene),

I miss you every day

Thank you for waiting for me to say goodbye

I hope I have made you proud

This is all for you.

Summary

Antimicrobial resistance is a global crisis that requires urgent attention. Although there is significant investment into drug development few of these drugs actually make it to market. There are shortcomings with some of the currently available testing methods, as these do not often take into account the *in vivo* environment in which the infections form. This requires a better understanding of the parameters that affect resistance and susceptibility, such as hypoxia or the recalcitrance of biofilms. Biofilms (communities of surface associated microorganisms) present a significant challenge for researchers and clinicians alike, as they are reported to be up to 1000 times more resistant to antimicrobials (Van Acker *et al*, 2014). These complex communities have a wealth of factors that induce increased recalcitrance to antimicrobials, and are reported to be present in up to 80% of chronic infections (Uruén *et al*., 2021). Yet, antimicrobial testing of biofilms is not yet widely adopted, and the use of biofilm testing in drug development is often not reported. This work aims to investigate these parameters to gain insight on how antimicrobial discovery and testing can be refined.

The first aim of this project was to determine the influence of culture method and hypoxia on *S aureus* susceptibility in several *in vitro* antimicrobial susceptibility testing methods. This compared broth microdilution methods (BMM), the Calgary Biofilm device (CBD), and the biofilm microtiter assay (MTA). Hypoxia was revealed to impact *S aureus* susceptibility in the BMM and CBD methods, and there was a large difference in the density of biofilms formed in the CBD and MTA.

The second aim of this project was to set up an *ex vivo* ovine wounded skin model infected with *Staphylococcus aureus*, which is simple, cost-effective, high throughput, and reproducible. The establishment of wound infection was confirmed by an increase in viable bacterial counts compared to the inoculum. This enabled the final aim to assess if the *ex vivo* model formed biofilms and if hypoxia would have an impact on the susceptibility of the biofilms within the *ex vivo* model. The presence of biofilms was confirmed through scanning electron microscopy, histology, and antimicrobial challenge of the biofilms. Hypoxia elicited an impact on the susceptibility of the *ex vivo* associated biofilms in an antimicrobial specific manner.

This work provides insight into the complex environment that is an ischemic infected wound, and has given insight into the parameters that can affect antimicrobial drug development in the preclinical stages.

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Abbreviations

Abbreviation	Definition
°	Degree
°C	Degrees Celsius
>	More than
<	Less than
%	Percentage
~	Approximately
µg	Microgram
µg / mL	Micrograms per millilitre
3D	Three-dimensional
3Rs	Replacement, Reduction and Refinement
Agr	Accessory gene regulator
ALI	Air liquid interface
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
AST	Antimicrobial susceptibility testing
Au	Gold
BMM	Broth microdilution method
BslA	Biofilm-surface layer protein A
CARB-X	Combating antibiotic-resistant bacteria biopharmaceutical accelerator
CBD	Calgary biofilm device
CDC	Centres for Disease control
c-di-GMP	Bis-(3', 5')-cyclic dimeric guanosine monophosphate
CFU	Colony forming units
CFU/explant	Colony forming units per explant
CFU/g	Colony forming units per gram
ClfA	Clumping factor A
CLSI	Clinical and Laboratory Standards Institute
cm	Centimetres

CO2	Carbon Dioxide
CV	Crystal Violet
CWA	Cell wall anchored protein
DAMPs	Damage associated molecular patterns
DFU	Diabetic foot ulcer
dH_2O	Distilled water
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
ECDC	European Centre for Disease Prevention and Control
ECOFF	Epidemiological cut-off value
EGF	Epidermal growth factor
EPS	Exopolysaccharide
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FBS	Foetal bovine serum
FDA	U.S. Food and Drug Administration
g	gram
G	G-force
h	Hours
H&E	Haematoxylin and Eosin
HaCaT	Human epidermal keratinocytes
HGT	Horizontal gene transfer
HIF	Hypoxia inducible factor
ISO	International Standards Organisation
kPA	Kilopascal
kV	Kilovolt
LMIC	Low-middle-income-countries
MBC	Minimum bactericidal concentration
MBEC	Minimum biofilm eradication concentration
mg/mL	Milligrams per millilitre

MHA	Mueller-hinton agar
MHB	Cation adjusted Mueller-hinton broth
MIC	Minimum inhibitory concentration
Min	minute
MK	Medium-199 with additives for explant culture
mL	Millilitre
mm	Millimetre
mmHg	Millimetres of mercury
MRSA	Methicillin resistant *Staphylococcus aureus*
MSCRAMM	Microbial surface components recognising adhesive matrix molecules
MTA	Microtiter assay
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n	Number of samples
NC3Rs	National Centre for the Replacement Refinement & Reduction of Animal in Research
NHS	National Health Service
nm	Nanometers
O2	Oxygen
OD	Optical density
OD_{600}	Optical density measured at 600 nm
PAMPs	Pathogen associated molecular patterns
PBP	Penicillin binding proteins
PBS	Phosphate buffered saline
PD	Pharmacodynamics
pH	Potential of hydrogen
PIA	Polysaccharide intercellular adhesin
PK	Pharmacokinetics
ppm	Parts per million
Psl	Polysaccharide synthesis locus
PSM	Phenol soluble modulins
PVC	Polyvinyl chloride

RPM	Rotations per minute
SCV	Small colony variants
SEM	Scanning electron microscopy
TS	Tryptic soy agar
TSB	Tryptic soy broth
UKAS	United Kingdom accreditation service
UV	Ultraviolet radiation
v/v	Volume per volume
VPSEM	Variable pressure Scanning Electron microscopy
WHO	World Health Organisation
w/v	Weight per volume
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfonphenyl)-2H-tetrazolium-5-carboxanilide

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Chapter 1: Literature Review

1.1 Antimicrobial resistance

1.1.1 An Introduction to Antimicrobial Resistance

Antimicrobials are one of the most transformative medicines that have ever been developed. They have changed the way we live and extended the average lifespan by decades (Cook and Wright, 2022; Podolsky, 2015). Before antibiotics were discovered, infections and communicable diseases were one of the highest causes of deaths worldwide, with over half of reported deaths being attributed to infections (Armstrong *et al.*, 2020; Cook and Wright, 2022; Adedeji, 2016). The discovery of penicillin lowered mortality associated with pneumococcal pneumonia from 40% to 5%, and average life expectancies went from 47 years in the early 20th century to 78 years post 1950 (Podolsky, 2006; Dowling and Lepper, 1951; Tomasz, 1997). Antibiotics have enabled a revolution of other drugs and procedures that have advanced the medical field in terms of surgeries, organ transplants and chemotherapies (Laxminarayan *et al.*, 2016). However, the widespread overuse of antimicrobials driven by economic growth and prosperity has allowed the emergence of antimicrobial resistance. Antimicrobial resistance is the development of the ability to survive exposure to an antimicrobial that was previously effective at either stopping the growth or killing the microorganism.

Antimicrobial resistance (AMR) represents a global public health crisis that has been acknowledged for decades (O'Neill, 2016). The World Health Organization (WHO) has listed AMR as one of the top 10 threats to global health, highlighting its potential to cause significant harm to humanity (Hu, Logue and Robinson, 2020). This threat is substantiated by multiple reports, such as a report by WHO stating that 30% of neonates with sepsis die as a result of infections caused by bacterial AMR, or from a systematic review by Murray *et al*, that demonstrated an estimated 1.27 million deaths can be attributed to bacterial AMR in 2019 alone (Murray *et al.*, 2022). WHO and the International Agency for Research on Cancer report that the world's most prevalent cancer- breast cancer- accounts for 685000 deaths globally in 2020, this number pales in comparison to the 1.27 million for AMR (Murray *et al.*, 2022; Wild, Weiderpass and Stewart, 2020). This is an increase on previous reports of deaths attributable to bacterial AMR of around 700,000 per year worldwide (Huemer *et al.*, 2020; Aslam *et al.*, 2018). Furthermore, it is estimated that by 2050 the annual global cost of AMR will cause 10 million deaths and cost $100 Trillion annually, and that for the first time deaths caused by AMR will outweigh the predicted deaths from all cancers (8 million) (O'Neill, 2016).

AMR is the result of both overuse and over-prescription of antimicrobial agents driving the emergence of new resistance mechanisms (McEwen and Collignon, 2018). The challenge that we face today is the need for new classes of antimicrobial drugs, strategies for drug delivery, ways of targeting and mitigating resistance mechanisms, as well as the need for better education and awareness on the appropriate use of antimicrobial agents (Dadgostar, 2019). It is clear that this requires an interdisciplinary approach, involving researchers, medical professionals, and policymakers working together to address the different aspects of this problem. By taking a comprehensive and collaborative approach to tackling AMR, it is possible to curb this global crisis and ensure that we have the tools to fight bacterial infections in the future. These factors have pushed through the development of a significant amount of new collaborations, such as the European One Health Action plan against AMR first initiated in 2017 by the European Commission (Mestrovic *et al.*, 2022). This is a collaborative effort throughout multiple industries and researchers to drive forward better health for people, animals, plants and the environment (McEwen and Collignon, 2018). This one health approach is necessary as the drivers of AMR exist within human, animal and environmental overuse of antimicrobials around the globe. Interestingly, this is not the only organisation or funding package that has been developed. In 2018, Novo Holdings launched Replenishing and Enabling the Pipeline for Anti-Infective Resistance (REPAIR) which was a $165 million initiative for investment in tackling AMR. Equally there have been the Combating Antibiotic Resistant Bacteria Biopharmaceutical Accelerator organisation (CARB-X) established in 2016, and a $500 million programme from the Bill and Melinda Gates Foundation. In addition to this, the AMR action fund was launched by the International Federation of Pharmaceutical Manufacturers and Associations with a $1 billion fund to support new research (Plackett, 2020).

The era of readily available, effective antibiotics is long gone, and it is now essential that new solutions are developed to address this increasing threat to human health. AMR has a significant and far reaching impact on the socioeconomic structures within society. Since we so often rely on antimicrobials to enable the day-to-day essential processes that were developed on the back of the antimicrobial revolution in the mid 1900's, the potential impacts from this increasing problem could be catastrophic.

1.1.2 Why are there not more drugs coming to market to combat AMR?

Although antibiotics have revolutionised modern medicine by enabling the treatment of serious communicable disease, few new drugs are coming to market. The impact of antibiotic development on society is evidenced in longer life expectancies (increase of 30 years) than in the early 20th century due to lowered risks associated with invasive medical procedures (Dutescu and Hillier, 2021). Although there is significant interest in AMR with WHO, the World Bank, and governments all over the world investing in resources, little has been seen in terms of new drugs to market (Dutescu and Hiller, 2021).

To make matters worse, 15 of the big 18 global pharmaceutical companies have abandoned their antibiotic research and development. This is due to the poor returns on investment antimicrobial development has. Antimicrobials are a low profit drug and aren't prescribed for long periods of time- which means that returns are generally low or even negative (Miethke *et al.*, 2021; Dutescu and Hillie, 2021; Brogan and Mossialos, 2013; Council of Canadian Academies, 2019). Short courses of prescribed antibiotics combined with strict antimicrobial stewardship further loses the interest of large corporations looking for high profit margins (Dutescu and Hillie, 2021; Brogan and Mossialos, 2013; Renwick and Mossialos, 2018). It is also likely that if new drugs are developed and used regularly in clinical practice, that the causative agents would develop resistance and render the drug ineffective over time. Equally if the cost of treatment was increased to entice the large companies this would cause further exacerbation of the disparity of LMICs and the prevalence of AMR there.

Innovation in the early stages of antimicrobial development is often shouldered by the academic sector through research grants and funding. Partnerships with external pharmaceutical companies are a significant advantage, however this is often only possible after a target compound has been selected and validated for use (Miethke *et al*, 2021). The drug selection process is long and costly, with some estimates of the costs on average being $1.4 billion. There is little chance for academic grants to support the full validation of novel compounds (DiMasi *et al*, 2016). There are a large number of global antimicrobial resistance organisations that have been developed to combat these issues and introduce public-private partnerships (Theuretzbacher, Årdal and Harbarth, 2017). However, even with the increased engagement and investment in partnerships there is not enough variability and availability to support potential candidates through to market. In addition, the amount of reward needed to entice multinational pharmaceutical companies is substantial, with an estimated $0.8 to $1.3 billion over a five-year period, it is simply not feasible for the public sector to fund these kinds of incentives (Theuretzbacher, Årdal and Harbarth, 2017, O'Neill, 2016).

So, are financial incentives the way forward to push through better antibiotic drug development? The question remains on the best ways to encourage antimicrobial drug development and what is most commonly recommended, as the current methods are proving ineffective, with no new compound classes developed for Gram-negative treatment in over 50 years (Miethke *et al*. 2021). There are many different incentives available, such as: grants, subsidies, tax incentives, advance market commitments, patent extensions, and market entry rewards. These incentives are classed as either push incentives (funding innovation and promotion of innovation) or pull incentives (rewards and benefits post drug development). A systematic review conducted by Dutescu and Hillier (2021) found that the most recommended incentive was a hybrid model, the Options Market Award model, which combines aspects of push and pull incentives including discounted prices for early purchase by stakeholders

(Dutescu and Hillie, 2021). There is no clear consensus on which model (push, pull or hybrid) is most effective in motivating innovation in antimicrobial development. It is on the right track to focus on incentives as most studies identify that the true barrier to innovation and development of antibiotics is the unattractive market, not scientific or regulatory barriers. To effectively combat this barrier, significant financial reward is essential to prevent further abandonment of antimicrobial development research (Theuretzbacher, Årdal and Harbarth, 2017). Therefore, the need for new economic models as incentives is essential in combating antibiotic resistance.

With financial incentives now covered, how difficult is it to overcome antimicrobial resistance by targeting new pathways for bacterial killing? Although it has been widely suggested that the central problem of the lack of new antibiotics is the economic issues, concerns surrounding the scientific challenges are still an essential consideration (Theuretzbacher *et al.*, 2019; Theuretzbacher, Årdal and Harbarth, 2017). As of 2021, there were 27 new antibiotics in clinical development that were targeted at the WHO classification of priority pathogens, sadly this number is a decrease compared to the number of drugs in the clinical development pipeline from 2017 (WHO, 2017). In support of the disappointment of new drugs to market, only 20 new antimicrobials have been approved by the Food and Drugs Administration (FDA) since 2010 (Theuretzbacher *et al.*, 2019; Theuretzbacher, Årdal and Harbarth, 2017). It is thought that these new antibiotics may lack diversity in their cellular targets, with a big push for new classes to be developed - not just the tweaking of targets and mechanisms within current classes (Hu Robinson, Logue, 2020).

1.1.3 Antimicrobials, their actions and the resistance developed to them

With a vast array of current antimicrobials, the ways in which bacteria evade these chemicals is far reaching. It is important to view these resistance mechanisms in the context of the initial mechanisms of action of antimicrobial, however in the context of this work, two groups will be discussed, cell wall synthesis and protein synthesis. Firstly, there are drugs that interfere with cell wall synthesis, this consists of β-lactams (including penicillin and carbapenems) and glycopeptides (including vancomycin and teicoplanin) (Nikolaidis, Favini-Stabile and Dessen, 2014; Tenover, 2006). These affect the bacterial cell wall, and in particular a component called peptidoglycan. Peptidoglycan consists of N-acetylmuramic acid and N-acetylglucosamine cross linked by peptides, and forms a complex mesh-like structure protecting the cells contents (Shaku *et al.*, 2020). Glycopeptide antibiotics target the biosynthesis of peptidoglycan by binding the terminal D-Ala-D-Ala dipeptide of nascent peptidoglycan terminating transpeptidation through substrate sequestration and blocking transglycosylation through associated steric hindrance (Zeng *et al.*, 2016; Georgopapadakou, Hammarstrom and Strominger, 1977; Bush and Bradford, 2016). Peptidoglycan synthesis is also targeted by β-lactam antimicrobials which

covalently bind to penicillin binding proteins (PBP), PBP are enzymes that are involved in the terminal steps of peptidoglycan cross-linking to support cell wall stability (Bush and Bradford, 2016; Cole and Riordan, 2013). There is a structural similarity of penicillin G to the D-Ala-D-Ala dipeptide which normally would bind into the serine site of PBPs resulting in cross-linking. However, this similarity enables the binding of penicillin and other β-lactams to the active serine site blocking cross linking resulting in cell wall instability and subsequent cell death (Georgopapadakou, Hammarstrom and Strominger, 1977; Bush and Bradford, 2016).

Secondly, there are drugs that inhibit protein synthesis, these include macrolides, aminoglycosides, tetracyclines, and chloramphenicol. Bacterial translational apparatus such as bacterial ribosomes are vital components for bacterial life. Since they are so essential, they make ideal targets for antimicrobial drugs (Brandi *et al.*, 2008). Bacterial translation is initiated by the binding of protein precursors with the 30S ribosomal subunit and the subsequent formation of a stable 30S initiation complex, this complex then associates rapidly with the 50S ribosomal subunit to form the 70S initiation complex (Gualerzi *et al.*, 2001; Gualerzi and Pon, 2015; Laursen *et al.*, 2005; Hennelly *et al.*, 2005; Tomšic *et al.*, 2000; Grigoriadou *et al.*, 2007; Brandi *et al.*, 2008). Aminoglycosides can gain access to bacterial cells through several different mechanisms, through electrostatic binding of the aminoglycoside to the negatively charged components of the bacterial membrane and the removal of magnesium ions causing membrane disruption increasing permeability and energy-dependent uptake of the aminoglycosides (Wilson, 2014; Garneau-Tsodikova and Labby, 2016; Krause *et al.*, 2016). The antimicrobials then diffuse through the intracellular space and attach to the A-site of the 30S subunit causing incorrect mRNA-tRNA pairing (Wilson, 2014; Garneau-Tsodikova and Labby, 2016). With regard to targeting the 50S ribosome, macrolides and chloramphenicol can bind between the nascent peptide exit tunnel and peptidyl transferase centre to prevent the addition of newly synthesised polypeptides from passing through the nascent peptide exit tunnel (Ban *et al.*, 2000; Vazquez, 1974).

There are also drug classes that target nucleic acid synthesis, as bacterial DNA synthesis requires the use of topoisomerases, which are enzymes that catalyse the topological forms of DNA that are essential as part of the replication machinery for transcription which are a typical target for these drug classes (Bush *et al.*, 2020). All types of topoisomerases can relax DNA, but only DNA gyrase can induce negative supercoiling, DNA gyrase is present and essential in bacteria and is not found in eukaryotes making it an excellent target for antibiotics (Gellert *et al.*, 1976; Wang and Lynch, 1993; Berger, 1998). Quinolones inhibit DNA supercoiling and relaxation by binding both DNA gyrase and DNA to stabilise the gyrase-DNA cleaved complex (Gellert *et al.*, 1976). When this is not resolved replication and transcription cannot happen and this causes bacterial death (Gellert *et al.*, 1977; Sugino *et al.*, 1977). Topoisomerase IV is the primary target in Gram-positive species. Once the topoisomerase is

sequestered by the antibiotic it leads to chromosomal fragmentation and rapid cell death (Drlica and Zhao, 1997; Hooper and Jacoby, 2016; Ferrero *et al.*, 1994; Khodursky, Zechiedrich and Cozzarelli, 1995; Redgrave *et al.*, 2014; Correia *et al.*, 2017; Aldred, Kerns and Osheroff, 2014; Bush and Bradford, 2016).

There are a significant number of intrinsic ways in which bacteria can resist antimicrobials. These include the differences between Gram negatives and Gram positives cell envelopes where due to the different cell wall structures certain classes of antibiotics inherently do not work. However, the bulk of the literature instead delves into acquired resistance, mechanisms in which bacteria have mutated or transferred to confer resistance to antimicrobials. Resistance mechanisms are often acquired through mobile genetic elements, such as plasmids which can be incorporated into different species for the enhancement of resistance to antimicrobial treatments (Munita and Arias, 2016). Additionally, there are mechanisms in which bacteria can pass across genetic information called horizontal gene transfer, this requires bacteria to be in close proximity to each other and contact must be initiated for the conjugation of genetic information to be acquired. Both of these mechanisms of mobile genetic elements and horizontal gene transfer have been well documented with resistance genes appearing in strains and species previously not associated with the newly acquired resistance mechanisms (Munita and Arias, 2016; McManus, 1997; Christaki *et al*, 2019). This Darwinian selection process has enabled the development of robust resistance mechanisms that enable the successful evasion of bacteria from antimicrobials.

Resistance to cell wall-targeting antimicrobials is conferred through the altering of the PBP structure, reducing the cell surface permeability through altered porin function or inactivation of the antimicrobial by secreted enzymes such as β-lactamases (Sapun *et al*, 2008; McManus, 1997). β-lactamases are prolific enzymes found in both Gram-positive and Gram-negative bacteria and are overproduced when in the presence of certain antimicrobials (McManus, 1997; Neu, 1992; Bryan, 1988; Shafran, 1990). These enzymes can be associated with the chromosome or borne on plasmids. With genes encoding β-lactamases being predominantly present on conjugative plasmids, the spread of resistance is a significant problem (McManus, 1997; Shafran, 1990). The alteration of porins decreases the influx of antimicrobials into the cell. This is conducted through downregulation of porins, structural modifications of the porins themselves, or even deletion of the genes encoding porin production (Delcour, 2010). Porins enable the passage of some antimicrobials into the cell, therefore a decrease in the amount entering the cell can provide resistance.

Bacteria have also developed several mechanisms to overcome the blocking of protein synthesis. This includes the alteration of membrane permeability, alteration or mutation of the target site, active removal of drugs from the cell through utilisation of efflux pumps, drug modification and degradation,

and production of target mimics (Poole, 2005; Poole, 2005; McManus, 1997; Bryan, 1988; Neu, 1984; Nikaido and Vaara, 1985; Wilson, 2016). Resistance to nucleic acid targeting drugs has been evidenced by alterations in the antimicrobial target, such as with the mutation of the quinolone-resistance-determining region in DNA gyrase which confers resistance to fluoroquinolones in both Gram negative and Gram positives (Ruiz, 2003; Redgrave, 2014).

Finally, when considering resistance to antimicrobials it would be remiss to not discuss the nuances of bacterial lifestyle. When existing in the environments in which they are naturally found bacteria have been observed to live on a variety of surfaces in multicellular communities (Donlan, 2002). These communities enable far better protection against antimicrobials and enhance the exchange of resistance mechanisms for better group survival. These communities have been called biofilms and have been implicated in a variety of clinical infections and observed to confer significant amounts of resistance to antimicrobials on a scale that had not been previously demonstrated

1.1.4 AMR and bacterial biofilms

Resistance to antimicrobials is a well-established field with significant investment and research into the molecular mechanisms, stewardship and novel targets. Yet still there are increasing amounts of therapeutic failure. As early as 1683 aggregated microbes have been observed by scientists, and despite these initial observations the bulk of research in microbiology has been conducted on planktonic cultures (Hoiby, 2017). Finally, in 1975 the first biofilm reports were published and the number of publications grew significantly as the term biofilm was embedded into medical nomenclature. Research has begun to shift focus towards the biofilm phenotype of bacteria as biofilms are estimated to be present at the site of 80% of chronic and recurrent infections (Uruén *et al*., 2021). It is often thought that biofilms present at the site of infection are the underlying reason for therapy failure (Van Acker, 2014). Biofilms are communities of bacteria adhered to a biotic or abiotic surface that form protective extracellular polymeric substances (EPS) to protect the community from the environment, this in turn can reduce the impact of antimicrobials on the bacteria (Amorena *et al*., 1999). The cells that exist within a biofilm are phenotypically and physiologically different to cells in planktonic culture (Costerton, Stewart and Greenberg, 1999a). As a result of this, biofilms are widely reported to be up to 1000 times more resistant to antimicrobials (Van Acker *et al*, 2014).

The biofilm secretion of EPS can quench the antimicrobials, making biofilms much more difficult to treat than their planktonic counterparts. However, there are a multitude of factors associated with biofilms that induce increased tolerance and resistance to antimicrobials. Despite this increased resistance, the use of biofilms as a standard for antimicrobial testing is still not widely adopted. There is a significant

degree of variability in the methods used for biofilm testing, which range from microtiter plate assays and flow models to bioreactors (Hassan *et al.*, 2011; Stepanović *et al.*, 2007; Høiby *et al.*, 2011). To address this issue, there has been an increase in accredited biofilm testing, guidelines and standardised methods in order to provide more consistent and reliable results (Coenye *et al.*, 2018; Malone *et al.*, 2017; Stepanović *et al.*, 2007; Høiby *et al.*, 2011). The biofilm phenotype of bacteria represents a significant barrier to the effective treatment of clinical infections, and further research is needed to understand how these communities of bacteria can be targeted and eliminated.

1.2 Biofilms

1.2.1 What is a biofilm?

When considering that humans exist in communities bound together to take advantage of natural resources and social connection for our survival, it is understandable that the community structures which benefit us would also benefit other species. It is then no surprise that microbial life, when found in natural habitats, benefits from these same community structures, and these structures enhance their own survival. There are many definitions for biofilms, however it is generally accepted that a biofilm is: A community of aggregated microbial cells (bacterial, viral, fungal and yeasts) surrounded by a self-produced matrix (Percival *et al.*, 2011; Neopane *et al.*, 2018). The importance of the bacterial biofilm was initially elucidated by the "Father of the Biofilms" Bill Costerton, it was in 1978 he introduced 'an extraordinarily new microbiological paradigm called: The Biofilm Theory' (Renata Arciola *et al.*, 2015). This discovery changed bacteriological research, with a new focus on how researchers and clinicians alike treated patients with infections due to the different micro-physiology seen in biofilms. Micro-organisms can form complex relationships with other cells and molecules within their environment, consistently adapting to new changes (Flemming *et al.*, 2016). The transition undertaken by bacterial cells in a planktonic state into the biofilm phenotype is a dynamic multistep process, often initiated in response to environmental changes (Kostakioti, Hadjifrangiskou and Hultgren, 2013). Biofilms can be both beneficial and detrimental for human health, they are crucial in industrial wastewater treatment, yet when associated with chronic infections can cause significant morbidity and mortality (Yan and Bassler, 2019; Neopane *et al.*, 2018).

1.2.3 Role of biofilms in antimicrobial resistance and tolerance

Resistant bacteria have the ability to multiply and grow in the presence of antimicrobials at concentrations that without resistance would have inhibited or killed them (Hall and Mah, 2017). In

contrast, antimicrobial tolerance is the ability to withstand similar levels of antimicrobial, without replication or growth for a transient period of time wherein the bacteria will resume replication following the removal of the antimicrobial (Hall and Mah, 2017; Crabbé *et al.*, 2019; Høiby *et al.*, 2010; Ciofu *et al.*, 2022). Tolerance involves an intrinsic slowing down of bacterial processes or other mechanisms to withstand antimicrobial treatment, but not to increase the minimum inhibitory concentration (MIC) as is seen with resistance (Brauner *et al.*, 2016; Crabbé *et al.*, 2019). The minimum inhibitory concentration is the concentration of a given antimicrobial that halt (inhibits) the growth of a chosen bacterium, these values are often used within antimicrobial research and clinical treatments to determine the appropriate treatment dose (EUCAST, 2023).

Biofilm formation is often recognised as a driving factor in the development of persistent infections that do not respond to standard antimicrobial interventions (Macia, Rojo-Molinero and Oliver, 2014). Biofilms are intrinsically far more tolerant to antimicrobials, and this is evidenced by the far reaching reports that biofilms are up to 1000 times less susceptible to antimicrobial treatment *in vitro* (Crabbé *et al.*, 2019; Haney *et al.*, 2018; Costerton, Stewart and Greenberg, 1999b). These aggregated communities are capable of surviving transient high doses of antimicrobials without the utilisation of inheritable traits as is seen in resistance (Ciofu *et al*, 2022). There are a large number of factors that facilitate tolerance such as; antimicrobial chemistry, strains of bacteria within the biofilm, environment surrounding the biofilm, age of the biofilm, heterogeneity of the biofilm, oxygenation of the biofilm, nutrient availability and many other environmental factors (Stewart *et al.*, 2016; Yan and Bassler, 2019; Ciofu *et al.*, 2022; Crabbé *et al.*, 2019).

Biofilm associated cells are phenotypically very different from their planktonic counterparts, and as a result reduced susceptibility to antimicrobials has been widely reported (Crabbé *et al.*, 2019; Brauner *et al.*, 2016). Particularly, tolerance has been observed with biofilm phenotypes such as small colony variants and persisters, as these cells have low metabolic activity therefore, the targets for antimicrobials are often found to be inactive (Crabbé *et al.*, 2019; Conlon, Rowe and Lewis, 2015; Garcia *et al.*, 2013). The initiation of these phenotypes is often in response to environmental pressures, such as lack of nutrients, anoxic conditions, or exposure to sub-inhibitory levels of antimicrobials. Biofilm formation is often in response to the same environmental pressures, subsequently causing the upregulation and downregulation of different genes to enhance adherence, and the secretion of EPS (Pratt and Kolter, 1998; Mah and O'Toole, 2001; O'Toole, Kaplan and Kolter, 2000; Friedman and Kolter, 2004a; Prigent-Combaret *et al.*, 2001; Parsek and Singh, 2003; Lenz *et al.*, 2008; Monds and O'Toole, 2009).

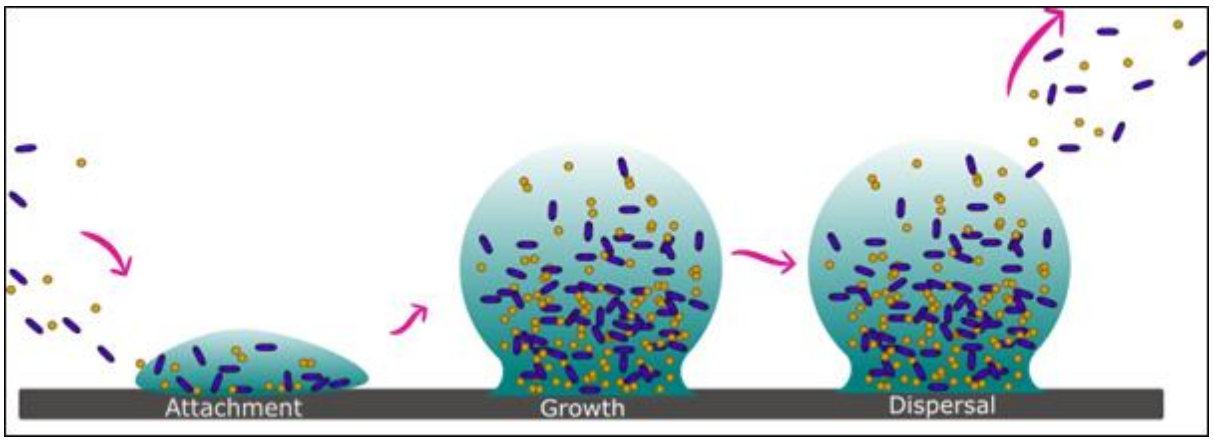


Figure 1 Diagram of the steps involved in the formation and development of a biofilm. Initial surface attachment by planktonic cells to a surface and initiation of EPS synthesis is then followed by a growth stage where bacteria proliferate, secrete more biofilm EPS and other matrix components. Finally, the dispersal stage allows the spread of microbial cells to other sites and new nutrient opportunities for bacteria. Created using Inkscape.

1.2.2 Biofilm development: Attachment

Biofilms develop in two main stages, attachment and development (Figure 1). Once attached biofilms are often very difficult to remove and can grow and diversify into incredibly complex and diverse communities (Tolker-Nielsen, 2015). Once attached and developed they disperse parts of the biofilm to enable further attachment and utilisation of nutrients at a different source (Boles and Horswill, 2008b; Sauer *et al.*, 2004). There are a multitude of ways for attachment to a surface occurs, and this is often dependent on the environment, the surface type and the bacterium (Donlan, 2002).

It has been demonstrated that the initiation of attachment occurs in response to an increase in the amounts of c-di-GMP (Valentini and Filloux, 2016; Rabin *et al.*, 2015; Ha and O'Toole, 2015). This intracellular secondary messenger is synthesised by diguanylate cyclases and degraded by phosphodiesterases, it is suggested that there are a number of environmental cues and transducer mechanisms that lead to increases in c-di-GMP activating the production of adhesins and EPS to facilitate adhesion (Tolker-Nielsen, 2015; Ha and O'Toole, 2015). Bacterial attachment to a surface can happen using a multitude of methods; receptor-ligand interactions, flagella and pili mediated attachment, gravitational forces, hydrodynamic forces, Brownian motion, and surface protein attachment (Donlan and Costerton, 2002; Beloin, Roux and Ghigo, 2008; Macia, Rojo-Molinero and Oliver, 2014; Wu, Cheng and Cheng, 2018). Motile bacteria can utilise their flagella and pili organelles to forcibly facilitate attachment and induce a cascade activation of different molecular processes such as chemosensing, mechanosensing, nutrient utilisation, and upregulation of survival based virulence

factors to aid the development of a robust biofilm (Whiteley *et al.*, 2001; Schembri, Kjaergaard and Klemm, 2003; Stanley *et al.*, 2003; Bagge *et al.*, 2004; Vuong *et al.*, 2004; Beloin, Roux and Ghigo, 2008; Lenz *et al.*, 2008; Zhang and Mah, 2008; Klebensberger *et al.*, 2009; Wu, Cheng and Cheng, 2018).

It has widely been established that *Pseudomonas aeruginosa* is an exemplary organism for studying biofilm formation, therefore it would be prudent to discuss the well characterised methods of biofilm attachment seen in *P. aeruginosa*. The intercellular c-di-GMP is shown to have significant effect on biofilm formation and dispersal in *P. aeruginosa*, with over 41 c-di-GMP proteins encoded within the genome that are expected to participate in the metabolism of c-di-GMP (Kulesekara *et al.*, 2006; Ryan *et al.*, 2009). Most of the proteins have been linked to sensory input domains on their N-terminus indicating that interaction with the wider environment is an essential stimulus to the cellular response (biofilm formation) (Galperin, Nikolskaya and Koonin, 2001; Galperin, 2004). Following on from c-di-GMP metabolism there are two distinct mechanisms that control and initiate flagellar motility in the early stages of biofilm formation and attachment. The first of which encodes the biosynthesis of the flagellar machinery by FleQ, a tightly regulated mechanism that binds to the upstream activation sequence of *flhA* to begin the cascade of flagellar expression through either sequestration of FleQ by FLeN or through conformational change of FleQ when bound to c-di-GMP (Liang *et al.*, 2007; Dasgupta, Arora and Ramphal, 2000; Hickman and Harwood, 2008; Claudine and Harwood, 2013). The second mechanism that responds to the increase in intercellular c-di-GMP is that of YcgR, this contains a PilZ domain that is common in c-di-GMP responsive proteins and once bound YcgR can interact with motors and stator proteins to increase or decrease the motility of the flagellum when there are high levels of c-di-GMP (Ryjenkov *et al.*, 2006; Boehm *et al.*, 2010; Paul *et al.*, 2010). It is through the flagellar activation and motility that *P. aeruginosa* can 'swim' around in a well-documented swarming phenomenon along with surfactant secretion to migrate through a substratum and find a suitable surface to attach to and form a biofilm. The polar flagellum then initiates reversible attachment which is the first step, however, *P. aeruginosa* can also utilise twitching motility enabled by the type IV pili to explore the reversibly bound surface. The next step is irreversible attachment, this step involves the repression of surface motility components and initiation of the production of exopolysaccharides. Pel and Psl have also been identified in *P. aeruginosa* to promote irreversible attachment. They have been demonstrated to act as adhesins in the early stages of biofilm development, and the secretion of Psl has been implicated in micro colony formation, as it is secreted as a trail around the bacteria to enhance the attachment of other cells (Friedman and Kolter, 2004a, 2004b; Zhao *et al.*, 2013; Monds and O'Toole, 2009).

C-di-GMP is often implicated with regards to the GGDEF protein domains, this domain has been identified in a wide spread of bacteria, suggesting that many species and phenotypes are targeted and

modulated by c-di-GMP (Karaolis *et al.*, 2005). However, it has been demonstrated that application of c-di-GMP to *S aureus* cultures impacts the cell-to-cell interactions, preventing cell-cell clumping and biofilm formation (Karaolis *et al.*, 2005). Interestingly, there appears to be some debate about the effect of c-di-GMP on *S aureus* biofilm formation, with Ishihara *et al*. suggesting importance for biofilm formation as there is a mutation in the GdpS protein which contains the GGDEF domain which was observed to be aided by the addition of external c-di-GMP (Ishihara *et al.*, 2009). In direct contrast to this, Holland *et al* reported that GdpS did affect biofilm formation but that this mechanism was independent of c-di-GMP (Holland *et al.*, 2008). More recent studies have observed that application of c-di-GMP to methicillin resistant *S aureus* (MRSA) did not show direct antimicrobial properties, yet when c-di-GMP was added to HaCaT cells prior to MRSA inoculation, colonisation of the HaCaT cells by MRSA was inhibited (Gao *et al.*, 2022). Although this inhibition is likely due to heightened immune response, it yields further conflicting information as to the role of c-di-GMP and *S* aureus biofilm formation. These discrepancies may be due to strain differences, yet it is unclear as to the purpose of c-di-GMP in *S aureus* biofilm formation (Wolska *et al.*, 2016).

Quorum sensing is another important factor in the initiation of biofilm formation. The processes that are undertaken in the formation of a biofilm are irrelevant unless undertaken in a coordinated way (Mukherjee and Bassler, 2019). Quorum sensing is a process in which there is the production and detection of extracellular signalling molecules (autoinducers) enabling cell-to-cell communication (Mukherjee and Bassler, 2019; Miller and Bassler, 2001; Papenfort and Bassler, 2016; Rutherford and Bassler, 2012). However, these autoinducers can have significantly different effects on the biofilm of various pathogens. *P aeruginosa* biofilms form at a higher cell density in response to autoinducer accumulation and detection, whereas *S aureus* has been shown to form lower cell density biofilms with autoinducer accumulation and detection impeding biofilm formation (Mukherjee and Bassler, 2019; Bronesky *et al*. 2016; Kievit and Iglewski, 2000).

1.2.3 Biofilm development: The EPS matrix

Once attached, bacteria encase themselves within a matrix of EPS accounting for roughly 90% of the total biomass of the biofilm (Flemming and Wingender, 2010). This EPS matrix consists of exopolysaccharides, proteins, extracellular DNA, pili, flagella, adhesive fibres and other macromolecules that enhance the structural stability and nutrient availability of the biofilm (Flemming and Wingender, 2010; Karygianni *et al.*, 2020). The composition of the EPS is dependent on the environment that the biofilm exists within, and the types of microorganisms that are included in the biofilm (Karygianni *et al.*, 2020; Flemming *et al.*, 2016). EPS enhances adhesion to surfaces and cements the microbial cells in close proximity encouraging intercellular interactions within the biofilm (Karygianni *et al*, 2020).

Matrix components allow nutrient and water 'trapping' of the biofilm by hydrogen bond interactions with hydrophilic polysaccharides that are readily found within the EPS. This allows highly hydrated parts of the biofilm to develop into a non-rigid structure with different zones of viscosity (Zogaj *et al.*, 2001; Tielker *et al.*, 2005; Branda *et al.*, 2006; Diggle *et al.*, 2006; Pinkner *et al.*, 2006; Cegelski *et al.*, 2009; Neopane *et al.*, 2018; Flemming *et al.*, 2016). It is important to microbial survival that the desiccation of the microbial cells is prevented, as bacteria frequently experience water stress in natural environments (Flemming *et al*, 2016). It has been observed that biofilms can actively respond to this water stress through increased production of some EPS molecules which protect the cells from desiccation by acting as a hydrogel as a result of water trapping (Flemming *et al.*, 2016; Flemming and Wingender, 2010). Within the components of the EPS, there is utilisation of flagella, pili and other larger components support the structural stability of the biofilm, enhancing the scaffold-like structures that support the highly hydrated components (Karygianni *et al.*, 2020; Flemming *et al.*, 2016; Persat *et al.*, 2015; Billings *et al.*, 2015). It has also been observed that host proteins and molecules can be utilised by the EPS, supporting the matrix scaffold, enhancing attachment to host surfaces and to use as nutrient sources (Marsh *et al.*, 2016; Karygianni *et al.*, 2020).

Nutrients are kept within the biofilm structure, in particular within the EPS, and an increase in enzymes into the EPS can result in sequestration of these trapped nutrients in response to a change in the nutrient availability in the local biofilm environment (Sauer *et al.*, 2004; Evans *et al.*, 2020). This process can be referred to as cross-feeding or external digestion, where subpopulations of bacteria have contrasting metabolism processes in the different areas of the biofilm, resulting in the production of specific metabolites that benefit bacteria residing in other areas of the biofilm (Giri *et al.*, 2019; Evans *et al.*, 2020). For example, bacteria within the innermost layers of the biofilm may be using fermentative metabolism as a consequence of the anoxic environment, this will introduce different waste products that subsequently can be utilised by the aerobically metabolising cells nearer the surface of the biofilm (Evans *et al*, 2020). Additionally, the secretion of extracellular components into the EPS has been shown to be partially under the control of quorum sensing as a way to ensure that there is an even share of the nutritious extracellular components (Mukherjee and Bassler, 2019; Popat *et al.*, 2012; Darch *et al.*, 2012; Cordero *et al.*, 2012). This enables tailored sequestering of nutrients on a needs basis from the EPS nutrient reserves and production of nutrient sources from waste products (Sauer *et al.*, 2004; Gjermansen *et al.*, 2005). Overexploitation of these communal nutrient sources must be mitigated as this can impact the fitness of the biofilm population negatively (Mukherjee and Bassler, 2019; Popat *et al*, 2012; Darch *et al*, 2012). This brings into play the structure of the biofilm along with the policing of the bacterial community to prevent the over utilisation by non producing bacteria in a phenomenon called cheating (Popat *et al.*, 2012; Cordero *et al.*, 2012; Waters and Bruger, 2015). Cheating is where

bacteria benefit from the production of these cooperative components, yet do not contribute themselves and are therefore deemed cheaters (Ghoul, Griffin and West, 2014; Wechsler, Kümmerli and Dobay, 2019). The policing of cheaters is a method by which quorum sensing bacteria can impact cheaters by imposing additional costs on them to enforce cooperation with the community (Wechsler, Kümmerli and Dobay, 2019; Inglis, West and Buckling, 2014). These sanctions can be as serious as the production of toxins to selectively target cheaters (Wang *et al.*, 2015; Wechsler, Kümmerli and Dobay, 2019).

The EPS facilitates the immobilisation of microbial cells for the maturation and growth of long-term mixed-species mature biofilms (Figure 2) (Flemming and Wingender, 2010). This long-term, close-quarters microenvironment of pathogenic and non-pathogenic microbes will encourage the transfer of genes. It is well known that horizontal gene transfer (HGT) occurs in increasing amounts within the biofilm environment (Luo *et al.*, 2021). With many different bacteria implicated in the conjugation of plasmids and transposons, as seen with *Veillonella despar* transferring its conjugative transposon Tn916 to several *Streptococcus* spp., or the acquisition of the IncP1 plasmid into *Stenotrophomonas rhizopilia* from *Pseudomonas putida* (de la Cruz-Perera *et al.*, 2013; Hannan *et al.*, 2010). Transformation has been observed in biofilms due to the eDNA secreted into the EPS and transduction has been seen with bacteriophages carrying Shiga toxin genes implicated in the promotion of the emergence of a Shiga toxin producing strain of *Escherichia coli* (Solheim *et al.*, 2013). The increased transmission of genes within biofilms promotes better survival to stressful conditions such as nutrient and oxygen limitation as well as antimicrobial treatment. The survival of the biofilm is governed by its environment and adaptation to it, as a result the biofilm can terminate its existence in response to certain environmental signals (Tolker-Nielsen, 2015) . Quorum sensing has been linked to the dispersal of biofilms by reducing the synthesis of one of the main polysaccharides in *P aeruginosa.* It can also initiate the induction of rhamnolipids and utilise their surfactant like properties to aid the degradation of the biofilm (Singh *et al.*, 2013; Irie, O'Toole and Yuk, 2005). Rhamnolipids can provide protection against the host innate immune system by inducing cell death of some immune cells therefore enabling released bacteria to encounter a less immunologically active local environment (Solano, Echeverz and Lasa, 2014). In *S aureus* it has been observed that the Agr system can downregulate expression of surface adhesins encouraging biofilm dispersal (Boles and Horswill, 2008a). Agr was also identified to induce the production of phenol-soluble modulins (PSMs) which can mediate biofilm dispersion due to their surfactant-like properties (Periasamy *et al.*, 2012; Wang *et al.*, 2011).

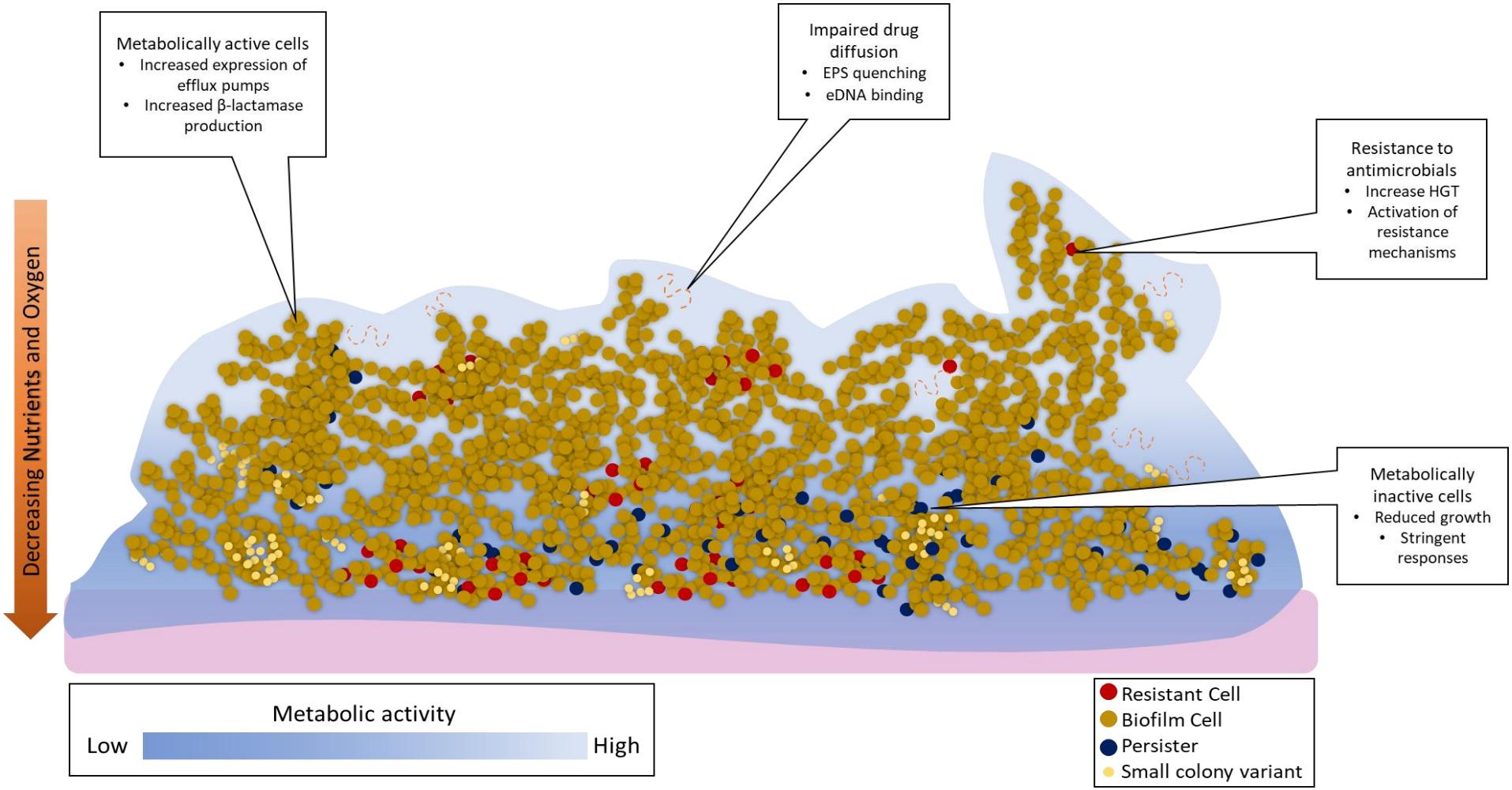


Figure 2 Schematic of a biofilm with components that increase recalcitrance to antimicrobial treatment and removal of biofilm. There are a many different phenotypes of bacterial cells in the biofilm that contribute to its recalcitrance. This includes resistant cells that can confer their own resistance to other susceptible cells through HGT, Persisters and Small colony variants that are able to shut down their own metabolic processes to avoid antimicrobial attack, a gradient of nutrients, oxygen and metabolic activity of the cells which enhances the heterogeneity of the biofilm conferring resistance and tolerance by employing a range of metabolically active cells. Additionally, there are secreted factors such as eDNA or β-lactamases to quench antimicrobials in the EPS. Created using Powerpoint

1.2.4 Biofilm Recalcitrance to Antimicrobials: EPS

The EPS is commonly implicated with reference to antimicrobial tolerance or resistance in biofilms, within the hydrated matrix there are gradients (oxygen, nutritional and density) that enable the secretion of EPS based factors associated with the impedence and inactivation of antimicrobials to then allow contact and subsequent inactivation of organisms within the biofilm (Donlan and Costerton, 2002). The EPS matrix slows the antimicrobial activity through several different processes; reduced diffusion, structural protection from physical removal, chemical reactions, and interference with antimicrobials. In *P. aeruginosa* biofilms, it has been observed that secretion of alginate (which has a net negative charge) can bind the positively charged aminoglycosides inactivating them, equally the secretion of β-lactamases into the EPS can quench antimicrobials before reaching their target cells (McManus, 1997; Neu, 1992; Bryan, 1988; Shafran, 1990; Cao *et al.*, 2016). Not only does EPS allow retardation of antimicrobial diffusion, it also enables avoidance of immediate immune action from the host through the covering of microbial cells (Cerca, Oliveira and Azeredo, 2007). Bacterial cells within the biofilm can utilise host derived matrices, such as fibrin and present these factors to allow masking of bacterial cells within the biofilm, mimicking the host and evading immune detection (Jesaitis *et al.*, 2003; Walters *et al.*, 2003; Jefferson, Goldmann and Pier, 2005; Leid *et al.*, 2005; Cerca, Oliveira and Azeredo, 2007). Once established the EPS provides stability through its structure and this can provide resistance to mechanical removal (Karygianni *et al*, 2020). The EPS structure not only prevents attack from host immune cells due to the size of the biofilm, it is additionally unable to be broken apart for neutrophil ingestion as there is a maximum ~1 kPA stress exertion that neutrophils can elicit (Kovach *et al.*, 2017).

Another factor implicated in the recalcitrance to antimicrobials is the spatial heterogeneity observed within the EPS. EPS can sequester and trap many different substances and therefore influence the dispersion of said components across the biofilm (Karygianni *et al*, 2020). This creates gradients of nutrients, pH and oxygen, this can be seen in figure 2. These gradients can be utilised and modified by the resident microbial populations, for example in *B subtilis* biofilms, it was observed that the modulation of matrix protein BslA included redox-dependent bifunctionality. BslA presented as a dimer in the oxygen rich areas of the biofilm, yet within the anoxic areas there was increased nutrient uptake as the dimerisation of BslA was inhibited by the low oxygen concentrations (Karygianni *et al,* 2020)

Extracellular DNA released from bacterial cells form an integral part of the matrix, with some (such as *P. aeruginosa*) forming grid-like networks throughout, enhancing the structural stability of the biofilm (Donlan and Costerton, 2002). It was originally thought that the extracellular DNA was only residual

from lysed cells, however bacterial cells within the biofilm can release fragments of DNA to allow for enhanced and passive horizontal gene transfer (HGT) (Donlan, 2001). Many different types of mobile genetic elements are used in HGT for acquisition of antimicrobial resistance (plasmids, transposons, and integrons), and within a biofilm these are horizontally transferred by many orders of magnitude that when compared with planktonic cells (Sultan *et al.*, 2018; Donlan and Costerton, 2002). The rapid acquisition of resistance genes could explain the use of some traditional planktonic cell mechanisms of multidrug resistance biofilms display against antimicrobials such as; β-lactams, aminoglycosides and fluoroquinolones (Høiby *et al.*, 2010) For example, resistance to β-lactams can be acquired through plasmid-mediated AmpC enzymes, where the transmissible plasmids initially encoded in the Enterobacteriaceae have emerged in *Escherichia coli, Klebsiella pneumoniae* and *Proteus mirabilis*, whereas plasmid-mediated resistance is also seen with qepA, that encodes an efflux pump that is able to expel fluoroquinolones (Jacoby, 2009; Sultan *et al.*, 2018).

Within the biofilm the physiological conditions support a higher bacterial mutation frequency than when compared with planktonic cells (Savage, Chopra and O'Neill, 2013). Increased mutation frequencies can allow acquisition of new resistance mechanisms from strain to strain, if resistant bacteria are present within the biofilm. The biofilm is a privileged environment with respect to horizontal gene transfer (HGT), with conjugation between cells up to 700 times more efficient in the biofilm compared with standard planktonic cells (Bowler, 2018). *Staphylococcus aureus* biofilms have exhibited increased mutability, allowing a drastic increase in the emergence of resistant strains. The increased rate of plasmid transfer events through mobilisation of the genome and conjugation have facilitated an accelerated spread of antimicrobial resistance genes in a heritable way (Savage, Chopra and O'Neill, 2013).

1.2.7 Growth in the Biofilm

Although antimicrobial resistance and tolerance in biofilms is often attributed to EPS there are also many other factors associated within the bacterial biofilm that are linked to antimicrobial tolerance (Singh *et al.*, 2009). Slow growth rate, spatial heterogeneity and biofilm-specific physiology are all factors that influence resistance and tolerance (Singh *et al.*, 2009). The biofilm-specific physiology of cells can be referred to as persisters and small colony variants (SCV). Persisters are a specific phenotype observed within bacterial life that can survive lethal concentrations of antimicrobials without the active utilisation of specific resistance mechanisms (Lewis, 2007). Persisters will often pre-exist within a bacterial population and can be activated through overexpression of certain regulatory genes, for example, chromosomal toxin-antitoxin modules can often shutdown cellular functions in times of environmental stress initiating cell dormancy to conserve the bacterial population (Keren *et al.*, 2004).

This cell dormancy enables tolerance of antimicrobials that may target metabolic activity such as, β-lactams that target bacterial growth (Percival *et al.*, 2011). Persisters allow temporary resistance to drugs and this was first named by Bigger et. al, who estimated that persistent cells make up around 0.1 to 10% of the biofilm population (Percival *et al.*, 2011; Bigger, 1944).

The environmental pressures that may activate persisting cells are likely to do with growth conditions, high cell density, limited nutrients, and limited oxygen (Waters *et al.*, 2016). As a response, bacteria will enter a low energy state where metabolic processes are largely stopped, resulting in stationary or persistent phase cells.

Small colony variants are different from normal bacterial colonies with respect to; size, reduced growth rate, and pigmentation (Proctor *et al.*, 2014). Defects in the electron transport chain that can be brought on due to environmental stress such as antimicrobial treatment, with some studies indicating that higher concentrations of antimicrobials yielded a higher amount of SCVs (Proctor *et al.*, 2014). *S. aureus* appears to produce a proportion of SCVs without prior exposure to antimicrobial treatment, and these cells appear physiologically similar to *S. aureus* biofilm cells (Batchelor *et al.*, 1997). This recalcitrant population of persisters and SCVs can then be used to reseed and re-infect clinical sites following antimicrobial treatment (Percival *et al.*, 2011).

Persistence within biofilms and various other static bacterial cells often associated with environmental pressures. These environmental pressures typically involve gradients within the biofilm. For example, gradients are formed with high concentrations of available nutrients and oxygen towards the outside of the biofilm as this is at the interface with the environment allowing sequestration and uptake of nutrients more readily. In the centre of the biofilm there is a lower concentration of nutrients and oxygen where there is a high cell density and therefore a higher waste product and a lower amount of nutrients. This endogenous oxidative stress and nutrient stress seen within the centre of the biofilm allows for genetic adaptation and evolutionary changes of bacteria. The various oxygen pressures induce conservation of energy from the cells and allow subsets to develop within the population. Waters *et al*., (2016) reported low energy levels are one of the major determinants of antimicrobial tolerance within biofilms. Resilience to antimicrobial treatments is impacted by the environmental gradients within the biofilm biomass as subpopulations of bacteria (persisters, small colony variants, static cells, anaerobes, facultative anaerobes, and aerobes) will differentially select gene expression to suit the local micro environment enabling either resistance mechanisms or cessation of metabolic processes (Lewis, 2007; Percival *et al.*, 2011)

1.3 *Staphylococcus aureus*

1.3.1 *Staphylococcus aureus* a notable human pathogen

Staphylococcus aureus is a notable nosocomial pathogen commonly implicated in an impressive array of serious infections such as endocarditis, pneumonias, septicaemia and wound infections (James *et al.*, 2008; Mashruwala, Guchte and Boyd, 2017). Estimated to colonise anywhere between 20-50% of the population as a part of the microbiota with colonisation seen in the nasopharynx, throat and skin as a ubiquitous commensal acting as an innocuous member of the microbiota (Mashruwala, Guchte and Boyd, 2017). It is a particularly virulent pathogen with regards to resistance, becoming increasingly problematic to treat clinically due to; acquiring methicillin, vancomycin and, most recently linezolid and daptomycin resistance (Lovering, Safadi and Strynadka, 2012; Mashruwala, Guchte and Boyd, 2017). *S. aureus* colonisation is often associated with a much higher risk of development of blood infection in the hospitalised immunocompromised population (Krismer *et al.*, 2017). These increased risks are often mitigated by the routine screening for methicillin-resistant and sensitive strains when in hospital.

1.3.2 Virulence

Commensal *S. aureus* can become more pathogenic following genetic expression to enhance the virulence factors, and it is through that these changes may be initiated by gaining access to host tissues (such as through a breach in the skin) (Jenkins *et al.* 2015). Therefore, as *S aureus* has many virulence factors, there are different genetic systems to regulate virulence factor expression.

The best studied of all *S. aureus* regulatory systems is the accessory gene regulator (*agr)*, through which the master virulence regulator quorum sensing systems are encoded (Jenul and Horswill, 2018). These systems enable *S. aureus* to sense surrounding bacteria and change the expression of virulence factors as a result (Novick *et al.*, 1993). There are a multitude of other virulence factors that are regulated by *agr*, inducing an increase in the production of virulence factors including; hemolysins, toxic-shock syndrome toxin-1 and proteases. However, a*gr* can also suppress the production of other factors, such as protein A and coagulase therefore causing a switch between adhesive and toxigenic growth phases (Sakoulas *et al.*, 2003; Novick *et al.*, 1993). The other systems regulating expression virulence factor expression are *saeRS*, *sarA* and *rot*. Interestingly, both *rot* and *sarA* have been demonstrated to play a role in the suppression of proteases that have been implicated in the degradation of biofilms and virulence factors (Mootz *et al.*, 2015). The importance of *rot* across multiple strains of *S. aureus* was shown, with loss of *rot* leading to a drop in biofilm formation therefore, showing the key regulator status of *rot* in *S aureus* biofilm formation (Mootz *et al.*, 2015).

Besides immune attack and immune cell mediated killing, there are also protein-mediated ways of killing *S. aureus*. If taken into consideration the fact that 20% of the human population are colonised by *S. aureus* and yet are not showing signs of infection, it indicates that the innate mechanisms of mediation of opportunistic pathogens are successful at keeping *S. aureus* at bay (Mashruwala, Guchte and Boyd, 2017). However, *S. aureus* is a potent pathogen, and still maintains the ability to upregulate its virulence and invade and overcome these defences (Jenkins *et al.* 2015). Biofilms can resist phagocytosis from neutrophils and macrophage due to their size, and can also utilise host proteins, such as those previously discussed to shield itself from immune detection.

One such mechanism of immune defence is the ability of human skin to produce unsaturated fatty acids that have antimicrobial properties (Takigawa *et al.* 2005). *S. aureus* has been shown to resist the antimicrobial effect of these fatty acids by expressing a surface protein called iron-regulated surface determinant A. (Takigawa *et al.*, 2005; Clarke *et al.*, 2007). Increased expression of this protein decreases bacterial hydrophobicity, rendering the cells resistant to the skin produced bactericidal fatty acids (Clarke *et al.* 2007). It has also been shown that *S. aureus* can utilise and incorporate these skin fatty acids into its cell wall lipoproteins (Nguyen *et al.*, 2011). After incorporation, the fatty acids act as an immunostimulatory component and increase the production of cytokines, resulting in increased inflammation and a more potent immune response. This shows that there are a variety of mechanisms relevant to skin infections that *S aureus* can employ to evade the innate defence mechanisms to allow for successful infection.

1.3.3 Biofilm formation as a virulence factor

S aureus attaches to biotic and abiotic surfaces in different ways, partially due to the environmental cues it encounters. When encountering biotic surfaces, the host matrices (collagen, fibrinogen, fibronectin) activate surface binding mediated by cell wall anchored proteins (CWA) (Schilcher and Horswill, 2020). There are over 20 CWA proteins that have been demonstrated in *S aureus*, and these are characterised by the presence of a sorting signal at the c-terminus responsible for coupling the protein covalently to the peptidoglycan (Foster *et al.*, 2014). There are 5 distinct groups of CWA proteins; microbial surface components recognising adhesive matrix molecule (MSCRAMMs), NEAr iron transporter motif protein family three-helical bundle motif protein A, G5-E repeat family and the legume-lectin cadherin-link anchorage domains (Foster *et al.*, 2014; Foster, 2019; Gross *et al.*, 2001; Holland, Conlon and O'Gara, 2011). The most well characterised of these groups is the MSCRAMMs, these are CWA proteins that bind host extracellular matrices using either a dock lock latch mechanism or collagen hug (Foster, 2019).

The dock lock latch mechanism (Figure 4) has been clearly demonstrated for *S aureus* and clumping factor A (ClfA) and the A domains of fibronectin binding proteins A and B (Rohde *et al.*, 2007; Foster *et al.*, 2014; Foster, 2019). These bind to the γ-chain peptide of fibrinogen using an unfolded peptide ligand within the open form of the molecule that sits within a hydrophobic trench that is located between the N2 and N3 subdomains (Foster, 2019). Binding initiates a conformational change that enables the unfolded extension of the N3 subdomain to lay over the peptide and lock it into place. Finally, the latch is created by the formation of an additional β-strand in the β-sheets of the N2 subdomain by a process called β-strand complementation (Deivanayagam *et al.*, 2002; Foster, 2019; Ganesh *et al.*, 2011; Ponnuraj *et al.*, 2003).

The collagen hug (Figure 3) facilitated binding to collagen using a variation of the dock lock latch mechanism. Here, the N1 and N2 subdomain structures in the collagen hug act similarly to the N2 and N3 domains in the dock lock latch mechanism. The target ligand docks into a shallow trench in the N2 domain initiating a conformational change for the N1 domain to wrap around the collagen molecule forming the 'hug' followed by latching by β-strand complementation stabilising the complex. (Zong *et al.*, 2005).

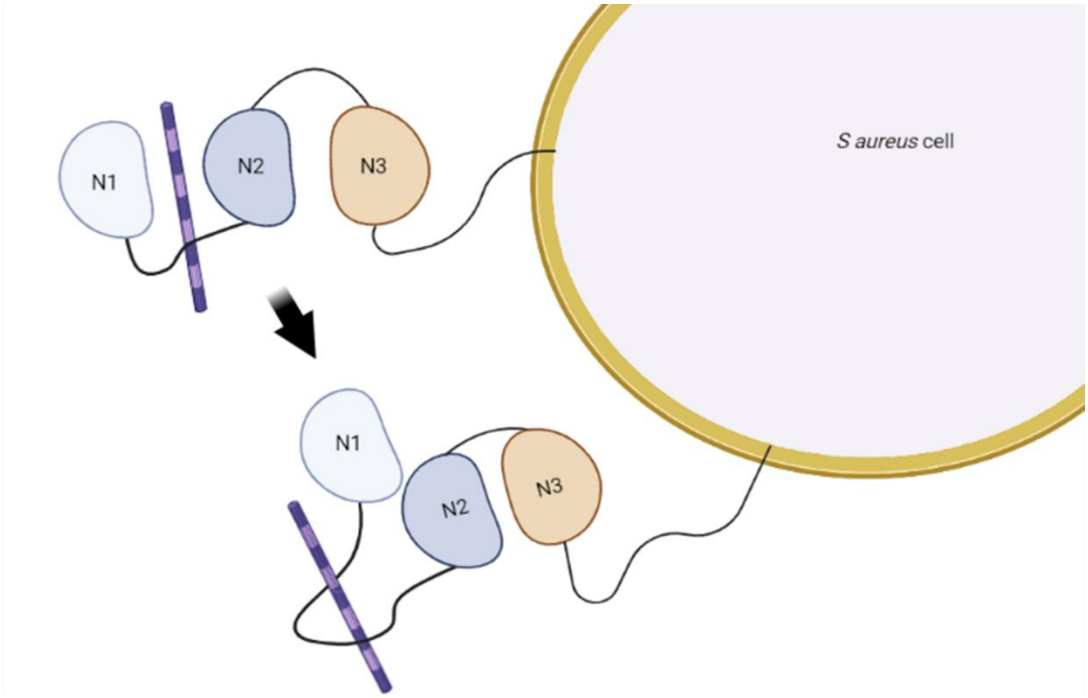


Figure 3 Schematic of collagen hug of *S aureus* attachment to host surfaces. The collagen hug binds collagen into the N1 and N2 subdomains where the N1 subdomain wraps around the collagen 'hugging' it (Created using BioRender)

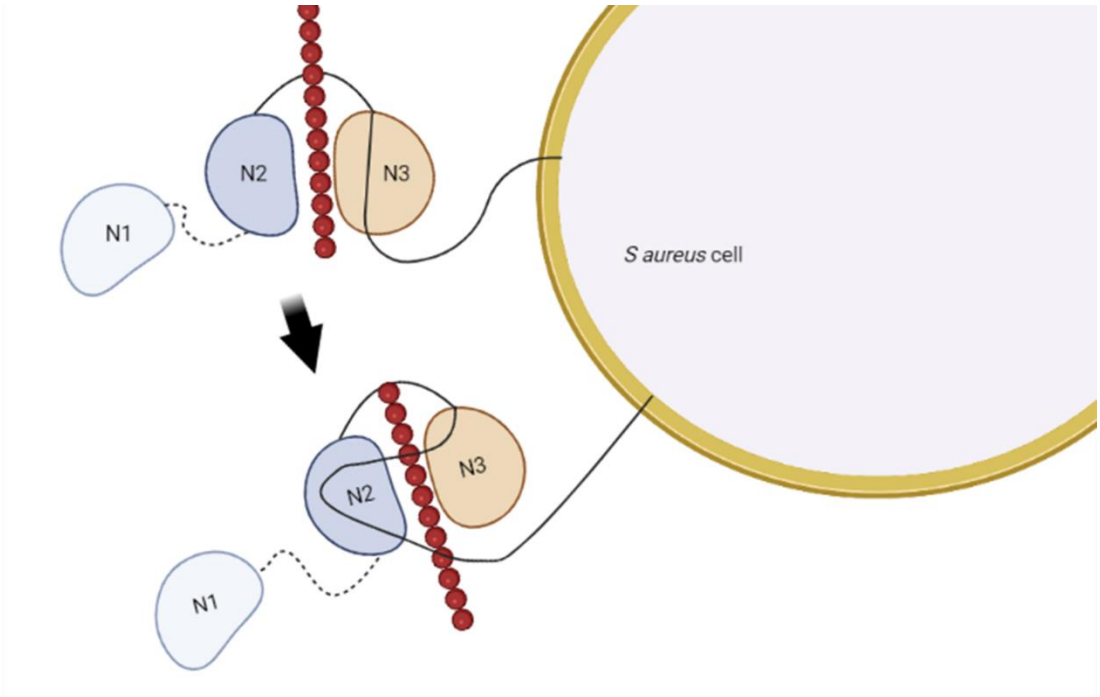


Figure 4 Schematic of MSCRAMM Dock, Lock, and Latch method of *S aureus* attachment to host surfaces. Dock Lock Latch mechanism utilises the binding of γ-chain peptide of fibrinogen into a trench between the N2 and N3 subdomains which subsequently initiates the conformational change allowing the locking of the N3 subdomain over fibrinogen. (Created using BioRender)

1.3.4 *S aureus* biofilms in chronic wounds

Skin infections caused by *S aureus* are more likely to be caused by the patient's own endogenous strain via long-term or environmental contamination (Fritz *et al.*, 2012). This means that recurrent and chronic *S aureus* skin infections are a problem due to the colonisation and subsequent reinfection however recurrent chronic infections have been attributed to the formation of persister cells and small colony variants. These phenotypes can also be triggered by exposure to antibiotics (Tuchscherr, Löffler and Proctor, 2020). Persister cells therefore remain following antimicrobial treatment and can act as a reservoir for resistance and further infection once treatment is completed.

S. aureus is a commonly seen isolate from chronic wounds, Percival (2010) identified colonisation of *S. aureus* in 88% of presenting chronic leg ulcers, with Rahim (2017) isolating *S. aureus* in 37% of chronic wounds analysed with *Pseudomonas aeruginosa* being the second most commonly isolated at 17% of isolates. Interestingly it seems that *S. aureus* colonises chronic wounds more readily, with around 48% of isolates from chronic wounds, it is likely that the isolates are of the biofilm forming phenotype, Neopane *et al*. (2018) analysed the *in vitro* biofilm formation of clinical isolates of wounds and found over 60% of isolates analysed formed biofilms *in vitro*, thus underlining the prevalence of the biofilm forming phenotype in wound research. James *et al* (2008) found that when studying bacterial isolates from chronic wounds that Staphylococcus *spp*. was the predominant at 65% of the wound bioburden. It has also been reported by Bessa *et al.* (2015) that through the systematic analysis of 312 wounds from 213 patients that the most common bacterial species was *S. aureus* (37%). Thus underlining the importance of *S. aureus* as a formidable chronic wound pathogen and significant barrier to normal wound healing. *S. aureus* is a Gram positive bacterium that is a commensal organism and opportunistic pathogen with an average population-wide carriage rate of 20-32% (Shopsin *et al.*, 2000; Wertheim *et al.*, 2005; Kinkel *et al.*, 2013). Often implicated in a wide array of infections, such as in soft tissue infections, skin infections, endocarditis and chronic medical implant infections, which comes as no surprise when considering the previously mentioned population wide carriage rate (Mootz *et al.*, 2015; Neopane *et al.*, 2018).

1.3.5 Molecular markers and mechanisms of wound obstruction by biofilms

The formation of dynamic microenvironments within biofilms is down to an intricate network of circuits and sporadic genetic change for example, if the global regulators *sarA* and sigma factor B are inactivated this restricts biofilm formation and development (Mootz *et al.*, 2015). In contrast, when the regulator *agr* is either inactivated or minimally active, the opposite has been observed i.e biofilms formed much

more readily and had a higher propensity for biofilm maturation when the *agr* system was inactivated (Anderson *et al.*, 2006; Boles and A Horswill, 2008). *Agr* system reactivation is essential for the virulence and dissemination of biofilm throughout the body, as Boles and Horswill (2008) indicated that *agr* system reactivation allowed dispersal of cells. It is not only gene expression and regulation of gene loci that control the biofilm phenotype. Many environmental factors can influence the phenotype that is expressed when a biofilm is forming and maturing. Regarding environmental conditions within chronic wounds that can aid or hinder biofilm development, Cramton *et al*. (2001) investigated biofilm formation under anaerobic conditions and observed induction of polysaccharide intercellular adhesin (PIA). PIA is one of the main components of exopolysaccharide (EPS) otherwise known as the matrix that surrounds and protects the biofilm (Mack *et al.*, 1996; Singh *et al.*, 2010). As PIA has been implicated in the hypoxic stress response it would be pertinent to further research the pathways that induce this overexpression to investigate the differences a hypoxic wound environment impacts biofilm formation and eventual wound resolution.

The role and utilisation of oxygen in a wound healing environment is a much debated subject, with some reporting that high amounts of oxygen administered as a preventative method (during surgery) reduces the amount of infection prevalent in a patient population (Belda *et al.*, 2006). Healing tissue evidently requires more oxygen due to the increased energy demand as a direct result of the tissue restructuring. However, immunological factors involved in wound healing cause hypoxia within the tissues to induce further healing pathways, such as keratinocyte motility and activation of reactive oxygen species (ROS) (Kimmel, Grant and Ditata, 2016). It is not fully understood how this initially beneficial process of hypoxia to activate pathways that aid wound healing then interacts with bacterial biofilms and becomes uncontrollable and detrimental for the healing process.

1.4 Skin wounds- a worldwide problem

1.4.1 Humanistic and Socio Economic cost

Chronic wounds are a serious and life-threatening condition that has widely been under-researched and often under-documented due to the cross-disciplinary nature of the disease (Nussbaum *et al.*, 2018). Chronic wounds are often classified as a comorbidity to other conditions yet have significant humanistic and economic burdens at personal and societal levels, and therefore it is understandable that these non-healing wounds are referred to as a silent epidemic (Graves, Phillips and Harding, 2022). In particular, chronic wounds are often a symptom of patients that are older, nonambulatory and unable to provide care for themselves. The wounds themselves are often as a result of an underlying

condition such as diabetes, renal impairment, autoimmune disorders or paralysis (Nussbaum *et al*. 2018). In the US, chronic nonhealing wounds impact 8.2 million Medicare recipients and in the UK, account for almost 75,000 annual deaths (Sen, 2009, 2021; Han and Ceilley, 2017a; Rahim *et al.*, 2017). A well known risk factor for chronic wounds is that of increased age. With the population of the aged in the US predicted to outnumber children by 2030, the potential for an increasing burden of chronic wound care on our healthcare systems is almost a certainty (US Census Bureau, 2017). When considering that currently 3% of over 65s are suffering with open wounds, the significance and impact of wounds is massive (Sen, 2021). Not only in terms of numbers of patients, but the cost of treatment on health care systems is astronomical.

In the US, wound care is estimated to cost up to $96.8 billion (Nussbaum *et al*, 2018). Whereas in the UK, average NHS cost of wound treatments was estimated to be around £7,345 per wound, however the estimation of cost per non-healing infected surgical wounds increases to £11,200 per wound, yet in the US cost per hospital stay was reported to be in a range of $12,851 and $16,267 (Guest, Fuller and Vowden, 2018; Graves, Phillips and Harding, 2022). The financial impact that is currently reported is huge, accounting for 1-3% of total healthcare costs in developed countries with all of these associated costs increasing (Sen, 2009; Olsson *et al.*, 2018).

Additionally, chronic wounds cost a significant amount of money, and they are also extraordinarily burdensome on patients and their lives. Looking into the human costs in terms of morbidity, mortality and quality of life, the outlook is bleak for those with chronic non-healing wounds. Although there is an increasing amount of novel interventions and research into chronic wounds the morbidity associated with them has not improved, Diabetic foot ulcers (DFUs) have a comparable 5-year mortality rate to cancer (30.5% to 31% respectively) which has not improved since 2007 (Sen 2021, Armstrong *et al*, 2020). Then, when taking into account the cost of patients' daily lives, with pain, odour and purulent discharge there is an impact on the quality of sleep, reduction in food intake and dependency on nursing staff for dressing changes. Chronic pain and fatigue can affect the ability for patients to eat a regular diet, and lack of nutrition may cause worsening in the chronic wounds, creating a vicious cycle continuously perpetuating chronic disease (Renner and Erfurt-Berge, 2017; Erfurt-Berge and Renner, 2020). These factors can cause withdrawal and isolation, impacting patients' social and professional lives. There are a high proportion of patients with chronic wounds reporting depression and anxiety as a result of their condition, (Renner and Erfurt-Berge, 2017; Olsson *et al.*, 2018). Treatments for chronic wound infections are often aggressive and detrimental to the patients' quality of life; amputations, debridement of tissues, long stays in hospital and multiple wound dressing changes all take a toll on the patient (Finlayson *et al.*, 2017; Sen, 2009).

1.4.2 The skin: when things go right

The skin is a dynamic organ that has evolved and developed to become a highly adaptive defence from external threats. It protects the vulnerable understructures from chemical, physical, thermal, and photic damage in addition to the regulation of thermostability and prevention of desiccation to achieve homeostasis (Wong *et al.*, 2016; Schilrreff and Alexiev, 2022). This impressive defence even extends to the immune system and barriers to pathogenic invasion, with the maintenance of beneficial microbiota whilst successfully fending off opportunistic pathogens. The skin consists of three main layers; the epidermis, dermis and subcutaneous tissues (Wong *et al*. 2016). The epidermis is the barrier to external penetration and invasion; it also contains the sebaceous glands, sweat glands and hair follicles (Maheswary, Nurul and Fauzi, 2021). The dermis holds the vasculature that supplies the entire skin along with connective tissues which give the skin its elastic characteristics. Finally, the subcutaneous layer with its high fat content supplies thermal and mechanical protection to the underlying tissues and structure (Schilrreff and Alexiev, 2022; Wong *et al.*, 2016). These layers and the underlying immunological and cellular processes create a well-balanced and intricate environment, however what happens when that delicate balance is disrupted?

During the early stages of a wound there is immediate activation of early inflammatory responses which results in the infiltration of macrophage and other immune cells (Aitcheson *et al.*, 2021). Blood vessels contract and a blood clot begins to form with the recruitment of erythrocytes, platelets, and formation of eschar (Figure 5). The cascade of immunological recruitment to the wound bed induces the second stage of acute wound healing i.e. inflammation (Aitcheson *et al,* 2021). Inflammation is the primary defence against pathogenic wound invasion, and is induced through signals such as damage associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs) (Schilrreff and Alexiev, 2022). These molecular patterns activate resident immune cells such as Mast cells that start a cascade of inflammatory pathways that is complex and modulated by many intrinsic and extrinsic factors (Figure 5). The cascade recruits macrophages, neutrophils, and monocytes to the site of injury and they all play different interwoven roles in the finely balanced process of wound healing. The balance in inflammatory response is a fine one, with excessive inflammation causing damage to surrounding tissues and delaying healing but insufficient immune response can also hinder repair by inadequately controlling infection (Schilrreff and Alexiev, 2022; Gonzalez *et al.*, 2016). The proliferative stage of healing is often characterised by activation of keratinocytes, fibroblasts, macrophages and endothelial cells to begin the process of wound closure and angiogenesis (Figure 5). Migration of keratinocytes that have been activated as a response to the mechanical tension in the wound then begin the reformation of the epidermal layer and subsequent re-epithelialization of the wound (Gonzalez *et al*, 2015; Sen,

2008). This highly proliferating tissue also stimulates new blood vessels to be created to meet these increased metabolic demands. Angiogenesis is triggered by hypoxia as microvascular endothelial cells proliferate and migrate into the wound bed following the induction of hypoxia-inducible factors (Gonzalez *et al*, 2015; Sen, 2008; Aitcheson *et al*, 2021). Finally remodelling takes place, with the replacement of collagen type III with collagen type I to increase the strength of the newly forming scar along with the formation of elastin and myofibroblast contraction to draw the matrix together during contracture (Aitcheson *et al,* 2021). This completes the wound healing process and the remaining immune cells dissipate or apoptose leaving only a scar.

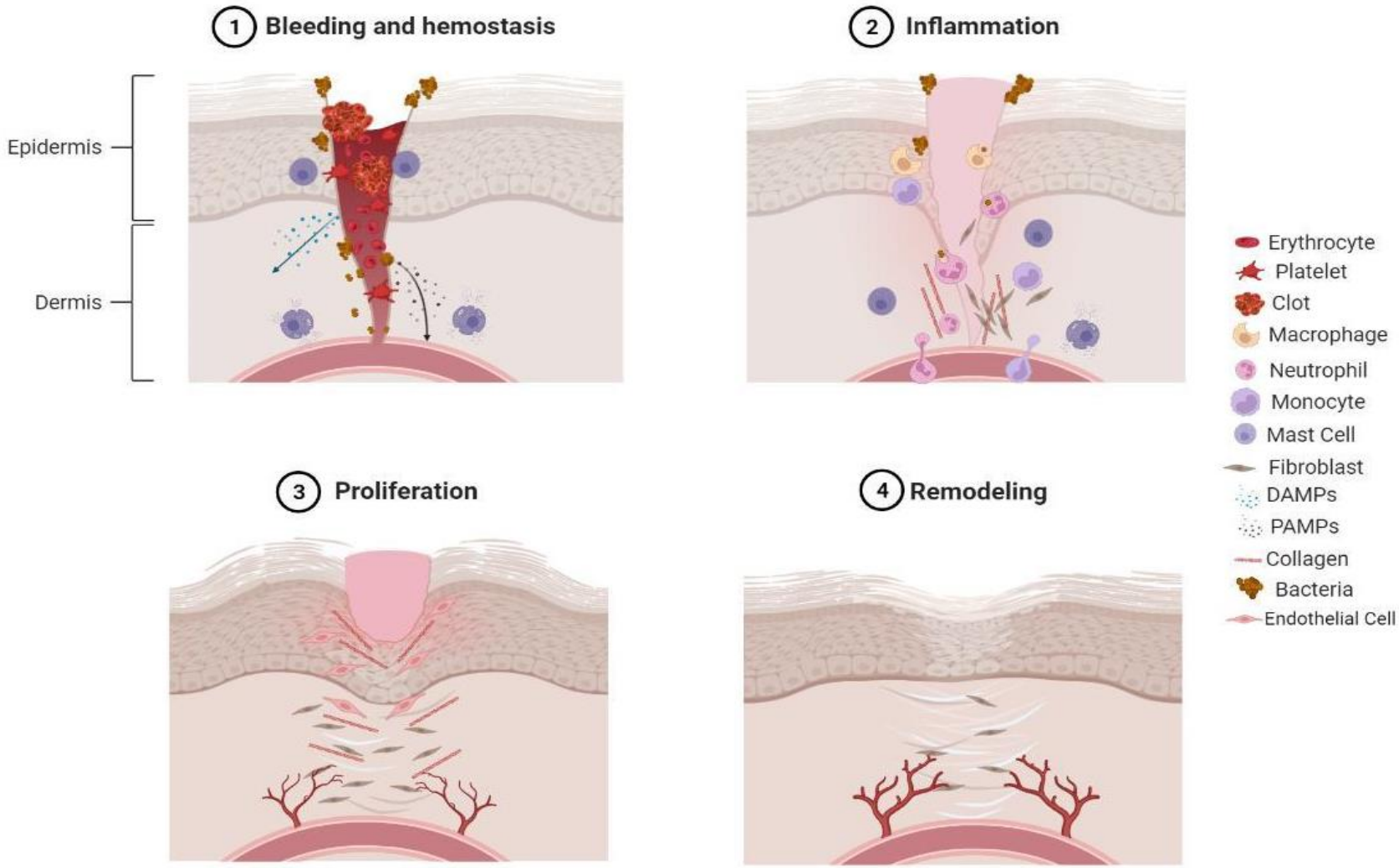


Figure 5 Schematic of standard wound healing process. 1.Immediate activation of immune cell recruitment and inflammation is initiated using PAMPs and DAMPs, along with haemostasis and clotting. 2. Macrophages, neutrophils, and monocytes are recruited to the wound site, and cause inflammation and progress wound healing. 3. Keratinocytes, fibroblasts, and endothelial cells are then recruited and activated to initiate angiogenesis and re-epithelialisation. 4. Changes in the collagen types happen to increase the strength of the scar along with contracture of the wound as the final steps. (Created using BioRender)

1.4.3 The process of wound healing: where does it go wrong?

Wound healing is a complex process with standard healing going through a tightly controlled sequence that regulates contamination, infection, inflammation, regeneration, angiogenesis and skin resurfacing (Ganapathy *et al.*, 2012; Hurlow *et al.*, 2015; Rahim *et al.*, 2017). There are many different theories and classifications of the wound healing process. Some report only 3 stages of repair: inflammation, proliferation, and remodelling whereas, others claim it is a close control of many regenerative and degradative pathways including chemotaxis for immune cell recruitment for the initial 'clean up' of the wound followed by subsequent stimulation of epithelial cells and fibroblast mitogenesis inducing and prepares for angiogenesis (Han and Ceilley, 2017a; Wu, Cheng and Cheng, 2018; Guest, Fuller and Vowden, 2018; Kalani *et al.*, 1999; Rahim *et al.*, 2017; Sen, 2021). Many of these steps depend on adequate oxygen perfusion and availability, as it is essential for the metabolism of contaminants, matrix synthesis, immune cell migration and proliferation, and angiogenesis (Shah, 2011; Sano, Ichioka and Sekiya, 2012).

Concentrations of oxygen in healthy tissue is reported to be around 2-19% oxygen or at least a 20mmHg oxygen tension for normoxic tissues and in compromised and non-healing wounds (infected and/or necrotic) it is estimated that the oxygen concentrations drop into hypoxic ranges (1% O_2 or a 5mmHg tension) and sometimes lower into anoxic conditions (<1% O_2) (Carreau *et al.*, 2011; Han and Ceilley, 2017a). Low oxygen tension in a wound is associated with higher amounts of necrotic debris although this is not always associated with halting of the wound healing process. A wound requires different amounts of oxygen throughout the healing process, it often cycles through normoxic and hypoxic oxygen tensions through all stages of wound healing (Sano, Ichioka and Sekiya, 2012). Initially, during the inflammation stage the vast recruitment of immune cells to the site of the wound uses large amounts of ATP causing an oxygen deficit and therefore requires an oxygen recovery period while the wound microenvironment stabilises (Kimmel, Grant and Ditata, 2016). During this stabilisation period, the hypoxic event will have initiated a cascade by which cytokines induced by the low oxygen recruit platelets and endothelial cells, along with stimulating growth factors to be secreted (Kimmel, Grant and Ditata, 2016). It has been reported by Sano *et al* (2017) that tissue hypoxia stimulates angiogenesis and angiogenic cytokines, this is done through activation of hypoxia induced factor (HIF) which can bind gene promoter regions upregulating glucose metabolism and control of vessel tone and subsequent angiogenesis. Although angiogenesis under hypoxia has been reported to aid healing, it may also allow the diffusion of infected material into the bloodstream and wider tissues. In cases where there is spreading necrotic debris, the standard healing process can be hindered. When subsequently combined

with the low oxygen in the wound bed the recruitment of immunological cells to clear this debris can be hindered and instead act as a nutrient rich environment for opportunistic pathogenic bacteria (Seth *et al.*, 2012b). Once established the bacteria will proceed to colonise the wound area and metabolise the nutrient rich necrotic debris, this process then releases microbial waste into the wound bed to stimulate strong inflammatory responses and halt the wound healing process in the inflammatory stage (Gompelman, van Asten and Peters, 2016a). The links between the tissue healing process and infection by a biofilm is not yet fully understood, it is thought that the opportunistic pathogens within the commensal microbiota reach the threshold of critical colonisation around 10^5 colony forming units which induces a wider infection (Han and Ceilley, 2017). This critical colonisation of the wound can lead to slowed progress to resolution and the initiation of a chronic inflammatory response, resulting in the establishment of a chronic wound. The hallmarks of this are high degradation of surrounding tissues, high levels of inflammation, increased anti-inflammatory cytokine production, and impaired proliferation and remodelling of the wound (Maheswary, Nurul and Fauzi, 2021; Ellis, Lin and Tartar, 2018; Larouche *et al.*, 2018; Smith and Sharp, 2019).

1.4.4 What is a chronic wound?

Chronic wounds can be seen as a loss of skin integrity, as absent or delayed healing over an 8-week period eventually requiring medical or surgical intervention for the resolution of the wound (Sen, 2009; Kimmel, Grant and Ditata, 2016; Han and Ceilley, 2017a; Parsek and Singh, 2003; Renner and Erfurt-Berge, 2017). Although due to a wide variety of wounds, there can be large discrepancies within the literature as to what the cut-off healing time periods are, with some specifying 4 weeks and others over 12 weeks (Graves, Phillips and Harding, 2022).

Chronic wounds are rarely found in healthy patients, they are often seen in those with underlying diseases such as diabetes (diabetic foot ulcers), obesity, vascular disease, surgical site infections, pressure ulcers and traumatic ulcers (Sen *et al.*, 2009; Rahim *et al.*, 2017) It is commonly reported that chronic wounds are non-healing due to the predominantly elderly patient population. This is likely as a result of the development of immunosenescence (the impedance of the immune system as ageing increases) as a person ages (Rahim *et al.*, 2017; Ventura *et al.*, 2017). There are many disease processes that cascade to result in impaired healing of wounds, these barriers range from poor perfusion to infection to poor immune response. Prolonged low oxygen tension in the wound and surrounding tissues has been implicated with increases of necrotic debris and compromised immune response. Equally co-morbidities associated with diseases such as diabetes have shown increased immune dysfunction that directly impacts the ability of skin wounds to heal (Aitcheson *et al*, 2021). These

implications aid the establishment of pathogenic bacteria within the wound, by creating an environment with high levels of nutrients and less disruption from the host immune response. This further impacts the capacity of the wound to heal (Han and Ceilley, 2017). Contamination and infection of a wound is highly associated with impede wound healing, and chronic infected wounds have little response from antimicrobial treatment and often are not resolved by the immune response alone (Gompelman, van Asten and Peters, 2016b; Han and Ceilley, 2017a).

1.4.5 The role of bacteria in wound healing

The normal pH of healthy skin can prevent the colonisation of pathogenic bacteria, as it is acidic at a pH of between 4 and 5 (Maheswary, Nurul and Fauzi, 2021; Choi, 2018; Lee and Jamil, 2020). This is important as most pathogenic bacteria associated with the skin have been shown to prefer an alkaline environment (Maheswary, Nurul and Fauzi, 2021; Blaak and Staib, 2018). The acidic environment also benefits commensal organisms and promotes their establishment (Blaak and Staib, 2018; Maheswary, Nurul and Fauzi, 2021)). Under normal wounding and healing processes the wound environment becomes increasingly acidic due to the accumulation of lactic acid and oxygen, this is also essential for the immune response seen in wound healing.

A critical factor in the delayed healing of wounds are bacterial presence and biofilm development. The microbiota is closely linked with skin health and disease, and wounds provide a rich opportunity for enhanced bacterial colonisation, particularly in the underlying tissues (Tomic-Canic *et al.*, 2020; Pastar *et al.*, 2013). The normal microbiota or normal flora can be broadly defined as the bacterial population that colonises the human skin without causing harm to the host (Maheswary, Nurul and Fauzi, 2021; Chen *et al.*, 2021). The commensal microbiota are thought to interact with skin cells to enhance normal wound healing and can help to modulate the innate immune response (Tomic-Canic *et al.*, 2020; Zeeuwen *et al.*, 2012; Harrison *et al.*, 2019; Lai *et al.*, 2009). This is mediated through various processes including substance release from the normal flora to maintain the standard skin barrier, protecting the skin from harmful microbes using phenol soluble modulins, bacteriocins and even secretome to reduce bacterial induced inflammation (Szabó *et al.*, 2017; Maheswary, Nurul and Fauzi, 2021). Despite these many beneficial processes pathogenic bacteria can establish and cause significant problems when the right opportunity arises.

These pathogenic bacteria have the propensity to colonise the nutrient rich wound bed and begin to multiply (Versey *et al.*, 2021). At the initial stages of colonisation, the innate immune response is often not triggered as no signs of clinical infection are yet present (Versey *et al*, 2021). It has been discussed

that the critical level of colonisation is 10^5 colony-forming units per gram of tissue therefore increasing the likelihood of infection (Alves *et al.*, 2021). However, when an infection establishes it has been shown that the wound environment becomes more alkaline which benefits pathogenic bacteria further supporting their establishment and invasion of the tissue (Bennison *et al.*, 2017; Kruse *et al.*, 2017).

Once bacteria have established an infection in a wound, the ability of the patient to clear the infection and heal decreases as the biofilm matures (Schierle *et al.*, 2009; Gompelman, van Asten and Peters, 2016b). Additionally, the bioburden of the wound increases over time, due to the production of wound exudate, necrotic debris and biofilm maturation causing impaired ability for re-epithelization (Schierle *et al.*, 2009; Gompelman, van Asten and Peters, 2016). This interaction between biofilm and healing processes has been shown by Pastar *et al* in 2013, that demonstrated polymicrobial infection of porcine cutaneous wounds can result in biofilm induced delayed epithelialization by down regulation of keratinocyte growth factors (Tomic-Canic *et al.*, 2020; Pastar *et al.*, 2013).

Additionally, bacterial biofilm waste products can induce strong immune responses, such as chronic inflammation which impedes healing further (Gompelman, van Asten and Peters, 2016b; Wolcott *et al.*, 2010). As microbial load increases, the inflammatory cascade observed in wound healing is sustained, and continued production of bacterial and host derived proteases degrade the extracellular matrix and growth factors which then disturbs normal wound healing processes halting wound closure (Schilrreff and Alexiev, 2022). As biofilms confer a multitude of advantages to the microbial community, they have a high probability of developing in wounds because, they can adhere to host components, are too large for phagocytosis from resident immune cells and can enhance the resistance of bacteria compared with planktonic cells (Percival *et al.*, 2011). Bacteria within a biofilm are hidden from immune attack, safe against some mechanical disruption and much more tolerant to antimicrobial treatment. So, how do wound based treatment methods affect biofilms and are they successful?

1.4.6 Treatment of Chronic wound biofilms

To date, antimicrobial susceptibility testing using biofilm models has indicated that biofilm are recalcitrant to antimicrobials over 1000 times higher than the MICs of the planktonic counterparts (Percival *et al.*, 2011; Ceri *et al.*, 1999; Hill *et al.*, 2010). The use of antimicrobials to treat chronic wound infections will work in the suppression of the target organisms however it will likely fail to penetrate the biofilm itself, leaving behind biofilm cells which will subsequently repopulate the wound site (Percival *et al*, 2011).

It has been observed that even at twice the therapeutic dose, antimicrobials have little impact on lowering the cell count, let alone eradicating the cells completely in a constant depth film fermenter wound model (Hill *et al*, 2010). The constant depth film fermenter was developed to model dental plaque biofilm formation allowing the generation of multiple biofilms of a uniform depth, and therefore can demonstrate the effect of antimicrobials within this model as being much reduced (Hill *et al.*, 2010). Additionally, bacterial wound infections that could be targeted by antimicrobial therapies tend to require lengthy treatment, this can cause serious complications in the patient such as kidney failure (Maheswary, Nurul and Fauzi, 2021; Tchero *et al.*, 2018). There are a wide range of topical therapies and antimicrobial dressings that can provide targeted therapy. Uckay *et al* demonstrated that the use of gentamicin impregnated sponges combined with systemic antibiotic treatment showed no significant improvement in patient outcome compared to those patients receiving systemic antimicrobial alone (Uçkay *et al.*, 2018). Additionally, the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) biofilm guidelines state that there is no reported evidence in support of the use of systemic antimicrobials for the treatment of chronic wound associated infections despite their common use in clinical practice (Høiby *et al.*, 2015). This certainly exposes a need for biofilm specific wound care and treatments that are not necessarily bombarding the patient with high dose systemic antimicrobials.

As a result, biofilm treatment is often only successful after complete removal from the patient, which is why some of the most common treatments for chronic infected wounds is aggressive debridement (Percival *et al*, 2011). Debridement can be defined as the removal of necrotic tissue, senescent cells and bacteria (Falcone *et al.*, 2021). This is often used as a last resort, with antimicrobial dressings such as silver, iodine and other antimicrobial impregnated dressings being applied to wounds in combination with systemic antibiotic prescribing (Han and Ceilley, 2017). However, debridement is essential for the successful healing in chronic infected wounds, whether through surgical intervention and removal of non-viable tissues or through the application of enzymatic treatments (Han and Ceilley, 2017). These treatments can be difficult to time correctly, as these vary greatly upon the type of wound and potential comorbidities the patient may have (Han and Ceilley, 2017). The importance of wound debridement has been clearly demonstrated in a study by Falcone *et al*, who recommended it should be used as a precursor to enhance the action of antiseptic and antibiotic application for the treatment of biofilm infected wounds (Falcone *et al.*, 2021).

The combination of physical removal and application of biofilm specific antimicrobials, such as those able to degrade the EPS is essential for the eventual healing of the wound (Høiby *et al.*, 2015). The management of biofilm-associated infections is an obvious and major challenge for healthcare, and the

development of new pharmacodynamic parameters to include the biofilm susceptibility testing is essential for the treatment of these infections. There is an urgent need for new antimicrobial classes that target biofilms, these may include; inhibition of quorum sensing by halogenated furanone compounds and the dispersion of EPS by DispersinB among many other new mechanisms (Roy *et al.*, 2018). There is also a significant amount of research being conducted into new perspectives in the eradication of biofilms. Some methods report using electrochemical methods which, when administered, can kill bacteria independent of their growth phase (Sultana *et al.*, 2015; Wolfmeier *et al.*, 2018). Alternatively repurposing current antimicrobials to enhance their delivery has been investigated in a variety of ways. These reformulations include encapsulated antimicrobials, nano embedded microparticles or hydrogels that are loaded with cationic biocides (Wolfmeier *et al.*, 2018). Unfortunately, most of these new perspectives are still within the early research stages of drug development therefore there is limited information as to which may achieve clinical success. However, the standardisation of biofilm specific antimicrobial testing is improving with the inclusion of minimum biofilm eradication concentration and minimum biofilm inhibitory concentrations as standards across the antimicrobial industry (Falcone *et al.*, 2021).

1.5 Laboratory methods in antimicrobial susceptibility testing

1.5.1 Planktonic Antimicrobial Susceptibility testing

Antimicrobial susceptibility testing (AST) has a multitude of different tests and methods of identifying the efficacy of a given antimicrobial. There are many planktonic methodologies that are widely used in AST, such as the disk diffusion assays, E-test strip methods, and broth microdilution methods (Figure 6). These methods are utilised to determine minimum inhibitory concentrations (MIC) and clinical breakpoint values. Clinical breakpoint values can be defined as the concentration of antibiotic used to determine whether an infection by a particular strain of bacteria is likely to be treatable in a patient; it relies on a grading system in which based on their MIC value strains are rated as susceptible, intermediate or resistant (EUCAST, 2023; Turnidge and Paterson, 2007). The classification of each means the following:

- Susceptible-High likelihood of therapeutic success using standard dosing.
- Intermediate- Susceptible with increased exposure. There is a high likelihood of therapeutic success if the dosing regimen is increased or by increasing concentration at the site of infection.

- Resistant- There is a high likelihood of therapeutic failure even with increased exposure to the selected antimicrobial.

MIC values of known isolates of each species inform the setting of breakpoint values for a selected microorganism. However, MIC values alone cannot give a specific concentration that allows for adjustments of antimicrobial treatment based on pharmacokinetic and pharmacodynamic evaluation (Wantia *et al.*, 2020). In addition to these values, there are also epidemiological cut-off values (ECOFFs) that are related to the distribution of MICs for wild-type strains lacking acquired resistance to an antibiotic. The ECOFF value is generally the highest acceptable MIC value for the wild-type that can detect resistance to antimicrobials as a biological phenomenon below the level of the clinical breakpoint. It can be used as an early warning system on potential resistance being developed (Idelevich and Becker, 2019).

The ECOFF and clinical breakpoints are determined by a set of standardised experimental methods that were developed in collaboration with the International Organisation for Standardisation (ISO) and the Clinical and Laboratory Standards Institute (CLSI) - an American organisation that provides standards and guidelines for medical professionals across America and the world (Humphries *et al.*, 2021). Other organisations for antimicrobial susceptibility research include the European Committee on Antimicrobial Susceptibility Testing (EUCAST), an international susceptibility testing committee jointly organised by European Society of Clinical Microbiology and Infection Diseases (ESCMID), European Centre for Disease Prevention and Control (ECDC) and European National breakpoint committees (EUCAST, 2023; Giske *et al.*, 2022). These organisations exist to determine, review and revise clinical breakpoints and ECOFFS for the surveillance of AMR, they have enabled standardisation of *in vitro* AST methods within Europe and the US, and have acted as educational and promotional agencies to detect and advise on AMR relevant to public health. Between these organisations methods have been developed on broth microdilution to be conducted in a reproducible way for bacteria, and therefore the ISO for broth microdilution became the international reference method: ISO 20776-1:2019 (CLSI, 2018; ISO, 2019). On the other hand, European agencies were tasked with harmonising methodologies across Europe, and a method based on Mueller-Hinton agar and zone diameter breakpoints were used (Idelevich and Becker, 2019; Giske *et al.*, 2022). So, there is standardisation in planktonic antimicrobial testing between, and CLSI. However, these values are generally only valuable when the reference methodologies are followed with no deviations, it is widely accepted that deviations from the recommended conditions have a critical impact on the test accuracy. Even using the same nutrient media from different manufacturers can elicit considerably different results in regard to the observed

antibiotic susceptibility (Idelevich and Becker, 2019; Idelevich *et al.*, 2016). Although these committees exist for general antimicrobial resistance surveillance and standardisation of methods, there is yet to be such a committee focused on biofilms. However, there are several methodologies that have been accredited by the United Kingdom Accreditation Service (UKAS) in line with ISO/IEC 17025 which provides assurance that results are accurate and reliable from laboratory testing (UKAS, 2022). These methods include the minimum biofilm eradication concentration method, the CDC biofilm reactor and the single tube method (UKAS, 2022).

It is largely debated on how successfully biofilm *in vitro* susceptibility testing will mimic and represent the clinical environment. Some antimicrobial susceptibility tests, such as the broth microdilution test may indicate bacterial susceptibility *in vitro* whereas in the relevant clinical setting, those same bacteria are resistant and do not respond to therapy (Donlan and Costerton, 2002). Clinical relevance of standard antimicrobial testing is largely disappointing regarding prediction of clinical success (Høiby *et al.*, 2011).

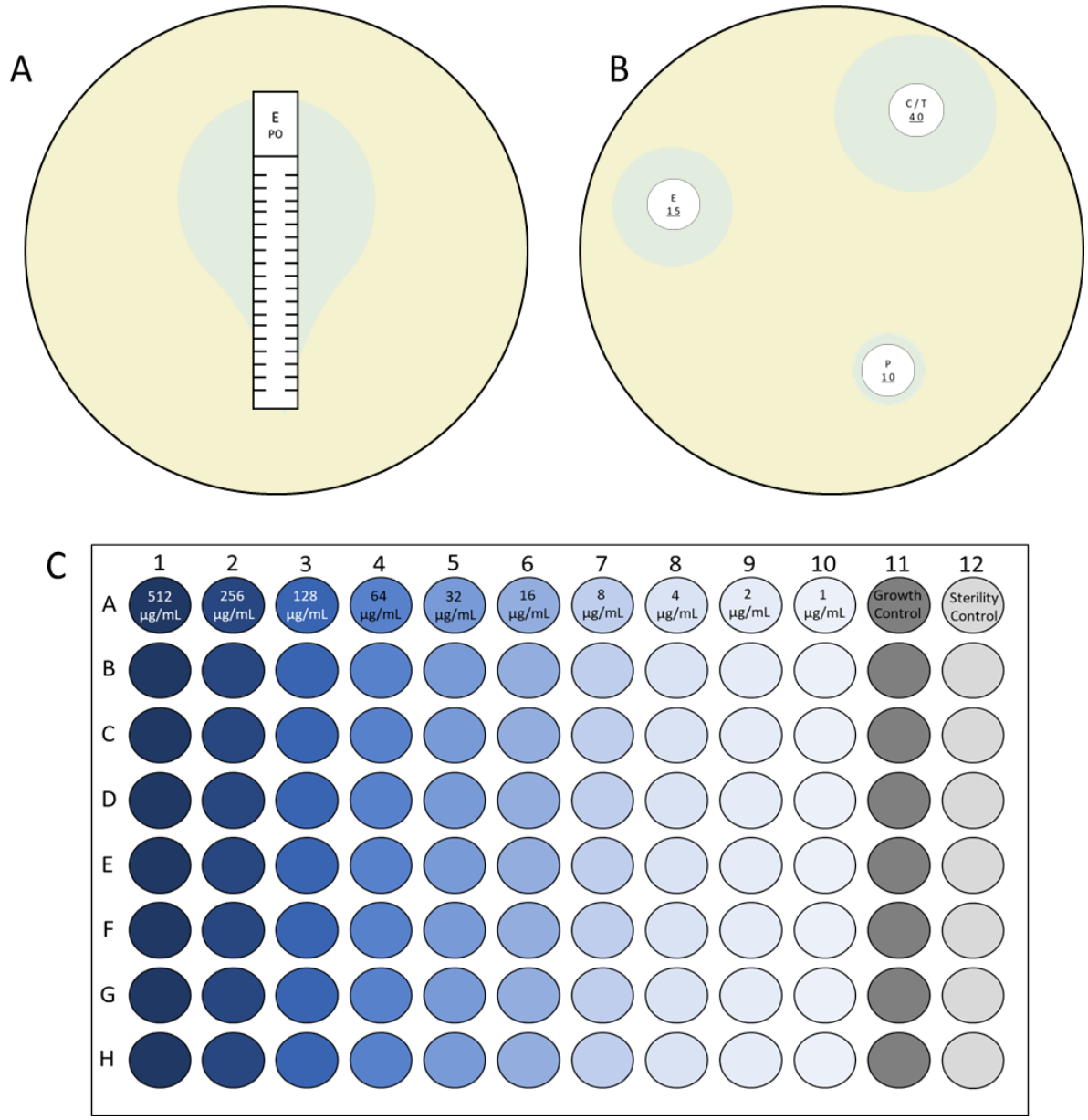


Figure 6 Schematic of planktonic antimicrobial susceptibility testing methods. (A) E test, which uses inoculated agar plates and an E test strip impregnated with increasing concentrations of antimicrobial (B) Disk Diffusion methods, which uses inoculated agar plates and impregnated disks with set concentrations of antimicrobials(C) Microdilution methods, which uses set concentrations of antimicrobial in broth and inoculated broth in a microtiter plate. Diagram made using Powerpoint.

1.5.2 Biofilm AST

Even within the biofilm susceptibility testing field there is a lack of reproducibility and robust methods among published studies (Goodman, Fanelli and Ioannidis, 2016; Allkja *et al.*, 2021). The development of biofilms has been extensively studied using various methods, including the Calgary biofilm device (CBD) and microtiter plate assays (MTA) for biofilm growth and susceptibility testing (Ceri *et al.*, 1999; O'Toole, Kaplan and Kolter, 2000; Merritt, Kadouri and O'Toole, 2005). However, questions remain regarding the optimal conditions for robust biofilm formation and the influence of experimental parameters on the Minimum Biofilm Eradication Concentration (MBEC) (Allkja *et al.*, 2021). The variation in results from different biofilm assays can be attributed to differences in handling, inoculum, or differences in the plates used for biofilm culturing (Kragh *et al.*, 2019). There are a variety of materials that are available in microtiter plates, with the most commonly used being polystyrene (Kavanagh *et al.*, 2018). There are also additives that can be used including treatments on the well surfaces such as a nonbinding surface treatment, or tissue culture treated plates. These differences in plates has been shown to have a significant impact on the MIC dependent on the antimicrobial and plate combination (Kavanagh *et al.*, 2018). Therefore, the type of plate used and any plate related treatments to the microtiter plate must be considered and investigated during experimental set up. In addition to this, the impact of media choice on biofilm growth and development has been well researched with media selection having a significant impact on the maturity of the biofilms and subsequently the recalcitrance to antimicrobials. Although those factors are considered in the wider research, certain additives that are included in these assays, such as Tween-20 or Tween-80 have also been shown to impact bacterial growth (Chen *et al.*, 2020; Kavanagh *et al.*, 2018; Miari *et al.*, 2020; Wu *et al.*, 2013).

Another important factor to consider is the static nature of these experiments, sessile biofilm formation is often referred to as a critical parameter in some research (Ceri *et al*,1999). There are important considerations to take into account using the MTA, as the biomass that forms at the bottom of the well may be due to settling of dead cells as well as sessile attachment. This may cause oxygen gradients to form within the wells, and planktonic cells may compete with biofilm bacteria for available carbon and oxygen (Kragh *et al.*, 2019). The impact of this is that the recalcitrance of the biofilm to antibiotics may be altered due to competition and oxygen deprivation initiating significantly more virulence factors in response, however as the amount of competition or oxygen deprivation in the wells hasn't been researched or standardised there is no measure of how these variables can affect replicates (Kragh *et al.*, 2019; Crusz *et al.*, 2012). The Calgary Biofilm device (CBD) was introduced as a suitable substitute for the MTA, as the method in MTA for removal of supernatant and planktonic cells is often either

pipetting out, scraping or inversion of the entire plate. This means that loosely bound biomass can often be disturbed which can in turn, introduce significant variances in biomass measurement (Kragh *et al.*, 2019). To mitigate the loss of biomass and inclusion of dead cells and debris, the CBD was designed to enable cell adhesion; this does, however, assume some degree of motility in the bacteria for successful attachment. The CBD was originally developed by Ceri *et al,* (1999) and was used to test antimicrobial agents against different strains by means of a high throughput biofilm attached antimicrobial assay (Figure 7). The system uses a 96 well plate lid that has 96 pegs attached that project downwards into the wells, enabling bacteria to adhere and grow in a biofilm. The pegs could then be removed, and placed in separate antimicrobial challenge plates (96 well plates with antimicrobial and media in pre-determined concentrations for the antimicrobial challenge stage of AST). It is the novelty of the removal of biofilm in a gentle manner that could allow loosely bound biofilm to be undisturbed during the changeover of media that was the main attraction of this revolutionary new method.

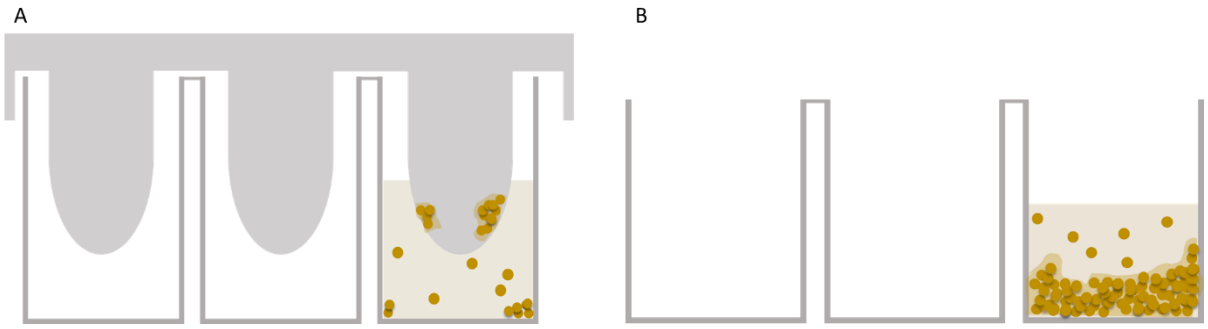


Figure 7 Diagram of the internal view of the CBD, with one well showing a schematic of the experimental conditions. Yellow circles indicate bacterial cells in broth and adhered to the pegs and wells. (B) Diagram of the internal view of the MTA with one well showing a schematic of the experimental conditions. Yellow circles indicate bacterial cells in broth and adhered to the wells. Diagram created in PowerPoint.

Other important factors when it comes to biofilm development and susceptibility testing is the media and the environment in which the biofilms are grown. Structure of the biofilm is dependent on the nutritional conditions surrounding the biofilm itself, with Klausen *et al* demonstrating a mushroom shaped microcolony development in glucose rich medium, yet in citrate based medium flat biofilms grew inside the same flow chamber (Klausen *et al.*, 2003). This difference has also been seen in dual-species biofilms when the biofilms were fed a medium that can only be metabolised by one species mixed species microcolonies were formed, however when fed a medium both species could metabolise separate microcolonies formed (Nielsen *et al.*, 2000). It seems that media has an important role in the structure of biofilms formed. When mixed species biofilms were cultured in the presence of diclofop, highly developed and patterned biofilms were formed, yet when grown in tryptic soy broth a biofilm lacking in variation in thickness and structure was observed (Wolfaardt *et al.*, 1994).

Although there is significant evidence that supports the flaws in using these methods with regards to clinical outcomes, they are still widely used within drug development. In fact, it has been reported that there is growing interest in the methods of antimicrobial activity screening and evaluation, due to global investment into tackling AMR. Examples of novel compounds utilising MIC and biofilm methodologies include a study by Smitten *et al.* (2020) demonstrating the antimicrobial activity of novel ruthenium based antimicrobials against *S aureus* (Smitten *et al.,* 2020). There, they used MIC as a method to identify the most promising lead out of their selected compounds, along with MBC and time-kill assays to determine the bactericidal properties of their compounds (Smitten *et al.,* 2020). Additionally, Song *et al*. (2019) demonstrated the antimicrobial activity of grapefruit seed extract by using MIC and biofilm adherence assays (crystal violet assay) (Song *et al.* 2019). This was to investigate the anti-biofilm activity of the preservative, and they demonstrated efficacy against *S aureus* and *Escherichia coli* (Song *et al.* 2019). Picoli *et al.* (2017) demonstrated the potential for melittin to inhibit biofilm formation of *S aureus*, *E coli,* and *P aeruginosa* (Picoli *et al.* 2017). They demonstrated these effects using MIC and MBC, along with a microtiter biofilm assay in conjunction with MTT solution (bromo-3- (4,5-dimethylthiazol-2yl) -2-5-diphenyl-2H-tetrazolate) to assess the viability of the biofilms post treatment (Picoli *et al.* 2017). So, there is a significant amount of evidence to indicate the use of both planktonic and biofilm methodologies to demonstrate antimicrobial activity in the early stages of drug discovery and development. Additionally, in support of the use of MIC in drug development, EUCAST has reported that novel drugs requiring testing can be preliminarily assessed by EUCAST as they advocate for MIC testing during these developmental stages (Giske *et al.*, 2022).

However, there are shortcomings with these standardised methods, as they can often be modified for the purposes of testing beyond the remit of standardisation. It has additionally been reported that it is imperative that changes do not affect the microbiological basics that underpin the testing - such as through diluting media or using highly concentrated inoculum (Balouiri *et al.* 2016). Although some antimicrobial drugs require specific solvents to achieve solution, it is then of the utmost importance that solvents be tested for an effect on the selected microorganism. This is to ensure that reported antimicrobial activity is due to the novel drug, as the drug may not be as effective once the solvent is removed or reformulated during the later stages of antimicrobial drug development (Balouiri *et al.* 2016).

When taking into consideration the large discrepancies reported between the MIC and MBEC, it brings into question if MIC can be enhanced to yield results that are more indicative of the MBEC (Mulla *et al.* 2016). This may use the inclusion of extra parameters such as oxygen concentration, pH or organic load,

as each of these additional challenges are often seen in later stages of drug development. These factors are often taken into consideration for the validation of antibiotic therapy with the utilisation of pharmacodynamics and pharmacokinetics (PK/PD) (Rodriguez-Gascon *et al.* 2021). The major indicator of the PD for antibiotics is the MIC, and it is relied upon as the indicator of susceptibility of the pathogen (Rodriguez-Gascon *et al.* 2021). The main issue with this reliance is that the clinical outcome is not conditioned by the MIC value alone- and as biofilm research has determined- may not be an accurate depiction of the pathogens susceptibility *in vivo*. Therefore, if we take into account additional parameters to the MIC and MBEC assays- we may be able to achieve a better picture on the future success of novel antimicrobials sooner. There are recommendations to include MBEC and further method development to discover better methods for the diagnosis and quantification of biofilm infection- however, this ethos is not often applied to the drug development market (Mulla *et al.* 2016).

As the medium, and method of AST (e.g. MBEC versus MIC), along with the potential utilisation of environmental specific bacterial mechanisms (such as the collagen hug and dock, lock, latch mechanisms by *S aureus*), has been shown to cause significant impact on the reported effect of an antimicrobial. It can then be hypothesised that using skin or a skin substitute may yield different results to *in vitro* biofilm models, and *in vitro* planktonic models.

1.5.3 Methods in Chronic wound or wound related AST

There are many different models that can be used to mimic skin infections such as 3D wound models, tissue engineered skin, human skin explants, porcine explants, murine *in vivo* models and porcine *in vivo* models.

The grouping of 3D models encompasses many different types of wound relevant models. Reddersen *et al*, 2022 developed a 3D biofilm model using agar and gelatin with nutrient rich medium to produce a haptic resembling chronic wounds, as there is a semi-solid permeable surface present therefore allowing formation of microclusters of biofilm (Reddersen, Tittelbach and Wiegand, 2022). The agar gelatine media mix was vigorously shaken to incorporate air bubbles to allow for the development of clusters of biofilm throughout the model, enabling the testing of antimicrobial wound dressings (Reddersen, Tittelbach and Wiegand, 2022). Townsend *et al*, 2016 developed an *in vitro* single layer cellulose based wound model consisting of cellulose matrix and horse serum hydrogel (Townsend *et al.*, 2016). This model enabled the study of topical antimicrobial treatments on polymicrobial biofilms. Interestingly, they found that biofilms formed in this method developed significantly increased resistance to povidone-iodine treatment and chlorhexidine treatment compared with the results from

standard *in vitro* biofilm testing. The increase in resistance to antimicrobial treatment from *in vitro* to a 3D model supported clinical reports of the antimicrobial agents having reduced efficacy against polymicrobial chronic wound infections (Townsend *et al*., 2016). There are increasingly complex models, such as those by Brackman *et al*, which used an artificial dermis to investigate the biofilm eradication of several topical gauzes and Chen *et al*, who developed a two layered model utilising an intentional skin breach to mimic a subcutaneous wound (Chen *et al.*, 2020). In the Brackman model, they assessed the efficacy of antimicrobial functionalized gauzes for their impact on quorum sensing, and found that there was a significant inhibitory effect observed in their functionalized gauze compared with control antimicrobial gauzes (Brackman *et al.*, 2016). This model consisted of chemically crosslinked hyaluronic acid and a second layer of hyaluronic acid with collagen. This was then partially immersed in wound simulating media, with Bolton Broth, plasma and laked horse blood. The Chen model utilised more wound relevant components in their substratum, which has pig fat to mimic the subcutaneous layers and introducing the use of a void to mimic dermis damage therefore exposing the nutrient rich subcutaneous fat layers (Chen *et al,* 2021). Whilst the 3D models are more useful than the CBD or MTA there are still shortcomings of the use of 3D structures for antimicrobial susceptibility testing as, there is limited or no immune response and systemic interaction in these models, which means there is limited alignment between these and clinical effects on the antimicrobial treatments (Steinstraesser *et al.*, 2010). There are also a large number of additives and microbial based media used in these models, which can impact the transferability of the findings to a clinical scenario.

With those factors considered, tissue engineered human epidermis can be utilised as a substrate for antimicrobial susceptibility testing. Companies such as Epiderm, Episkin and Labskin have developed human skin equivalents that are used for the reduction of use of animal testing (Lerebour, Cupferman and Bellon-Fontaine, 2004; Kandárová *et al.*, 2005; Roguet *et al.*, 1994). Although models such as Episkin and Epiderm were originally developed as irritancy testing for topical compounds (Roguet *et al.*, 1994). The Episkin model has since diversified to include microbial adhesion as part of their testing recommendations (Roguet *et al.*, 1994; Kandárová *et al.*, 2005; Lerebour, Cupferman and Bellon-Fontaine, 2004). Episkin has been used in microbiological research, Lerebour *et al*. investigated the adhesion of *S aureus* and *S epidermidis* to Episkin compared to a stainless steel substrate and found that adhesion differed between the strains, with *S aureus* showing a higher affinity to Episkin adherence than to the abiotic surface (Lerebour, Cupferman and Bellon-Fontaine, 2004). Other research models have been published including a skin equivalent dermal matrix that uses fibrin populated with fibroblasts with a stratified epidermis as a model to investigate the microbiota colonisation of the skin (Holland *et al.*, 2008a). There are also other 3D models of engineered human skin that have been

developed to observe bacterial infection, as demonstrated by Shepherd *et al*, who used two different forms of wounding to observe the course of infection of bacteria after 72 hours (Shepherd *et al.*, 2009). Interestingly they found that *P aeruginosa* migrated through the dermis into the lower dermal layers of the model, whereas *S aureus* stayed within the superficial epidermis (Shepherd *et al.*, 2009). There are shortcomings with these models as these engineered equivalents require refinement to the culture conditions to ensure the reproducibility in the tissue physiology (Couto *et al.*, 2021). This was evidenced in a study by Couto *et al*. who analysed the xenobiotic metabolism in a 3D human skin model called Labskin comparatively to *ex vivo* human skin. They found inconsistent results with regards to xenobiotic metabolising enzymes and auxiliary protein abundance in the Labskin, with variation between Labskin samples tested, and missing markers compared with the *ex vivo* human skin. The discrepancies between samples, and inconsistencies with regards to key markers identifies an area for improvement within tissue engineered skin, so that a reproducible model can be used to mimic the skin and wound environments.

To further reduce these discrepancies, *ex vivo* models have been widely developed to model a more clinically relevant environment for the use of antimicrobial susceptibility testing. *Ex vivo* models utilise tissues and organs collected from living animals for experimentation in a controlled laboratory setting (Andersson *et al.*, 2021). In a review by Parnell and Volk, it was observed that with over 25 years of wound model research only 3% were *ex vivo* and *in silico* models (Parnell and Volk, 2019). The same study found that 74% of wound models were *in vivo* models, and that these models have become the gold standard tool for experimental studies of wounding, utilising a wide range of species to observe the wound healing processes (Parnell and Volk, 2019). Studying wound infections is much more difficult using *in vivo* models due to infection variability, sampling challenges and application of treatments (Andersson *et al*, 2021). Additionally, there are many complex factors associated with the undertaking of animal studies, such as ethical considerations, animal welfare standards and high expenses (Andersson *et al*., 2021). To mitigate this, many studies have investigated *ex vivo* models, which can be developed to form robust, high throughput methods that are affordable to evaluate the efficacy of topical treatments for wounds (Andersson *et al.*, 2021; Schaudinn *et al.*, 2017; Yang *et al.*, 2013; Phillips *et al.*, 2015).

Schaudinn *et al*, developed a bacterial wound infection model using *ex vivo* human skin, human skin has been used for many years to study physiology, immune response and drug penetration (Schaudinn *et al.*, 2017). Here, they used *ex vivo* human skin with injected bacteria underneath a purpose-made wound and treated the subsequent infection using topical application of ciprofloxacin, and observed a

4 $\log_{10}$ reduction in the CFU compared with the untreated explants (Schaudinn *et al*, 2017). Interestingly, this was using 375 µg mL^{-1} of ciprofloxacin compared with a MBC of 0.125 µg mL^{-1} and MBEC of 16 µg mL^{-1} for comparable bacteria. The concentration required to reduce the bacterial count by the FDA minimum reduction of 4 $\log_{10}$ was 3,000 times higher than the MBC and 23 times higher than the MBEC. This demonstrates the importance of testing antimicrobials within a biofilm wound model as even MBEC values are underestimating the amount of antimicrobial necessary to elicit a significant reduction in CFU (Schaudinn *et al.*, 2017; Wang *et al.*, 2010). Although this model is valuable, there are many roadblocks in the way of scientific research with human tissues, such as ethical approval, adherence to strict procedure in accordance with the Human Tissue Act and the scarcity in availability of tissues (Andersson *et al*, 2021). Therefore, other models have investigated the use of animal tissues as a way to mitigate the challenges.

Andersson *et al* utilised skin from minipigs that was subsequently sterilised and frozen prior to experimentation. The tissues were then wounded by burning and infected by placing inoculum on the apical surface of the wound (Andersson *et al*, 2021). The wound beds were covered with parafilm to prevent evaporation and after the establishment of infection were treated with Levofloxacin or Prontosan gel. Andersson reported that the response to antimicrobial treatment was poor, and that the *in vitro* MIC of levofloxacin was not sufficient to eradicate the bacteria from the wounds in their study (Andersson *et al,* 2021). These results reiterate the findings by Schaudinn, that the susceptibility of bacteria in biofilms in *ex vivo* wound beds show significantly decreased susceptibility to the reported MIC. The disparity between *in vitro* findings in antimicrobial susceptibility with *in vivo* models has also been observed in a study by Garcia *et al*. They investigated the impact of chestnut honey and bacteriophage on an *ex vivo* wound model, using fresh porcine explants that were also frozen prior to experimentation (García *et al.*, 2018). The explants were prepared and inoculated on to the apical surface of the skin before infection development and subsequent treatment. The results indicated that the treatments conducted on *in vitro* biofilms, using polystyrene plates showed higher reduction of CFU compared with the porcine *ex vivo* model, where treatment was less effective. Additionally, in a study by Yang *et al,* who used frozen porcine skin that had been wounded and infected (Yang *et al.*, 2013). They determined that the fresh porcine skin obtained from local abattoirs had undergone scalding and boiling as a method to depilate the carcasses. As a result, they then took a section of skin from undamaged carcasses, prior to depilation and froze them for 1-3 months then compared the biofilm growth on the samples. Interestingly they found that when samples had been frozen for over one month there were 1-3 log reductions in the CFU/explant in the untreated controls. This implies that biofilms formed on tissues frozen for more than 1 month may be less dense than biofilms formed on

fresh tissues. Frozen porcine skin purchased from a commercial supplier produced thin explants which resulted in a 2-4 log reduction compared to the samples from the local suppliers, and these samples- even when thicker and yielding higher CFU/explant had a larger reduction in the biofilm after antimicrobial exposure this indicates that frozen tissues may produce more susceptible biofilms than fresh skin (Yang *et al*., 2013). The use of fresh frozen skin may have an impact on the integrity of the skin, Yang *et al* also reported that the commercial skin had a spongier texture than freshly harvested skin and was more prone to dehydration. However, the so-called fresh skin from this study had been frozen prior to use, meaning that it was a frozen skin model which may also have a potential impact on the biofilm development and susceptibility, as no fresh skin was tested for biofilm development.

Yang et al, (2017) then reported the use of fresh porcine skin without the reporting of the samples being frozen prior to use, and demonstrated a successful model for the testing of dressings. This model placed antibiotic agar underneath the explant reportedly to prevent the bacteria penetrating the explant (Yang et al., 2017). However, the study did not report the use of tissue culture medium to supply the tissues with nutrients for the duration of the experiment (Yang et al., 2017). This method of utilising agar as a support for explants is also seen in models developed by Phillips et al. in which an ex vivo biofilm porcine explant model using fresh porcine skin, was wounded and placed on soft TSA plates (Phillips et al., 2015). The wounds were infected with bacterial suspension left to develop infection then fully submerged in tryptic soy broth containing gentamicin for 24 hours. Gentamicin treatment was performed as a way to remove planktonic cells from the biofilm on the porcine explant before testing new treatments. It was explained that the amount of antibiotic was used as it was capable of restraining the biofilm to the surface of the explant (Phillips, Yang and Schultz, 2013; Phillips et al., 2015). This method of removal of planktonic cells through high levels of antibiotics for 24 hours can be seen in an explant study by Schultz et al, (2018) where *P. aeruginosa* biofilms were submerged in 50 × MIC gentamicin prior to removal of the biofilm and enumeration of the CFUs (Schultz et al., 2018). This antibiotic submersion method is also employed by Roche et al, 2019, who submerged their biofilm explants in 50 × MIC prior to recovery of biofilm bacteria. However, in this study they compared the explant results to an in vivo porcine model, yet did not use the same incubation in 50 × MIC broth for the removal of planktonic cells, instead incubated biopsies in neutralising DE broth to limit the antimicrobial activity of any treatments applied. These differences in methodologies can also be evidenced in the study results, when observing the log reduction of CFU, there was a 7 $\log_{10}$ and 4 $\log_{10}$ reduction for *P. aeruginosa* and *S aureus ex vivo* biofilms respectively, yet in the *in vivo* model, a 3.4 $\log_{10}$ (*P. aeruginosa*) and 0.84 $\log_{10}$ (*S aureus*) reduction was seen compared to the untreated controls. This may be due to the utilisation of an extended antimicrobial exposure on the biofilms in the *ex vivo*

model compared to the in vivo model. It is well reported that the transition between *ex vivo* and *in vivo* models is large, and experimental findings often are poorly extrapolated from *ex vivo* to *in vivo* (Roche et al., 2019a). This may be due to the variation in infection reproducibility seen in *in vivo* models, it is reported that there are often issues with sampling and inoculation when using live animals for antimicrobial testing (Andersson et al, 2021). Additionally, there are far more factors and variables to consider when utilising an *in vivo* model (Andersson et al, 2021).

The use of high levels of antimicrobials on the explant and biofilm may have a significant impact on the biofilm results. Although it is reported that the use of antibiotics to kill the planktonic organisms leaves biofilm cells unaffected, as the approach utilises the properties of the EPS to protect biofilm cells from antibiotics (Schultz *et al.,* 2018). This is contrary to a significant amount of *in vitro* biofilm work, in which antimicrobial treatment is undertaken on biofilm through the immersion of a biofilm into antimicrobial enriched broth to determine reduction of biofilm cells (Ceri *et al.*, 1999; O'Toole, Kaplan and Kolter, 2000). It is also important to consider that if a more clinically relevant model is intended that the high concentration of antimicrobial is almost never reported in chronic wound biofilms, therefore the impact of exposure to high level of antimicrobial prior or post exposure to *in vivo* treatment could be inducing significant changes within the biofilm microenvironment itself. Other researchers such as Lone *et al*, have investigated the impact that media choice can have on the biofilm and responses in *ex vivo* models. Lone *et al* investigated the ability for *S aureus* to grow in the tissue culture medium independent of the *ex vivo* model, to ensure that biofilm growth was dependent on the utilisation of the wound bed in the explants (Lone *et al.*, 2015). This method of checking the ability for a given test organism to grow within the culture medium may be an important step to ensure that the resulting biofilms are dependent on the utilisation of the explant and not through media contamination.

As animal models have been used in the bulk of studies, there may be more standardisation across these models that allow for better comparison of results and the clinical translation. In 1996, a study was conducted in a *S aureus* infected murine model, wherein only a few hours post inoculation biopsies taken from the mice there was the presence of membrane like structure and clusters with inflammatory cells around these clustered membranes (Akiyama *et al.*, 1996). It was also observed that the surrounding tissues were severely degenerated and become necrotic, which is highly suggestive of the presence of a bacterial biofilm in the tissues. Following on from these discoveries, many more experimental mice, rat, rabbit models went into studying the effects of the biofilm (Gompelman, van Asten and Peters, 2016b; Schierle *et al.*, 2009; Schaber *et al.*, 2007; Kanno *et al.*, 2010; Seth *et al.*, 2012a; Gurjala *et al.*, 2011).

For example, in 2010, Zhao *et al* produced a chronic wound murine model by using *P aeruginosa* within punch biopsies in diabetic mice. Although the majority of the uninfected control wounds closed within 28 days, none of the biofilm infected wound closed over the same time period (Zhao *et al.*, 2010). Thompson *et al* (2015) described a murine biofilm model, using directly applied a *Klebsiella pneumoniae* bacterial suspension in phosphate buffered saline (PBS) to the 6mm wound biopsy (Thompson *et al.*, 2015). The biofilms were challenged with a gallium salt antimicrobial at two concentrations (0.1% and 0.3%) on the model, and found a 0.44 log (0.1%) and 2.1 log reduction (0.3%) in the CFU/explant. When comparing this to the MIC from the gallium salt of <1 μg/ mL the same disparity evidenced in the *ex vivo* models can be seen, suggesting that *ex vivo* models may be able to predict *in vivo* success.

The lack of standardisation in these models means it is difficult to compare results and determine if a reduction in CFU or the efficacy of a drug is due to the drug itself or the tissue condition, additive, or prior antimicrobial exposure (Yang *et al*, 2013; Phillips *et al*, 2015;2013; Lone *et al,* 2015). Equally, the importance of new methods that also fulfil other areas for advancement in science is essential. As previously discussed, animal based research is costly, intensive and challenging with regards to the multitude of regulations and welfare standards that are essential to undertake such research (Andersson *et al.*, 2021). The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) is and independent organisation that pioneers the discovery and application of new approaches to minimise the use of animals in research (Percie du Sert and Robinson, 2018). The principles of the reduction of purpose-bred animals for *ex vivo* models is an exciting area of research. Much of the research reviewed within this work has indicated the value of the *ex vivo* model and the benefits it can yield with regards to antimicrobial susceptibility testing. However, as a lot of these models have used additives or utilised high levels of antimicrobial exposure there is a need for a new model that reduces the need for purpose-bred animals, models a wound environment, and can yield biofilm specific antimicrobial susceptibility results without the need for addition of extraneous nutrients. If this model was additionally high-throughput it may lead to a reduction in the need for purpose bred animals to be sacrificed and used for other *ex vivo* models. Herein lies the challenge for this thesis.

1.6 Project Aims

A significant amount of research has been conducted into biofilms and antimicrobial susceptibility. This project aimed to investigate the impact of several wound relevant parameters on biofilm recalcitrance to antimicrobial challenge. This was to be done with reproducibility and repeatability in mind, and to utilise an accessible ethical approach to wound model development with the NC3Rs in mind.

The main aims of the project were:

1. To determine if *in vitro* model choice has an impact on the antimicrobial susceptibility of *Staphylococcus aureus.*
2. To assess the impact hypoxic incubation has on *S aureus* in planktonic and biofilm culture in terms of antimicrobial susceptibility.
3. To develop a suitable infected skin wound model utilising waste tissues from food production.
4. To determine the ability of the infected skin wound model to facilitate the formation of biofilms in terms of the presence of clustered cells, EPS, and tolerance of antimicrobials.
5. To investigate the impact of hypoxia on the antimicrobial susceptibility of wound biofilms.

Chapter 2: *In vitro* susceptibility testing

2 Introduction

Staphylococcus aureus is known for its ability to cause a wide range of life-threatening biofilm associated infections, including wound infections and surgical site infections (Wertheim *et al*., 2005). The biofilm environment provides bacteria with an increased resistance to antibiotics, making it more difficult to eradicate using traditional methods. In addition to the inherent challenges in treating *S. aureus* infections, the conditions within clinical infection sites are substantially different from those that are typically studied *in vitro* and *in vivo*. This has led to a growing realisation that *in vitro* conditions may not accurately reflect the complexity of the bacterial microenvironment within the human body, and that new models and approaches are needed to better understand and combat *S. aureus* infections.

There are many standards available within the scope of global antimicrobial susceptibility testing. There are well documented discrepancies in reported susceptibility can also be attributed to the biofilm phenotype and slow uptake of standardised biofilm testing. Even within the biofilm susceptibility testing field there is a lack of reproducibility and robust methods among published studies (Allkja *et al*., 2021; Goodman, Fanelli, & Ioannidis, 2016).

Antimicrobial testing on *S aureus* biofilms has taken place in numerous devices, including the Calgary biofilm device (CBD) and the microtiter plate assay (MTA) for biofilm development and susceptibility testing, but there are still questions regarding optimal conditions for robust biofilm formation and influence of experimental parameters on the Minimum Biofilm Eradication Concentration (Allkja *et al*., 2021). The high variation of results from the MTA or CBD can be attributed to differences in handling, inoculum, and the impact of batch differences and the materials and coatings used in microtiter plates (Kragh *et al*., 2019; Kavanagh *et al.*, 2017). The impact of media choice has been investigated, and such research has demonstrated variability in biofilm formation (Chen *et al*., 2020). Tween-20 is included in the methodologies of the CBD as an additive to aid recovery of bacterial cells from the biofilm, yet it is implicated in a few studies as having an influence on the microbial cells themselves (Miari *et al*., 2020; Wu *et al*., 2013).

The work within the biofilm field has suggested that the use of standard MIC testing has very little relevance for the more clinically relevant biofilm models, therefore it was important to validate this suggestion and to consider if applying clinically relevant environmental stressors would yield a more

clinically relevant outcome. This is particularly important when considering that antimicrobial resistance is currently quantified using MIC (Coenye, Bové, & Bjarnsholt, 2022; Brauner *et al*., 2016).

In this work we aim to investigate the influence of hypoxia on *S aureus* biofilm growth and susceptibility and compare it to planktonic growth and susceptibility under hypoxia. We compare two different methods for biofilm growth and also the potential influence of Tween. This will aid in the development of standardised, more clinically relevant protocols for *S aureus* susceptibility testing.

2.4 Aims

The main aims of this study are.

1. To determine if hypoxic conditions have an impact on *S aureus* in planktonic antimicrobial susceptibility testing
2. To compare the biofilm formation in two static, high throughput biofilm devices in terms of:
    a. Biofilm formation and colony forming units.
    b. Antimicrobial response
    c. Impact of hypoxia on antimicrobial response and biofilm development
3. To observe the effects of Tween20 on CFU counts in biofilm antimicrobial susceptibility testing

2.5 Methodology

2.5.1 Bacteria and growth conditions

S. aureus ATCC 29213 (DSM 2569) was used for this study. Strains were grown from frozen stocks -80°C on Tryptic Soy Agar (TS) (14432-500G-F, Merck Life Science, UK) for 18-24 hours at 37°C. Overnight planktonic cultures were set up in cation-adjusted mueller-hinton broth (MHB) (90922, Sigma-Aldrich, UK) (MIC/MBC Method) or tryptic soy broth (TSB) (41298-500G-F, Merck Life Science, UK) (Biofilm methods). TSB with 1% tween20 (P1379-100ML, Sigma-Aldrich, UK) was used as recovery media in some experiments using the microtiter plate method to determine the impact of tween20 on cell recovery. TSB with no additives was used as recovery media in all other cases. Cultures were adjusted to a 0.1 OD_{600} using fresh media in a spectrophotometer (Jenway, 7310). Inoculum was standardised for a viable count of $1x10^6$ CFU/mL in MHB for the MIC/MBC methods and a 1 in 10 dilution in fresh media for a viable count of $1x10^5$ CFU/mL in TSB for the Biofilm Methods.

2.5.2 Antibiotic preparation

Gentamicin sulphate (Sigma-Aldrich, PHR1077-1G), vancomycin hydrochloride (Sigma-Aldrich, SBR00001) and clindamycin hydrochloride (Sigma-Aldrich, PHR1159-1G) were used in this study. Stocks of 10 mg/mL were made using sterile dH_2O and kept frozen at -20°C. Working concentrations were made on the day in cation adjusted Mueller-Hinton broth at 1024 µg/mL.

2.5.3 Environmental Oxygen Conditions

Normoxia was defined here as environmental oxygen concentration, 5% CO_2, 37°C incubating within a Heracell Vios160 Incubator. Hypoxia was defined here as 1% oxygen, 5% CO_2 37°C incubating within a Heracell Vios160 Incubator.

2.5.4 Minimum Inhibitory Concentration and Minimum Bactericidal Concentration Determination

This methodology was adapted from the ISO standard for the broth microdilution method (ISO 20776-1:2019(en), 2019). Polystyrene microtiter plates (Fisher Scientific, 10687551) were prepared using MHB and antibiotics were added in a doubled concentration to achieve a final dilution range of 512 µg/mL-1 µg/mL with an untreated control and sterility control. Once the antimicrobial dilution plate was ready, 100 µL of standardised inoculum (Section 2.1) was added to the desired wells to achieve final concentration of antibiotics within the previously specified range. The plates were sealed using a gas

permeable plate seal (Fisher Scientific, ESI-B-100) and incubated at 37°C at 120 rpm. The OD_{600nm} of the plates were read 24 hours after inoculation in a Tecan Spark multimode microplate reader. To determine the MBC 10-fold serial dilutions were performed in sterile PBS from 10^1 to 10^8 and 10 µL of each sample was plated onto a TS agar plate and air dried. Plates were incubated for 18 hours, and the colonies were counted and recorded following the incubation period. The following equation was used to calculate the CFU/mL:

$$CFU\ per\ mL = average\ no. colonies\ \times \frac{1}{0.01} \times dilution\ factor$$

Experiments were conducted in triplicate with 3 repeats.

2.5.5 Calgary Biofilm Device Method

The Calgary Biofilm device assay was performed as described by Ceri *et al*.1999 with some modifications (Ceri *et al*., 1999). Standardised culture was transferred in 150 µL aliquots into each well to be investigated in a Calgary Biofilm device plate (Polystyrene, Innovotech, 19112), and peg lids were inserted. Plates were incubated at 37 °C for 24 hours. Peg lids were then removed, placed into a wash plate with 200 µL of sterile PBS for 1-minute. Following the wash, an antimicrobial challenge plate was prepared with the antibiotics gentamicin, clindamycin and vancomycin in MHB, in decreasing concentrations from 512 µg/mL to 1 µg/mL. The lid was subsequently removed from the PBS wash and placed into the antimicrobial challenge plate. This was then incubated at 37 °C for a further 24 hours. The lid was then removed and washed in sterile PBS and then placed into recovery media-TSB. The plates were then placed into the Bransonic Sonicator 1800 using the dry insert for 10 minutes on high (40 kHz), to release the adhered biofilm cells. Individual wells were then serially diluted to 10^{-8} and 10 µL aliquots were plated on a LB agar plate. These plates were then incubated at 37°C for 18-24 hours. The colonies were counted by eye, and CFU/mL calculated. The following equation was used to calculate the CFU/mL:

$$CFU\ per\ mL = average\ no. colonies\ \times \frac{1}{0.01} \times dilution\ factor$$

Experiments were conducted in triplicate with 3 repeats.

2.5.6 Microtiter plate Biofilm Method

The Microtiter plate biofilm assay (MTA) was performed using overnight planktonic cultures that were adjusted in fresh media (TSB) and150 µL transferred into each well in a microtiter plate. Plates were sealed using a gas permeable plate seal and incubated at 37°C for 24 hours. Spent media was removed, the plates were washed with 200 µL of sterile PBS and replaced with the antibiotics gentamicin,

clindamycin, and vancomycin in decreasing concentrations from 512 µg/mL to 1 µg/mL was added into the corresponding wells. After antimicrobials were added the plate was then incubated at 37 °C for 24 hours. The plate was then removed and washed with 200 µL sterile PBS then recovery media-TSB or TSB with 1% Tween20- was added. The plates were then placed into the Bransonic Sonicator using the dry insert for 10 minutes on high (40 kHz), to release the adhered biofilm cells. Individual wells were then serially diluted from neat to 10^{-8} and 10 µL aliquots were plated on a TS agar plate and air dried. These plates were then incubated at 37°C for 18-24 hours. The colonies were counted by eye and recorded following the incubation period. The following equation was used to calculate the CFU/mL:

$$CFU\ per\ mL = average\ no.colonies\ \times \frac{1}{0.01} \times dilution\ factor$$

Experiments were conducted in triplicate with 3 repeats.

2.5.7 Crystal Violet Method

Biofilms were grown as stated, after incubation wells were washed with sterile PBS then fixed. Biofilms were fixed using 200 µL of methanol for 15 minutes after which the methanol was removed, and the plates were air-dried. Once dry, 200 µL of crystal violet solution (0.1%) was added to all wells for 20 minutes. The crystal violet solution was carefully removed, and a sterile PBS wash was added, plates were washed until the supernatant was clear. The bound crystal violet was released by adding 250 µL of 33% glacial acetic acid. The absorbance was measured at 570 nm. All steps were carried out at room temperature. This experiment was conducted in triplicate with two repeats.

2.5.8 SCANNING ELECTRON MICROSCOPY (SEM).

2.5.8.1 SAMPLE PREPARATION FOR SEM.

Biofilms were inoculated and prepared as described previously. Biofilms were grown for 12, 24, and 48 hours in normoxia and hypoxia. The specimens were fixed with 2.5% (v/v) glutaraldehyde in PBS for a minimum of three hours at 4°C. The specimens were then washed three times with PBS at 30-minute intervals at 4°C. Secondary fixation was performed in 2% (v/v) aqueous osmium tetroxide for 1 hour at room temperature. Dehydration was accomplished through a graded series of ethanol, with 75% ,95%, 100% (v/v) ethanol for 15 minutes, followed by drying over anhydrous copper sulphate for 15 minutes. All of the steps were performed at room temperature. The specimens were then placed in a 1:1 mixture of 100% (v/v) ethanol and 100% hexamethyldisilane (HEX, Merck) for 30 minutes, followed by 30 minutes in 100% hexamethyldisilane. The specimens were air-dried overnight, mounted on 12.5mm

diameter aluminium pin stubs (G301P, Agar Scientific) with Leit Adhesive Carbon Tabs (AGG3347N, Agar Scientific)

2.5.8.2 AU SPUTTER COATING.

Edwards S150B Sputter Coater was used to coat the specimen with 25 nm thickness of gold (Au).

2.5.8.3 SEM MICROSCOPY.

Specimens were examined with the Tescan Vega3 LMU scanning electron microscope (EM Facility – The University of Sheffield) at a 10 kV operating voltage and micrographs were captured with the SE (Secondary Electron) detector.

2.5.9 Statistical Analysis

Statistical analysis was performed in GraphPad Prism version 9 for Windows. Paired t-tests were utilised for the pairwise comparison of CFU counts in hypoxia and normoxia, and to determine difference in CFU of the untreated controls. Differences between CFUs were calculated using Ordinary one-way and two-way ANOVAs with post-hoc pairwise comparisons.

2.6 Results

2.6.1 Impact of Antimicrobial Challenge on planktonic *S aureus*

The MIC is a valuable tool in the investigation of the amount of antimicrobial required to inhibit the growth of planktonic culture, when combined with the MBC, which investigates the minimum antimicrobial concentration to kill the planktonic culture determinations on the susceptibility of a given strain can be decided. The MIC value as observed from this study was compared with the clinical MIC breakpoints from EUCAST to assess whether the strain was susceptible or resistant to the antibiotic.

MICs and MBCs were undertaken for *S aureus* using the broth microdilution method for cells in suspension conducted in triplicate (n=6) (*ISO 20776-1:2019*). MIC values were scored using the TECAN Spark to read OD values at the endpoint of the assay, then samples were taken from each well, diluted, plated and colony forming units counted by eye to determine the MBC.

When treated in normoxia, the MIC indicated that *S aureus* was susceptible to all antimicrobials tested, however an MBC of 16 μg/mL was observed for clindamycin and vancomycin (Table 1, Figure 8). In hypoxia, gentamicin and clindamycin show no inhibition of growth (Figure 8, Table 1), and therefore the MIC is reported as >512 μg/mL. However, the MBC results were determined as 8 μg/mL and 256 μg/mL respectively (Figure 8) this result is unusual, as the MICs are defined as the lowest concentration of antimicrobial that will inhibit growth, and MBCs are the lowest concentration that will prevent the frowth of the organism after subculture (Andrews *et al*. 2001).

Vancomycin elicited an inhibitory effect on *S aureus* in hypoxia with an MBC of 64 μg/mL, however, as can be seen in figure 8C there is variation in the groups of OD values for the values in the 512-64 μg/mL. As there was a large amount of variation in these values, further investigation was essential to determine if there was a significant difference in the values in the range of 64 μg/mL and 512 μg/mL. Statistical analysis that was conducted indicated that there is no significant difference between the OD values of MIC testing in normoxia to hypoxia at 64, 128, 256 or 512 μg/mL using a two-way ANOVA, this then confirmed that 64 μg/mL was the MIC value (Figure 8). Results show hypoxia has a significant impact on the susceptibility, eliciting increased resistance of *S aureus* to the antibiotics, especially when considering the EUCAST breakpoints as a point of reference (Table 1) (EUCAST, 2019).

Table 1 MIC, MBC, EUCAST breakpoints and MBEC values from the MTA and CBD of the antibiotics tested (N=Normoxia, H=Hypoxia) MIC values from this study were scored using the TECAN Spark.

	EUCAST Breakpoint	MIC		MBC		CBD		MTA	
		N	H	N	H	N	H	N	H
Gentamicin	2	2	>512	2	8	2	4	>512	>512
Clindamycin	0.25	<1	>512	16	256	16	>512	>512	>512
Vancomycin	2	2	64	16	64	2	32	>512	>512

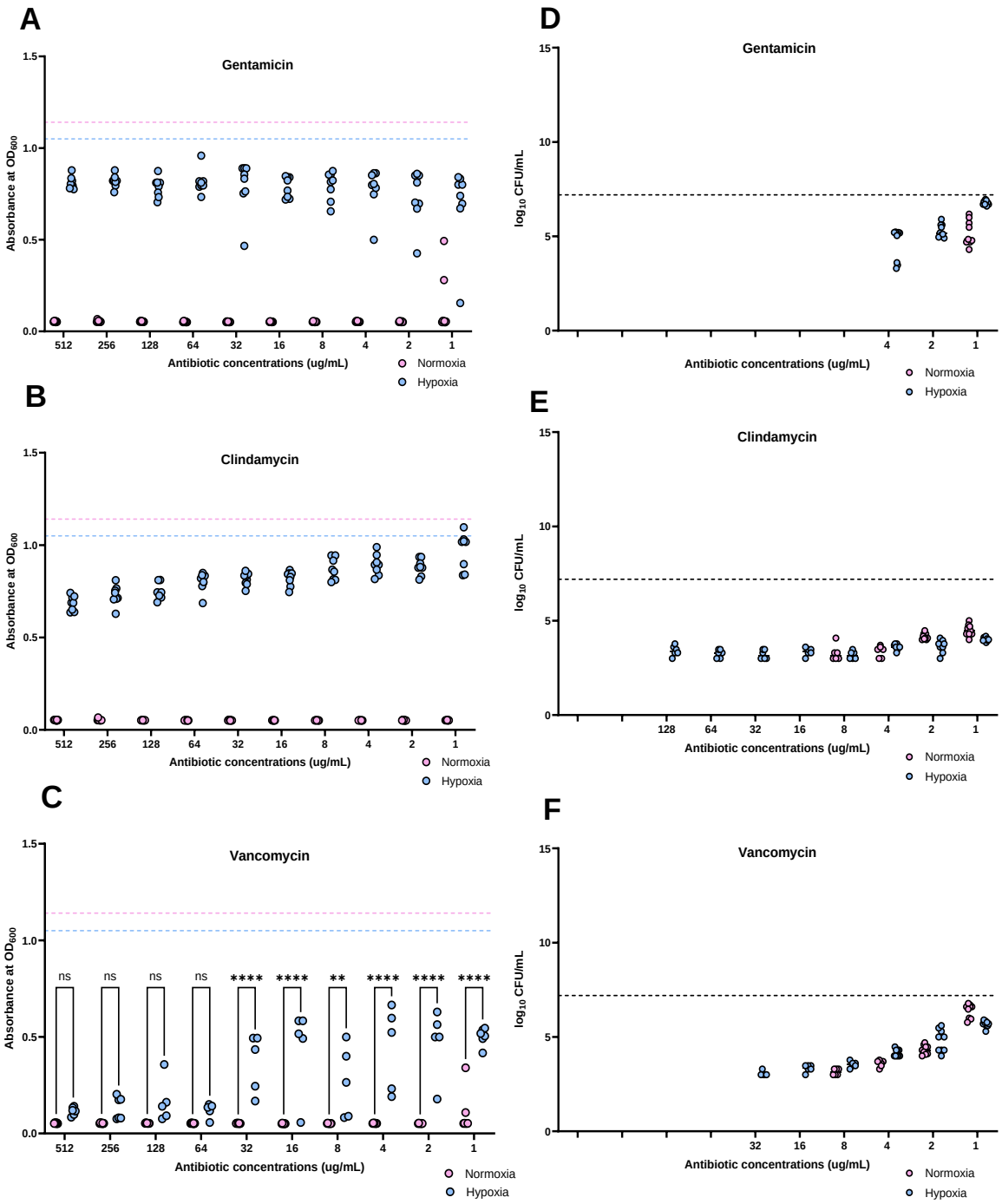


Figure 8 MIC and MBC results of *S aureus* 29213 using hypoxic and normoxic oxygen environments. (A) Gentamicin treated *S aureus* in hypoxia and normoxia (B) Clindamycin treated *S aureus* in hypoxia and normoxia (C) Vancomycin treated *S aureus* in hypoxia and normoxia. Pink dotted line indicates the untreated control after 24 hours incubation in normoxia. Blue dotted line indicates the untreated control after 24-hour incubation in hypoxia. (D) Gentamicin treated *S aureus* in hypoxia and normoxia (E) Clindamycin treated *S aureus* in hypoxia and normoxia (F) Vancomycin treated *S aureus* in hypoxia and normoxia. Black dashed line indicates the untreated control after 24 hours incubation. (n=6, from 3 repeated experiments)

2.6.2 Comparison of biofilm development methods

In order to determine the most suitable assay for *S aureus* biofilm testing, the CBD and MTA were used to compare the CFU of the biofilms formed over time. Biofilms were formed over 24 and 48 hours the cells recovered and plated to enumerate the CFUs (Figure 9). The CBD had an average of 4.9 (normoxia) and 4.7 (hypoxia) $\log_{10}$ CFU after 24 hours, which was a decrease from the inoculum (5.2 $\log_{10}$) after 48 hours the CFUs increased to 6.5 (normoxia) and 5.5 (hypoxia) respectively.

In contrast to this, the microtiter method yielded much higher CFUs with 8.0 (normoxia) and 7.9(hypoxia) $\log_{10}$ CFU after 24 hours and 10.7 (normoxia) and 10.7 (hypoxia) after 48 hours of incubation. Although the same inoculum was used for each assay there was a significant increase in recovered biofilm cells for *S aureus* biofilms in the microtiter plate compared with the CBD after 24h and 48h ($p<0.0001$, Figures 9 A and B). This indicates that model selection has a significant impact on the biofilm cell density when working with *S aureus*.

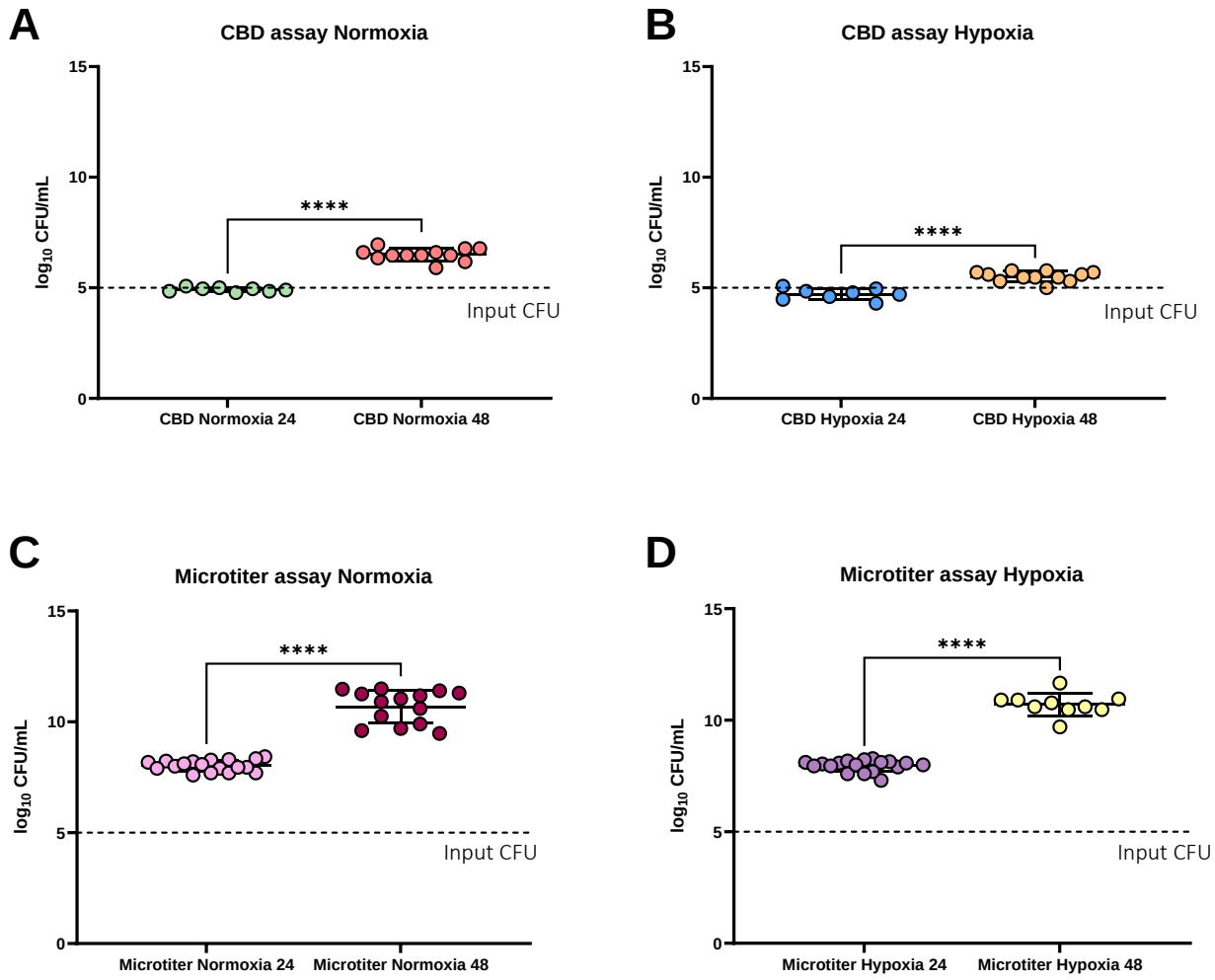


Figure 9 Biofilm growth after 48 hours of *S aureus* untreated controls on the Calgary Biofilm Device and the Microtiter plate method in hypoxia and normoxia. (A) CFU counts following 24 and 48 hours in Normoxia using the Calgary Biofilm Device (B) CFU counts following 24 and 48 hour incubation in Hypoxia using the Calgary Biofilm Device (C) CFU counts following 24 and 48 hour incubation in Normoxia using the Microtiter plate method. (D) CFU counts following 24 and 48 hour incubation in Hypoxia using the Microtiter plate method. (** P=<0.0001 From a two-way ANOVA) Dashed line indicates input CFU of 1x10^5 CFU/mL, error bars indicate standard deviation.CBD normoxia and hypoxia 24 hours n=8, CBD normoxia and hypoxia 48 hours n=12, MTA hypoxia 48 hours n=10, MTA normoxia 48 hours n=14, MTA normoxia and hypoxia 24 hours n=18. Results taken from 4 repeated experiments.**

Whilst there was a significant increase in the CBD CFU (1 $\log_{10}$) in normoxia compared with hypoxia at 48 hours ($p<0.0001$, Figure 9) no difference was seen at 24 hours (Figure 9). In addition, no significant differences were observed when comparing hypoxia and normoxia in the MTA at 24 h (P=0.9647) or 48h (P=0.9995). A lack of difference in hypoxia compared with normoxia is further supported by the crystal violet assay (CV) results (Figure 10).

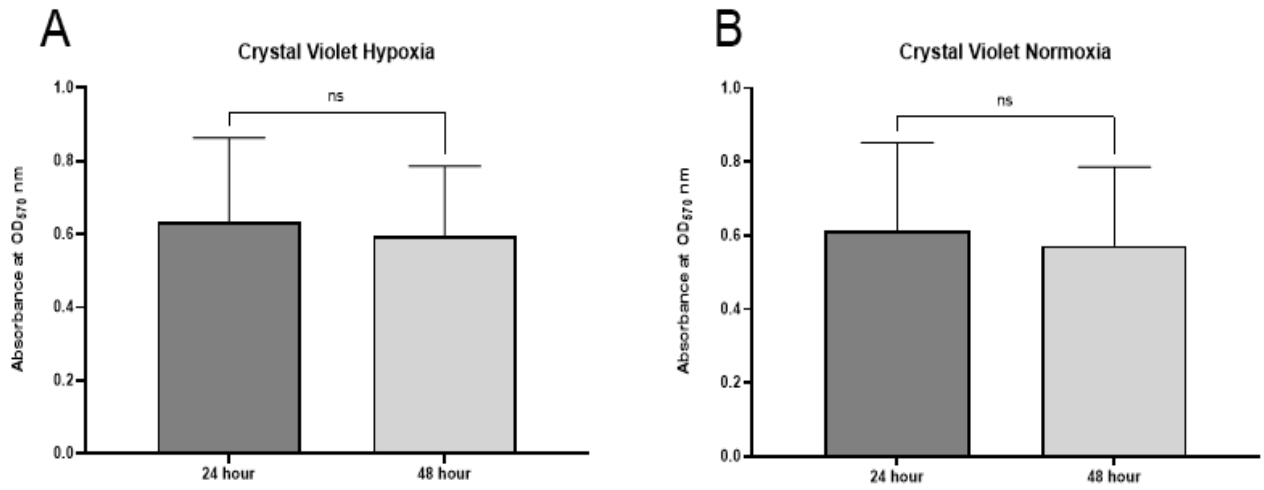


Figure 10 *S aureus* Crystal Violet absorbance at OD570nm of the bacterial biomass formed from 24 hour and 48 hour biofilm growth. (A) Hypoxic environmental conditions (B) Normoxic environmental conditions. Statistical tests: one-way ANOVA (ns=not significant) (n=78)

Interestingly, a significant difference was observed in the MTA CFU at 24h compared with 48h with increases of 2.7 $\log_{10}$ (normoxia) and 2.8 $\log_{10}$ (hypoxia), however this increase in cell number was not observed in the CV assay results. This assay was conducted to observe the biomass development of *S aureus* in the microtiter plate method, no significant difference was seen in crystal violet dye retention at 24h and 48h. In addition to this, SEM was undertaken to further compare the different assay types.

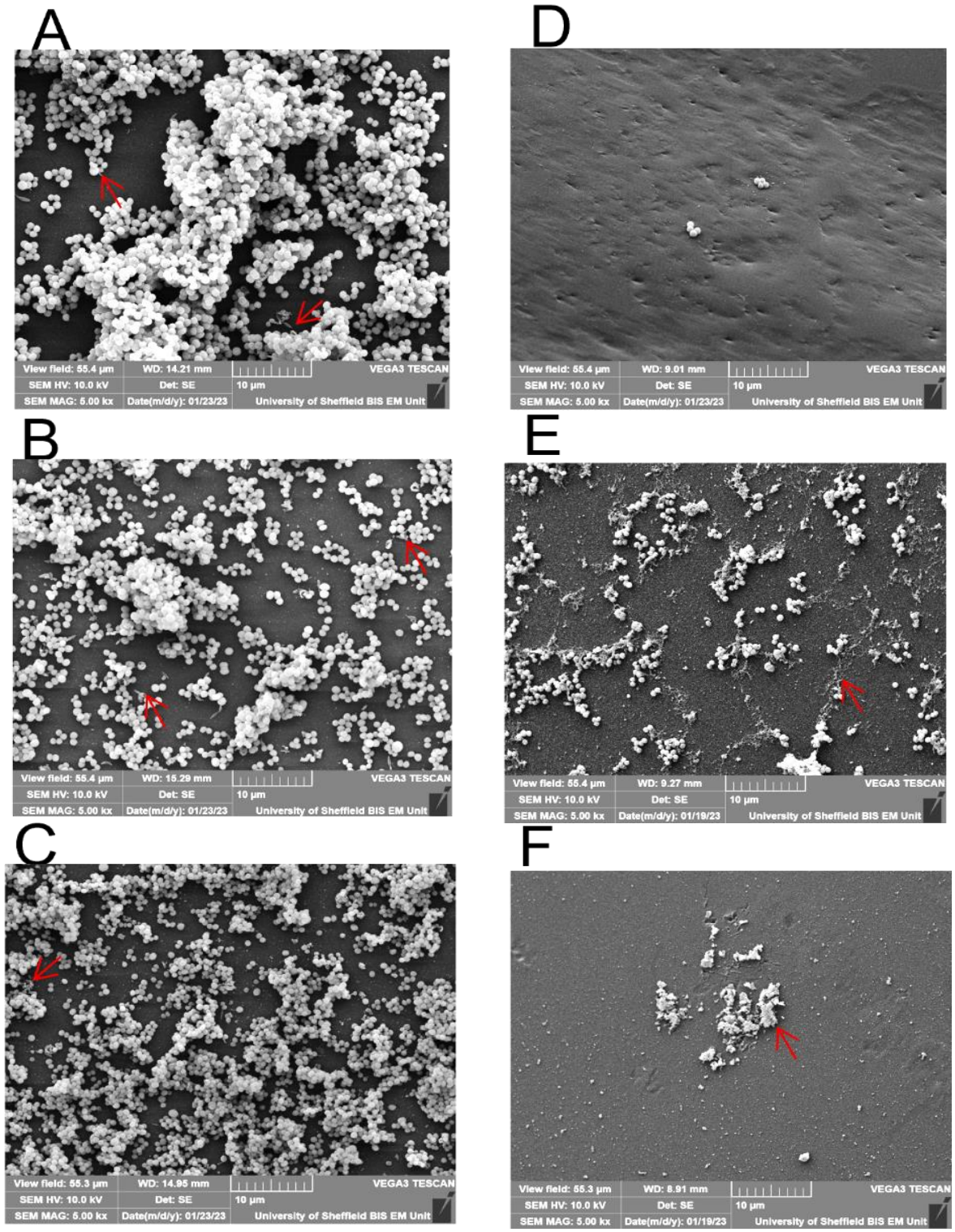


Figure 11 S aureus normoxic biofilms micrographs. (A) SEM, 5000x bottom well of the MTA grown for 12 hours. (B) SEM, 5000x bottom well of the MTA grown for 24 hours. (C) SEM, 5000x bottom well of the MTA grown for 48 hours. (D) SEM, 2000x surface of the CBD peg grown for 12 hours. (E) SEM, 5000x surface of the CBD peg grown for 24 hours. (F) SEM, 5000x surface of the CBD peg grown for 48 hours. Red arrows indicate EPS

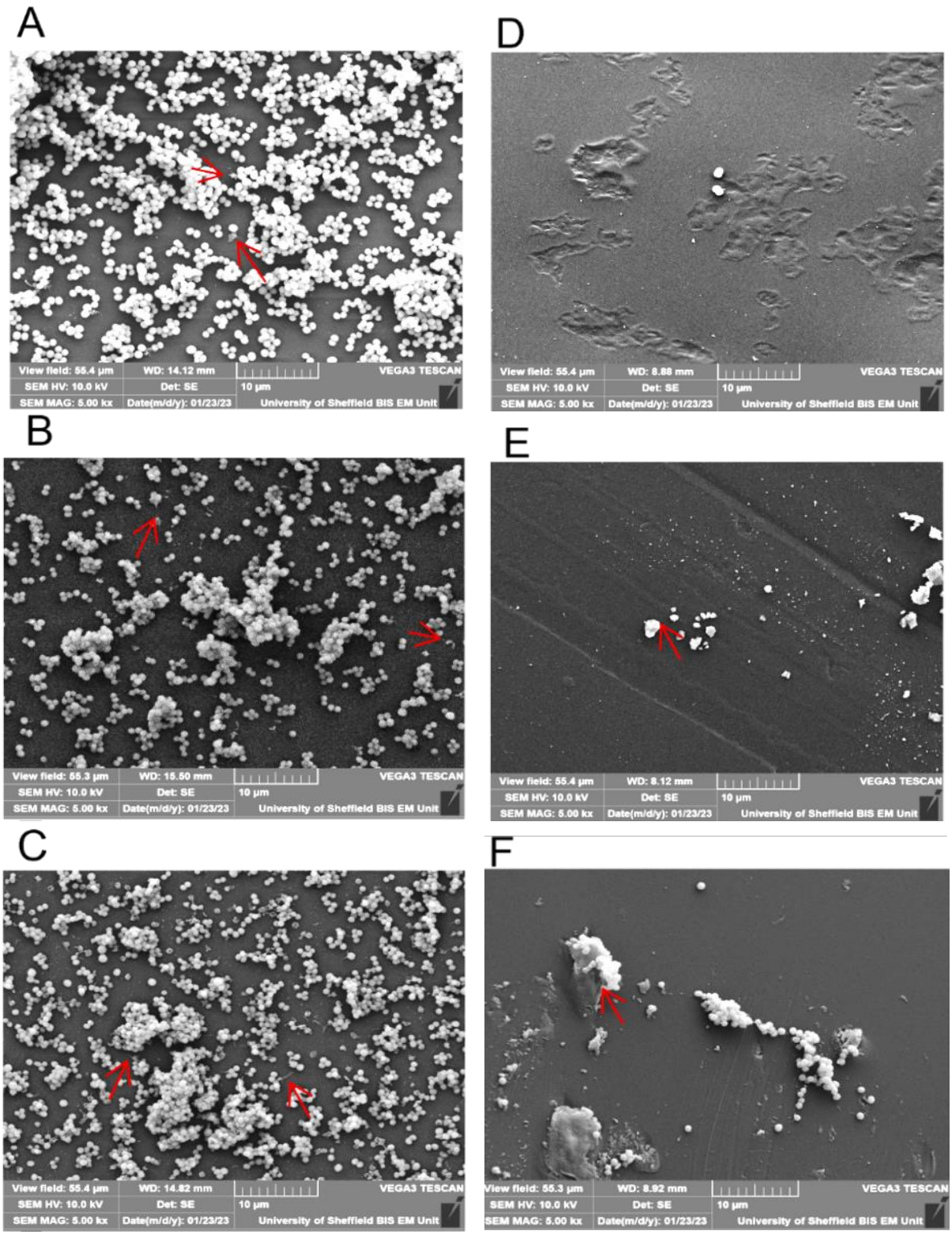


Figure 12 *S aureus* hypoxic biofilms micrographs (A) SEM, 5000x bottom well of the MTA grown for 12 hours in normoxia. (B) SEM, 5000x bottom well of the MTA grown for 24 hours. (C) SEM, 5000x bottom well of the MTA grown for 48 hours. (D) SEM, 2000x surface of the CBD peg grown for 12 hours. (E) SEM, 5000x surface of the CBD peg grown for 24 hours. (F) SEM, 5000x surface of the CBD peg grown for 48 hours. Red arrows indicate EPS.

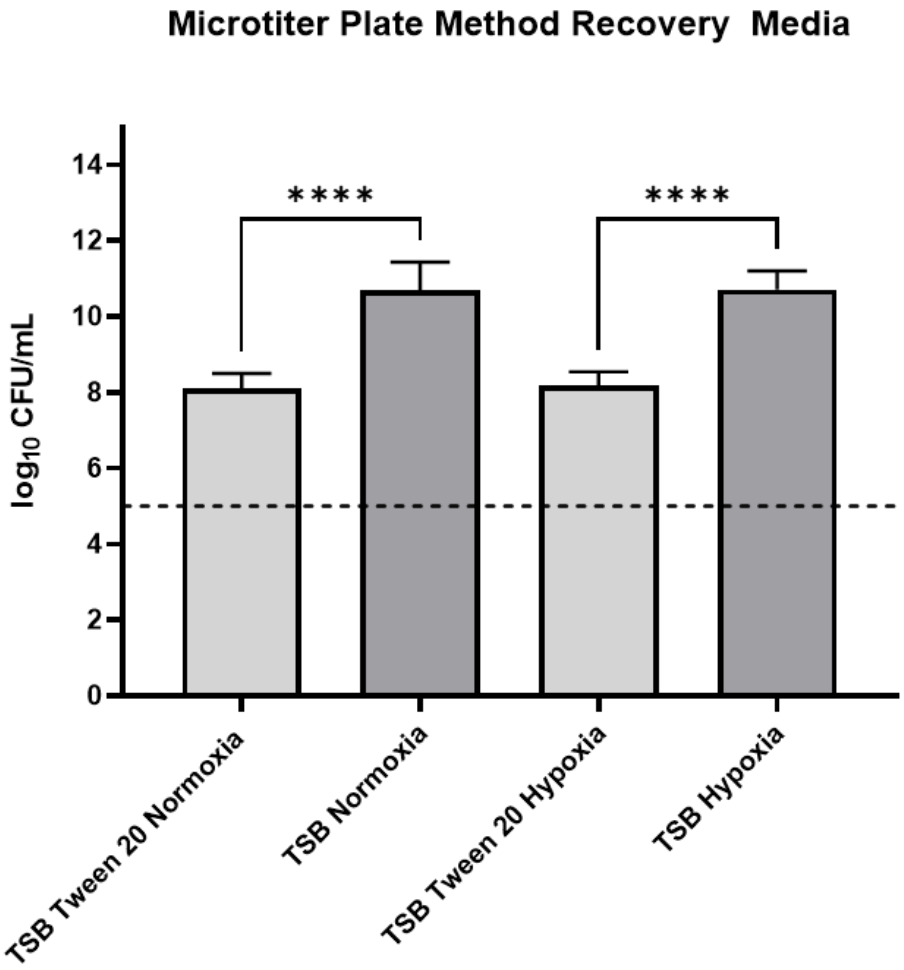


Figure 13 *S aureus* untreated control biofilm growth after 48 hours in the Microtiter plate method in hypoxia and normoxia using either TSB with 1% Tween20 of TSB as the recovery media. (** P=<0.0001 From One-Way ANOVA) Dashed line indicates input CFU of $1x10^5$ CFU/mL, error bars indicate standard deviation(n=6). Results taken from 3 repeated experiments.**

The SEM images shown in Figure 11 and Figure 12 constitute careful observation of the biofilm development over time in both the CBD and MTA. EPS can be observed in these images (red arrows) confirming the presence of biofilms in both models. EPS was not observed at 12 hours in the CBD however it was evident in the MTA 12-hour biofilms.

Biofilms in normoxia and hypoxia (Figures 11 and 12) were much more robust in the MTA than the CBD at 12, 24 and 48 hours in terms of coverage of biofilms cells and evidence of EPS production. In fact, very few cells were able to be identified from the CBD samples at all.

Tween20 is often recommended as an additive to aid the recovery of biofilm cells post incubation (Ceri *et al*., 1999). It has been often implicated as having an effect on cells or biofilm formation, so it was determined that an investigation into the potential effects of this additive may have on the strain used here. It was observed that there is a significant association between the addition of Tween20 and a decrease in the recovered biofilm cells (Figure 13, $p<0.0001$). For this reason, all antimicrobial challenge experiments are reported without the use of Tween20 in the recovery media.

2.6.3 Impact of Antimicrobial Challenge on *S aureus* Biofilm in CBD and MTA

To identify the MBEC of the chosen antimicrobials the CBD was used and 24h biofilms were treated with antimicrobials for 24h before sonication and enumeration of CFUs. In normoxia, gentamicin, clindamycin and vancomycin all eradicated the biofilms following 24h of drug treatment (Figure 14) at 2 μg/mL, 16 μg/mL and 2 μg/mL, respectively. In hypoxia, the minimum biofilm eradication concentration for gentamicin and vancomycin was much higher in hypoxia than normoxia (4 μg/mL and 32 μg/mL respectively) however clindamycin did not achieve eradication of the biofilms, therefore the MBEC is reported as >512 (Table 1).

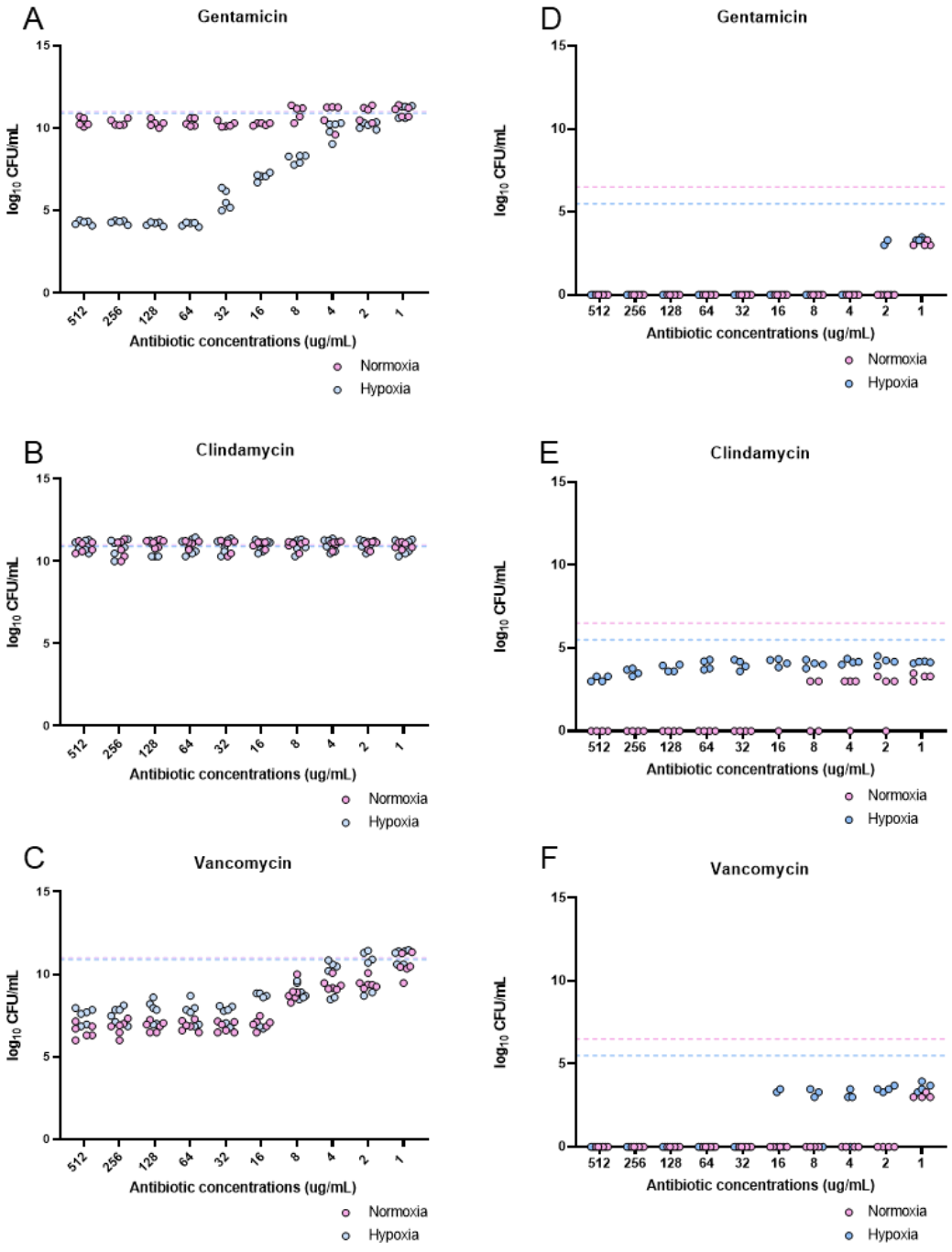


Figure 14 Colony forming units of *S aureus* 29213 following treatment with antibiotics in the MTA (A-C) and CBD (D-F). Twenty-four-hour-old biofilms were treated with selected antibiotics for 24 hours. (A) Gentamicin against *S aureus* in hypoxia and normoxia in the MTA(B) Clindamycin against *S aureus* in hypoxia and normoxia in the MTA (C) Vancomycin against *S aureus* in hypoxia and normoxia in the MTA. (E) Gentamicin against *S aureus* in hypoxia and normoxia in the CBD (F) Clindamycin against *S aureus* in hypoxia and normoxia in the CBD (G) Vancomycin against *S aureus* in hypoxia and normoxia in the CBD. Pink dashed line indicates the untreated control after 48 hours incubation in normoxia. Blue dashed line indicates the untreated control after 48 hour incubation in hypoxia. (n=6). Results were taken from 3 repeated experiments.

Next, the effect of antimicrobial on the biofilm grown in the microtiter plate method using the same conditions was tested (Figure 14). There was no MBEC achieved following treatment with gentamicin clindamycin or vancomycin in both environmental conditions. A reduction was seen in the vancomycin treated biofilms of 4 $\log_{10}$ at 64 μg/mL which did not further decrease up to 512 μg/mL. When testing the effect of hypoxia using the microtiter plate method, vancomycin and clindamycin had no significant difference in hypoxia compared with normoxia. Hypoxic biofilms treated with gentamicin had a decreased CFU count compared with normoxic biofilms, with a 6-log reduction at the highest antimicrobial concentrations (64 μg/mL and above), no eradication concentration was determined with the concentrations of antimicrobials tested here.

2.7 Discussion

The aims of this study were to investigate the impact of a single clinically relevant parameter (hypoxia) on the efficacy of antimicrobials in planktonic cultures, to determine the impact of different biofilm model choices on biofilm development, and if hypoxia has an impact on biofilm susceptibility to antimicrobial treatment *in vitro*.

Clinical environment considerations are particularly important when considering preclinical drug development to give an earlier result as to how well that drug may perform. A critical step during preclinical drug development is whether the drug will work in people. It is a key part in the development of novel compounds to ensure that the drugs work effectively. Unfortunately, it is commonplace for low amounts of novel drugs to succeed past the clinical trial stage, with only 17% of novel antimicrobials to enter market approval (Dheman *et al*., 2021). This, along with a significant amount of *in vitro* work suggests that there is a large disparity in bacterial physiology between infection *in vitro* and clinically, often with overestimations in the antimicrobial efficacy or inappropriate experimental methodologies used for clinical estimation of antimicrobial success (Antunes *et al*., 2010).

2.7.1 MIC and MBC

MICs and MBCs are a mainstay of antimicrobial susceptibility testing, with approved methodologies across the globe using broth microdilution methods, such as those found from EUCAST and CLSI (ISO 20776-1:2019; CLSI, 2022; EUCAST, 2022). The MIC is defined as the lowest concentration that inhibits visible growth in planktonic broth culture (bacteriostatic), whereas MBC can be defined as the minimum concentration that results in 99.9% killing of planktonic bacterial cells in broth culture(bactericidal) (Britt *et al*., 2016; Hess, Henry-Stanley and Wells, 2011). In normoxia, gentamicin, clindamycin and vancomycin effectively killed the planktonic cells at <2µg/mL, this finding is supported by the EUCAST MIC values and other papers which have widely reported planktonic susceptibility to this standard lab strain (Britt *et al*., 2016; EUCAST, 2019; Hess, Henry-Stanley and Wells, 2011). These methods are set as the gold standard due to their ease, repeatability, and reproducibility. However often these methods do not get an accurate enough picture of how drugs will perform in clinical scenarios. Clinical infection sites are complex environments with many different parameters to consider such as reduced oxygen availability, nutrient availability, and acidosis (Hull *et al*., 2022). Hypoxia is well documented throughout infection sites and in the human body, with healthy capillaries having oxygen tensions of 20-30 mmHg and non-healing diabetic foot ulcers <10 mm Hg (Hajdamowicz *et al*., 2019a; Kalani *et al*., 1999). The MIC data gathered under hypoxia displays increased resistance during decreased environmental oxygen

indicating that susceptibility to antimicrobials is dependent on the environmental oxygen availability. This may suggest that MICs conducted under normoxic laboratory conditions alone may overestimate the antimicrobial effect novel formulations may have. It has been suggested that underestimation of new-to-market drug efficacy could be improved in the pre-clinical stages by considering the implementation of physiological conditions such as hypoxia as an experimental parameter (Hajdamowicz *et al*., 2019b).

This decrease in susceptibility seen in the MICs conducted under hypoxia indicates the deployment of mutations to enhance the survival of *S aureus* in hypoxia. An observed *gyrB* mutation was implicated in causing an increase in the bacterial fitness in hypoxia which can enhance the survival of *S aureus* as seen in our MIC data (Hull *et al*., 2022). *S aureus* may utilise the two-component system *ssrAB* which regulates the production of virulence factors to resist oxidative stress and to enable metabolic response to hypoxia (Kinkel *et al*., 2013). This metabolic adaptation likely contributes to the increased resistance seen in this study, as both clindamycin and gentamicin target protein synthesis by binding the 50S or 30S ribosomal subunits respectively (Spížek & Řezanka, 2017; Krause *et al*., 2016). Genetic adaptation may also include the switching to a small colony variant or persister phenotype, which would also aid the resistance seen in hypoxia. It has been documented that exposure to hypoxia can influence some of the key biofilm formation and adhesion characteristics including the upregulation of the *ica* cluster increasing polysaccharide intercellular adhesin production (Ulrich *et al*., 2007; Cramton *et al*., 2001). This upregulation of the biofilm phenotype could also increase the resistance to antimicrobials.

2.7.2 Biofilm susceptibility

This study observed a significant reduction in the density of biofilms in the CBD, indicating that although biofilms form, it is likely that with non-motile bacteria such as the *S aureus* strain tested here, they are significantly less robust than in the MTA. The differences in the construction of each method are significant, whilst the MTA allows bacteria to 'settle' at the bottom of the wells the CBD and its peg lid relies only on the motility of the bacteria to attach and form biofilms. In the original Calgary Biofilm Device study by Ceri *et al.* the cell density reported was lower for *Staphylococcus* than other bacteria tested (Ceri *et al*., 1999). It can therefore be suggested that these lower cell densities seen in this study could be due to a lack of cell motility. Staphylococci are historically defined as non-motile bacteria, although *S aureus* has been observed to show some passive motility it does not possess the type IV pili or flagella to intentionally move to initiate attachment (Pollitt & Diggle, 2017; Jarrell & McBride, 2008; O'Toole, Kaplan, & Kolter, 2000). Our findings suggest that the type of motility in a bacterium is an important consideration when choosing a biofilm forming assay. The observation of different bacteria

forming different types of biofilms can be referenced back to the study by O'Toole and the ring and film biofilms produced by *P aeruginosa* and *S aureus* respectively (O'Toole, Kaplan, & Kolter, 2000). This study has observed that the choice of assay -particularly when reporting biofilm susceptibility- has a significant impact on the reported results, Table 1 shows a direct comparison of the large difference in MBECs from the CBD and MTA. For example, the MBEC for gentamicin in normoxia is 2 µg/mL in the CBD and is >512 µg/mL for the MTA, the only difference in conditions in those experiments were the actual plate used. This further compounds the importance of assay choice when conducting antimicrobial susceptibility; however, once a suitable assay has been chosen the question remains; how important are the reagents and other experimental design options? It therefore was decided that an investigation into the additives in MBEC testing would be useful due to the notable differences observed in assay choice.

In common with other studies, we have observed that reagent use has a significant impact on the density of biofilms as seen in Figure 9 (Chen *et al*., 2020; Stepanović *et al*., 2001). Interestingly this study has found that the use of Tween20 impacts the results from biofilm recovery by reducing the cell numbers. It has often been reported that reagents have an impact on the outcomes, particularly with biofilm studies (Chen *et al*., 2020; Kostenko, Ceri, & Martinuzzi, 2007; Stepanović *et al*., 2001, 2007). Chen *et al* found that biofilms cultured with TSB were much more difficult to eradicate compared with biofilms grown in ca-MHB. This highlights the importance of validation stages of susceptibility projects beyond the standardisation of inoculum.

The MTA has often been referred to as the gold standard for biofilm detection. It is understandable as a large amount of research has been undertaken into the validity of the model compared to other methods such as the tube method and the Congo red agar method for detection of biofilm cells (Hassan *et al*., 2011; Antunes *et al*., 2010; Stepanović *et al*., 2007; Knobloch *et al*., 2002). Interestingly the impact of model choice on *S aureus* antimicrobial challenge has also been documented by failure of treatment with different model choices, by changing from a single pass drip reactor to a static microtiter model (Alonso *et al*., 2018). It was previous comparisons that fuelled the decision to compare methods used in this study to observe if the CBD and microtiter plate had similar differences in the results, as the studies previously referenced had large changes (drip reactor to static MTA) and there were far fewer mechanical differences between the CBD and MTA. Both methods have often been used to observe significant resistance in susceptible strains (Antunes *et al*., 2010; Stepanović *et al*., 2007; Ceri *et al*., 1999). However, as this study has observed that there is a large discrepancy in the density of the biofilms formed in each culture method. The density of a biofilm, sometimes referred to as the maturity

of a biofilm, has a significant impact on the results of antimicrobial challenge. The impact of the cell number has been documented well, with inoculum impact studies showing increases in resistance correlate directly with CFU. Ryback *et al*. observed susceptibility of *S aureus* to vancomycin at 10^6 CFU/g however when increasing the inoculum to 10^9 CFU/g, little to no change in CFU was elicited for the biofilm cells (Rybak, 2006). Equally, Kragh *et al*. reported no significant killing of cells was elicited by tobramycin for 24- and 48-hour biofilms, therefore the reported resistance seen in this study is in accordance with previous reports of tolerance and resistance to antimicrobials in this method (Kragh *et al*., 2019; Mah & O'Toole, 2001).

To further investigate the impact model choice had on biofilms, we tested both the CBD and MTA using gentamicin, vancomycin, and clindamycin. We found significant differences in susceptibility of the biofilms to antimicrobials depending on the model alone (Table 1). The data from this study reflects previous findings that for *S aureus*, the MTA MBEC results are significantly higher than MBEC values using the CBD, with some studies reporting >8000 μg/mL as an MBEC using the MTA and <2 μg/mL for the CBD (Chen *et al*., 2020; Castaneda *et al*., 2016; Coraça-Hubér *et al*., 2012). This indicates that the same strain can be over 100 times more resistant to the same antimicrobial when tested in a different biofilm model. Suggesting that the choice of model is critical in the investigation of the antibiofilm effect of antimicrobial formulations, and that susceptibility may be overestimated when an inappropriate model has been chosen. The discrepancies between the CBD and MTA support that statement, and when hypoxia is considered, there are further differences in the responses not associated with antimicrobial treatment alone. Interestingly, the impact of hypoxia was greater on the perceived resistance in the CBD compared with the MTA, this may be due to a multitude of factors, such as the maturity and recalcitrance associated with mature biofilm formation- as the biofilms in the CBD are much less developed the impact of environmental pressures is larger than would be in a mature, robust biofilm.

The lack of susceptibility under oxygen limited conditions to vancomycin specifically in this strain is corroborated by a study by Larsson *et al*, that observed a decrease in the effectiveness of vancomycin under anaerobic conditions (Larsson *et al*., 1996). The CBD results would support this, with a normoxic MBEC of 2 μg/mL and hypoxic MBEC of 32 μg/mL reduction in environmental oxygen renders vancomycin treatment less effective. However, these differences are not seen in the MTA biofilms, where the recovered CFU counts were not significantly different from normoxic results. In contrast to this, gentamicin was seen to be resistant when challenged in the MTA in normoxia, this concurs with previous research that demonstrated planktonic susceptibility and biofilm resistance even with high

concentrations of gentamicin (Hess, Henry-Stanley, & Wells, 2011). In contrast when in hypoxia there is a significant reduction in the biofilm CFU, this is contrary to current research on gentamicin. Gentamicin relies on oxidative metabolism for transport into bacterial cells, which suggests that susceptibility will significantly diminish in hypoxia. There are many studies that have demonstrated reduction in the efficacy of gentamicin in anaerobiosis (Harrell & Evans, 1977; Reynolds, Hamilton-Miller, & Brumfitt, 1976). With that research considered, it can be suggested that the decrease in CFU seen in the MTA is a result of the hypoxic environment enabling oxidative metabolism to still occur within the mature MTA biofilm structure.

Biofilms in the CBD were reported to be much more susceptible to antimicrobials than the MTA results, one reason for this may be due to differences in biofilm density, the CBD facilitated much less dense biofilms which can be referred to as less mature biofilms as often density is associated with the maturation of the biofilm (Kragh *et al*., 2019). In this study, biofilms were far denser in the MTA than in the CBD, as can be seen in figures 11 and 12. There was a decrease from the MTA results for the vancomycin MBEC when using the CBD, which indicates that biofilm density (in terms of CFU) has a significant effect on the antimicrobial susceptibility (Figure 12, Table 1) (Rybak, 2006; Larsson *et al*., 1996). These findings are supported by results from previous studies that determined that dispersed biofilm bacteria have similar intrinsic susceptibility to planktonic cells and suggest that intact biofilm bacteria gain their recalcitrance from EPS and persisters (Hess, Henry-Stanley, & Wells, 2011). Clindamycin is a protein synthesis inhibitor for the treatment of Gram-positive infections, it has been shown that the presence of clindamycin in subinhibitory concentrations can induce increased production of phenol soluble modulins in *S aureus* (Schilcher *et al*., 2016; Yamaki, Synold, & Wong-Beringera, 2013; Joo *et al*., 2010). It has been observed that clindamycin in sub-inhibitory concentrations increases biofilm formation in terms of higher viable bacteria counts and increases in attachment, therefore the recalcitrance seen in this study could be as a result of previously observed cell wall thickening and increased biofilm formation in response to clindamycin (Schilcher *et al*., 2016; Nakao *et al*., 1972). This supports the results in this study wherein high amounts of clindamycin elicited no reduction in CFU of the biofilms tested here.

Overall, this study has demonstrated that MBECs and resistance to antimicrobials is variable dependent on the model selected. It is important to decide on the maturity of the biofilm within the model, and if the bacterium chosen for susceptibility testing is suitable for that chosen model. Biofilm resistance is indeed independent to the MIC profile - individual biofilm testing of clinical isolates is worth the time and effort from chronic biofilm infected sites as there is significant evidence to support discordant

results from MIC to biofilm susceptibility, as observed from the results shown here (Müsken *et al*., 2017).

2.8 Conclusion

The main aims of this study were.

1. To determine if hypoxic conditions have an impact on *S aureus* in planktonic antimicrobial susceptibility testing
2. To compare the biofilm formation in two static, high throughput biofilm devices in terms of:
    a. Biofilm formation and colony forming units.
    b. Antimicrobial response
    c. Impact of hypoxia on antimicrobial response and biofilm development
3. To observe the effects of Tween20 on CFU counts in biofilm antimicrobial susceptibility testing

The general findings from this study indicate that in the context of *S aureus* ATCC 29213: (1) Hypoxia is an important parameter to consider during planktonic antimicrobial susceptibility testing, often eliciting significantly different results. (2) The Calgary Biofilm device has a much-reduced biofilm formation compared to the microtiter plate method using *S aureus* as a model organism. (3) Hypoxia does not affect the cell count or biomass of untreated biofilms grown using either the CBD or MTA but affects the antimicrobial susceptibility in a drug specific way. (4) MBEC is variable dependent on the choice of model. (5) Tween20 reduces the CFU recovered from the MTA.

Antimicrobial resistance is a global crisis with a limited outlook in terms of novel antimicrobials arriving to market. Although the failure of these drugs is multifactorial, this study suggests that poorly considered models may have overinflated the success of new formulations, setting them up to fail. Including new standards to add relevant parameters such as hypoxic incubation can yield drastically different susceptibility profiles. This can give more control to the organisations and researchers developing drugs to see a better picture of how their drug may behave in a clinical setting.

Hypoxia and biofilms are often seen in clinical *S aureus* infections, and this study has shown that changing parameters to better mimic the host infection environment can yield significantly different results. The use of standardised testing can miss out on drug specific responses to environmental pressures, as seen in the case of the increased resistance in vancomycin, clindamycin and vancomycin in the CBD biofilms tested in hypoxia.

When considering the results of this study, it can be recommended that CBD should not be used for non-motile organisms such as *S aureus*, intead MTA should be used as the resulting biofilms are more dense and therefore may better represent the propensity of biofilms to resist or tolerate antimicrobial treatment. Additionally, it can be concluded that full panels with multiple parameters such as environmental oxygen and biofilm formation can enable researchers to have better control over the correct selection of model for a clear view on the resistance to antimicrobials going forward.

Chapter 3: A Novel High-Throughput *Ex vivo* Ovine Skin Wound Model for Testing Emerging Antibiotics

The work in this chapter is taken from published work in : Regan, H.C., Taylor, A.F., Karunakaran, E., 2022. A Novel High-Throughput *Ex Vivo* Ovine Skin Wound Model for Testing Emerging Antibiotics. Journal of Visualized Experiments. https://doi.org/10.3791/64041

3.1 Introduction

Skin infections are an important global issue, with large economic costs to healthcare providers around the world. The development of multidrug resistance and biofilm formation by pathogens plays a key role in the prevalence of non-healing wounds (Rahim *et al.*, 2017; Guest, Fuller and Vowden, 2018; Sen *et al.*, 2009; Claeys *et al.*, 2018). As a result of this, skin and soft tissue infections are one of the more common reasons for extended hospitalisation and subsequent readmission (Wilcox and Dryden, 2021). Delays in wound healing are costly for both the patient and healthcare providers, with some estimates suggesting around 6.5 million patients are affected annually in the US. In the UK, skin infections and associated complications result in approximately 75,000 deaths annually (Rahim *et al.*, 2017; Sen *et al.*, 2009; Han and Ceilley, 2017b).

Staphylococcus aureus (S. aureus) is a formidable wound pathogen frequently isolated from patient wounds (Sen *et al.*, 2009; Percival *et al.*, 2011). The rate of emergence of multidrug resistance increased drastically in the 2000s. During this time, around 60% of acute bacterial skin and skin structure infections were culture positive for methicillin-resistant *S. aureus* (Claeys *et al.*, 2018). The increasing number of multidrug-resistant strains among *Staphylococci*, and indeed other pathogens, within the last two decades indicates an urgent need for the rapid development of antibiotics with new modes of action that can overcome resistance.

However, since the early 2000s, antibiotic discovery programs have been dominated by longer developmental times and low success rates, with only 17% of novel antibiotics entering clinical trials in the US achieving market approval (Dheman *et al.*, 2021). This suggests a disparity between results from *in vitro* testing of emerging antibiotics and their clinical outcomes. It can be contended that this disparity is largely due to differences in bacterial physiology during infections *in vivo* and during conventional microbiological methods when testing the efficacy of antibiotics in the *in vitro* preclinical

stages. Therefore, novel laboratory methods that are more representative of bacterial physiology during infection are needed to improve the success rates in antibiotic discovery programs.

Current methods for studying skin infections include studies in live animals (e.g., mice), *ex vivo* skin models (e.g., porcine), and 3D tissue-engineered skin models (e.g., human) (MacNeil, Shepherd and Smith, 2011; Yang *et al.*, 2013; Malachowa *et al.*, 2013; Brandenburg *et al.*, 2018). Studies in live animals are strictly regulated and have relatively low throughput. In animal models, wounding and infection cause significant distress to the animals and raise ethical concerns. Human skin models, *ex vivo* or tissue-engineered, require ethical approval, compliance with local and global legislation (the Human Tissue Act, the Declaration of Helsinki), and there is difficulty in acquiring tissues, with some requests taking years to fulfil (Bledsoe and Grizzle, 2022; Danso *et al.*, 2015). Both model types are labour intensive and require significant expertise to ensure experimental success. Some current *ex vivo* skin infection models require pre-inoculated discs and additives for the wound bed to enable infection; although these models are incredibly useful, there are limitations in the infection process as additives limit the utilisation of the wound bed as a nutrient source (Yang *et al.*, 2013; Torres *et al.*, 2020; Zhao *et al.*, 2010; Schierle *et al.*, 2009). The model described in this study uses no additives to the wound bed, which ensures that the pathology of infection and viable cell counts are a result of direct utilisation of the wound bed as the only nutrient source.

Given the need for new laboratory methods, a novel high-throughput *ex vivo* ovine model of skin infections for use in evaluating the efficacy of emerging antibiotics has been developed. Skin infection studies face many challenges—high costs, ethical concerns, and models that do not show a full picture (Maboni *et al.*, 2017; MacNeil, 2007). *Ex vivo* models and 3D explant models allow for better visualisation of the disease process and the impact treatments can have from a more clinically relevant model. Here, the setup of a novel ovine skin model is described, which is simple, reproducible, and clinically relevant and has high throughput. Ovine skin was chosen as sheep are one of the large mammals commonly used to model responses to infections *in vivo*. Moreover, they are readily available from abattoirs, ensuring a steady supply of skin for research, and their carcasses are not scalded, ensuring good tissue quality. This study used *S. aureus* as the exemplar pathogen; however, the model works well with other microorganisms.

3.2 Aims

The main aims of this study are:

1. To investigate the suitability of ovine skin for use as an *ex vivo* wound infection model
2. To determine a suitable decontamination procedure for the ovine skin following collection and harvest
3. To investigate the ability of *ex vivo* ovine skin as a reproducible, high throughput infected wound model

3.3 Methodology:

Lambs' heads from the R.B Elliott and Son Abattoir were used as the source of skin explants in this project. All lambs were slaughtered for consumption as food. Instead of discarding the heads, these were repurposed for research. Ethical approval was not required as the tissue was sourced from waste discarded from abattoirs.

3.3.1 Media Composition

Antibiotic free medium was prepared by filter sterilising MK medium (Medium 199) with Hanks' salts, L-glutamate, and 1.75 mg/mL sodium bicarbonate (M0393, Sigma-Aldrich) with added Ham's F12 in a 1:1 ratio (N4888, Sigma-Aldrich), with added FBS (10% v/v), (F9665, Sigma-Aldrich), EGF (10 ng/mL) (E5036, Sigma-Aldrich) , insulin (5 µg/mL), 91077C, SAFC), and for the antibiotic media penicillin-streptomycin (100 U/mL), (P0781, Sigma-Aldrich), and amphotericin B (2.5 µg/mL), (A2942, Sigma-Aldrich) was added.

3.3.2 Assessment of skin sterilisation

To determine the most appropriate method to sterilise the explants a series of experiments were planned to investigate a range of chemicals and sterilisation times (Table 2). The following chemicals were made up according to the manufacturer's instructions: 1% (v/v) Distel (Tristel, TM305), 1% (v/v) Virkon (SLS, CLE1552), 3% (v/v) Videne (Ecolab, 3030440), 5% (v/v) Videne, 10% (v/v) Videne, 70% (v/v) ethanol, UV (254nm UV-C, Thermo Scientific Safe 2020 Class II Biological Safety Cabinet), 0.5% (v/v) sodium hypochlorite (Sigma Aldrich, S5881) and 200 ppm Vimoba solution (Quip labs, VMTAB75BX). Working dilutions were made in sterile distilled water.

Explants were processed as described in section 3.3.5 and placed into PBS following removal from the head. In triplicate, explants were placed into each disinfectant, for either 10, 30 or 60 minutes. After the disinfection time had elapsed, the explants were removed and washed again in PBS for 1 minute prior to placement into a 24 well plate containing antibiotic free medium. The explants were then incubated at 37 °C in a humidified 5% CO_2 tissue incubator for 24 hours. Following incubation, the explant media was examined for evidence of turbidity or fungal growth. Explant media that did not exhibit turbidity or fungal growth was determined as successful sterilisation. This experiment was conducted 3 times using biologically different lambs to ensure that any contamination, turbidity or fungal growth was due to the inefficiency of the chemical to sterilise the explant within the allotted time.

3.3.3 Assessment of explant condition post sterilisation

After appropriate chemicals were selected following the experimentation in section 3.3.2, the sterilisation chemicals and the timings of sterilisation were assessed for potential to cause damage to the explants. An untreated control was used, by placing the explant into PBS this would allow the comparison the effects of the sterilisation chemicals on the explants.

Explants were sterilised using the same methods used in 3.3.2, and after the sterilisation process was finished, the samples were washed in sterile PBS. The explants were then immediately placed into neutral-buffered formalin and subsequently sent for histological analysis (described in section 3.3.4).

3.3.4 Histology

3.3.4.1 Sample preparation

Explants were processed in the Department of Infection and Immunity in the Medical School. Explants were preserved in 10% (v/v) neutral buffered formalin for 24 hours prior to placement into cassettes and embedding in paraffin. Subsequently, sections were cut from the preserved explant and mounted onto slides. The slides were dewaxed by washing twice with xylene for 5 minutes, followed by graded rehydration at ethanol concentrations of 100%, 95%, and 70% (v/v) for 5 minutes each, and then rehydration in running water for 5 minutes

3.3.4.2 Haematoxylin staining

Gill's haematoxylin was applied to the slides for 2 minutes, followed by a rinse in running water. Scott's tap water was added for 10-20 seconds, then the slides were rinsed again in running water. Eosin was applied for 5 minutes, followed by another rinse in water. The samples on the microscope slides were drained and dehydrated through three dips in increasing ethanol concentrations of 70%, 95%, and twice at 100% (v/v). Finally, the samples on the microscope slides were transferred into xylene and protected with coverslips.

3.3.4.3 Gram staining

Crystal violet solution was applied to the samples on the microscope slides for 1 minute, then thoroughly rinsed with deionized water. The samples on the microscope slides were treated with Gram's iodine mordant solution for 5 minutes, then rinsed and blotted. Differentiation was performed using absolute alcohol and rinsed with deionized water once more. The samples on the microscope slides were stained with Safranin O for 60 seconds, then rinsed and blotted again. The sections were

treated with Tartrazine solution for 5 seconds, then blotted. The samples on the microscope slides were dehydrated through three dips in increasing ethanol concentrations of 70%, 95%, and 100% (twice), transferred into xylene, and protected with coverslips.

3.3.4.4 Imaging of Histology sections

Sections were imaged using an Olympus BX51 Fluorescence Microscope and ProgRes CapturePro software located in the Department of Chemical and Biological Engineering.

Following on from the initial investigation into the most suitable process for sterilisation to achieve sterile explants that maintain their initial tissue integrity, the following methods were developed.

3.3.5 Preparation of the explants

The forehead section of the lamb was disinfected by washing with 100 mL of 200 ppm chlorine dioxide solution onto the sample area. The sample area was then shaved and washed again, prior to further disinfection with ethanol and application of hair removal cream. The hair removal cream was left for 35 minutes before removal and a final wash step using 200 mL of Vimoba.

Sterile 8 mm biopsy punches were used to cut out explants from the sample area. Once explants were removed from the sample area all cutaneous fat was removed using a scalpel and samples were placed into sterile phosphate buffered saline (PBS). Explants were then transferred into 50 mL of 200 ppm chlorine dioxide solution, and left to sterilise for 30 minutes at room temperature.

Samples were removed from the Vimoba solution and washed in sterile PBS. Once washed, samples were placed into 24 well plates, and 350 µL of pre-warmed antibiotic medium was added, with care taken to maintain the explant at the air-liquid interface. Gas permeable plate seals were applied to the plates and explants were incubated at 37 °C in a humidified 5% CO_2 tissue incubator for 24 hours. After incubation, spent media was removed and explants were washed in 500 µL of sterile PBS. Following washing, antibiotic-free media was added to each explant and incubated at 37 °C for 24 hours to remove residual antibiotics in the explant ready for inoculation.

3.3.6 Preparation of the inoculum

S. aureus ATCC 29213 was cultured on Tryptic Soy agar (Vegitone: 22091, Millipore, UK) at 37°C overnight. Several colonies were taken from the cultures and inoculated in 5 mL of Tryptic Soy broth

(Vegiton: 14432, Millipore, UK) and incubated at 37°C in a shaking incubator overnight. Inoculum was prepared by centrifugation of overnight culture at 4000 × g for 3 minutes with the supernatant discarded and sterile phosphate buffered saline (PBS, P4417 Sigma-Aldrich) wash added. The wash was repeated 3 times then resuspended in sterile PBS, then was adjusted to 1 $\times 10^7$ CFU/mL using a spectrophotometer and sterile PBS to dilute, ready for inoculation onto the wounded skin samples.

3.3.7 Infection of skin explants

Explants were removed from the incubator, spent media was removed and washed in PBS. Explants were then placed one at a time into a petri dish for the wounding process. Explants were initially inspected to ensure removal of all cutaneous fat had been achieved at the collection stage. Then, a 4mm biopsy punch was used to puncture the sample whilst allis toothed tissue forceps (Rocialle, RSPU500-322) were used to secure the edge of the biopsy. A 15mm blade scalpel (Fisher Scientific, O305) was used to gently remove the top layer of skin from each sample. Wounded samples were then placed into Transwell inserts with a 0.4 µm membrane (VWR, 353095) and 200 µL of antibiotic free medium was placed into the posterior chamber of each well.

The infection of the explants with *S aureus* was performed by adding 15 µL of standardised inoculum (1.5×10^5 CFU/explant) to the apical wounded surface area of each sample. The 24 well plates were then incubated either normoxically in a Heracell Vios 160s with 5% CO_2 at 37 °C.

Antibiotic free media was changed at 24 hours post infection, taking care to not disturb the wound bed. Antibiotic treatment was added to the wounded surface of the sample in the apical chamber immediately following the media change.

3.3.8 Determination of the bacterial load

The bacterial load at 24 and 48 hours was investigated to determine which incubation time point would lead to a reproducible and robust infection that could be used for future antimicrobial susceptibility testing.

After 24 and 48 hours of infection samples were placed into 1 mL sterile PBS and vortexed for 30 seconds continuously to disrupt the biofilm on the wound bed. Each sample was then vortexed for 10 seconds immediately prior to a 20 µL sample being removed. This step was to resuspend the bacterial cells to ensure a more homogenous solution. The 20 µL was subsequently placed into a 96 well plate with 180 µL of sterile PBS, each sample was serially diluted 10^{-1} to 10^{-8}, and plated onto tryptic soy agar

plates and air dried. The plates were then incubated for 18 hours. Following incubation, the colonies were counted and colony forming units (CFU) per explant were recorded.

$$CFU\ per\ explant = average\ no. colonies\ \times \frac{1}{0.01} \times dilution\ factor$$

3.3.9 Statistical Analysis

Data was initially input into Excel spreadsheets to calculate the CFU/explant. CFU/explant data was then transferred into GraphPad Prism for statistical analysis and graph synthesis. An unpaired t test was used to compare the CFU of the wounding methods and time of incubation with each other.

3.4 Results

The identification of a route to sterilise the skin before setting up the wound infection model was challenging. The challenge lay in sterilising the skin without damaging the different skin layers, which may then go on to have unintended consequences in the outcome of infection. To identify an appropriate sterilisation regime, different treatments were tried for varying lengths of time, as outlined in Table 2. Contamination was recorded as the development of turbidity after 48 h in the MK medium used to maintain the skin samples. Tissue integrity was monitored by histology followed by staining with haematoxylin and eosin (H&E) immediately after treatment (Figure 15). A 30 min treatment with chlorine dioxide proved the most effective at reproducibly sterilising the skin tissue whilst preserving tissue integrity.

Table 2 Each skin sample was left in the disinfectant for the specified time. F denotes failure to sterilise the tissue, with bacterial contamination present in the media. P denotes passing, with no bacterial contamination present in the media. (n=6, from 3 repeated experiments)

Disinfectant	10 Minutes	30 Minutes	60 Minutes
1% Distel	F	P	P
1% Virkon	F	P	P
3% Videne	F	F	F
5% Videne	F	P	P
10 % Videne	F	P	P
10% Ethanol	F	P	P
UV	F	F	F
0.5% Hypochlorite	F	P	P
200ppm Vimoba tablets	F	P	P

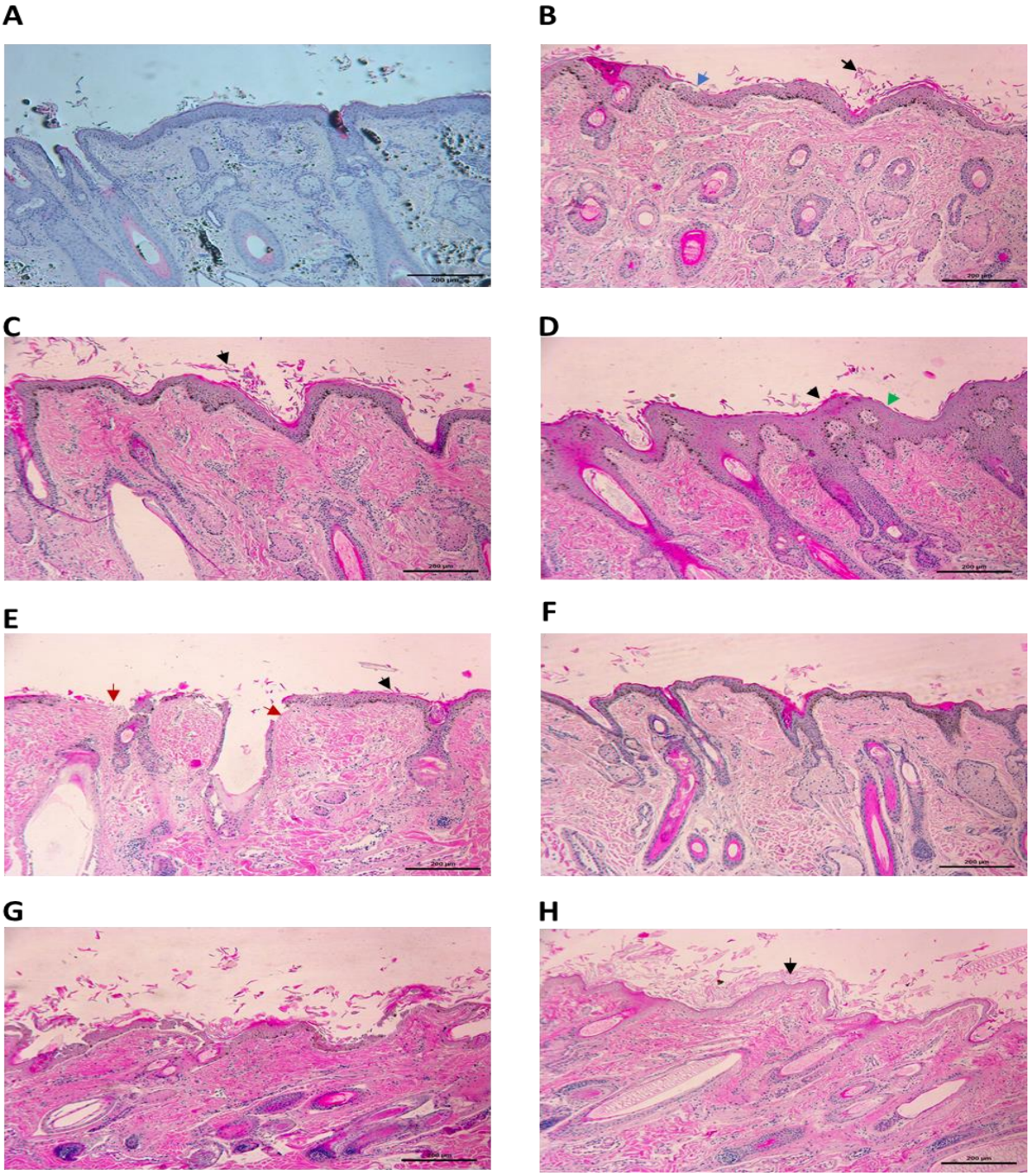


Figure 15 Histology of uninfected *ex vivo* ovine skin treated with disinfectants (H&E stain) at 100x magnification. (A) Control skin sample with 30 min treatment in PBS. Some epidermal shedding can be seen, but the epidermis is not disrupted. (B) Ovine skin treated with 1% Virkon for 30 min. Epidermal shedding (black arrow) along with some disruption to the stratum granulosum (blue arrow). (C) Ovine skin treated with 5% Videne for 30 min. Moderate damage to the tissue can be seen here, with epidermal shedding of the stratum corneum (black arrow). There is minimal disruption to the underlying epidermal layers. (D) Ovine skin treated with 10% Videne for 30 min. The top layers of the epidermis have been damaged, with evidence of shedding (black arrow) and thinning (green arrow). (E) Ovine skin treated with 1% Virkon for 30 min. Severe damage to the sample can be observed, with significant epidermal shedding (black arrow) and complete eradication of the stratum corneum (red arrow). (F) Ovine skin treated in 200 ppm Vimoba for 30 min. Some epidermal shedding can be seen, but the epidermis is intact. (G) Ovine skin treated with 0.5% sodium hypochlorite for 30 min. Severe damage to the epidermis is present, with a high level of epidermal shedding (black arrow) and eradication of the epidermis in places (red arrow). (H) Ovine skin treated with 70% ethanol for 30 min. Damage to the epidermis can be seen, with significant epidermal shedding (black arrow). Scale bar is 200 µm.

In the experimental setup, the wounded sterile tissue is placed in the apical chamber of a 24-well insert at the air-liquid interface (Figure 16). Two different wounding techniques were attempted—a flap removal technique, in which the tissue is wounded by a punch biopsy tool and the top layer of the wounded tissue is removed using a combination of a 15-blade scalpel and sterile toothed allis tissue forceps, and a scratch technique, in which the tissue is wounded by a punch biopsy tool alone. Although the scratch model harboured a higher average number of CFUs after 24 h, the results were variable. The flap removal technique produced more consistent results (Figure 18). After 48 h, approximately 100 times more bacterial CFUs were recovered from the wounded tissue compared to the inoculum (Figure 17). This was deemed to indicate a successful infection. A white film in the wound bed was evident on the tissue 48 h after infection (Figure 17).

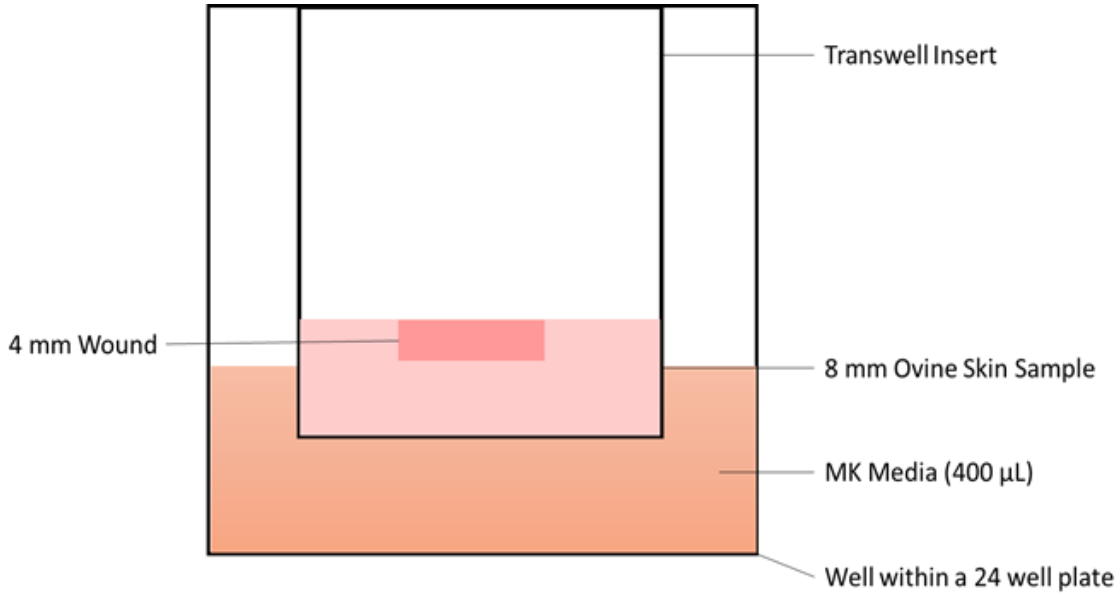


Figure 16 Schematic of the experimental setup.

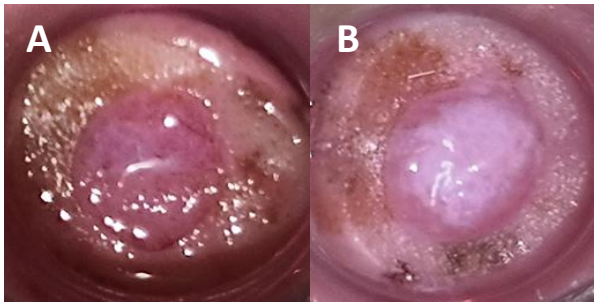


Figure 17 Pictures of ex vivo ovine skin prior to infection (A) and post 48 h infection (B). Note the white film present following 48 h incubation in infected tissue, which is absent in uninfected tissue.

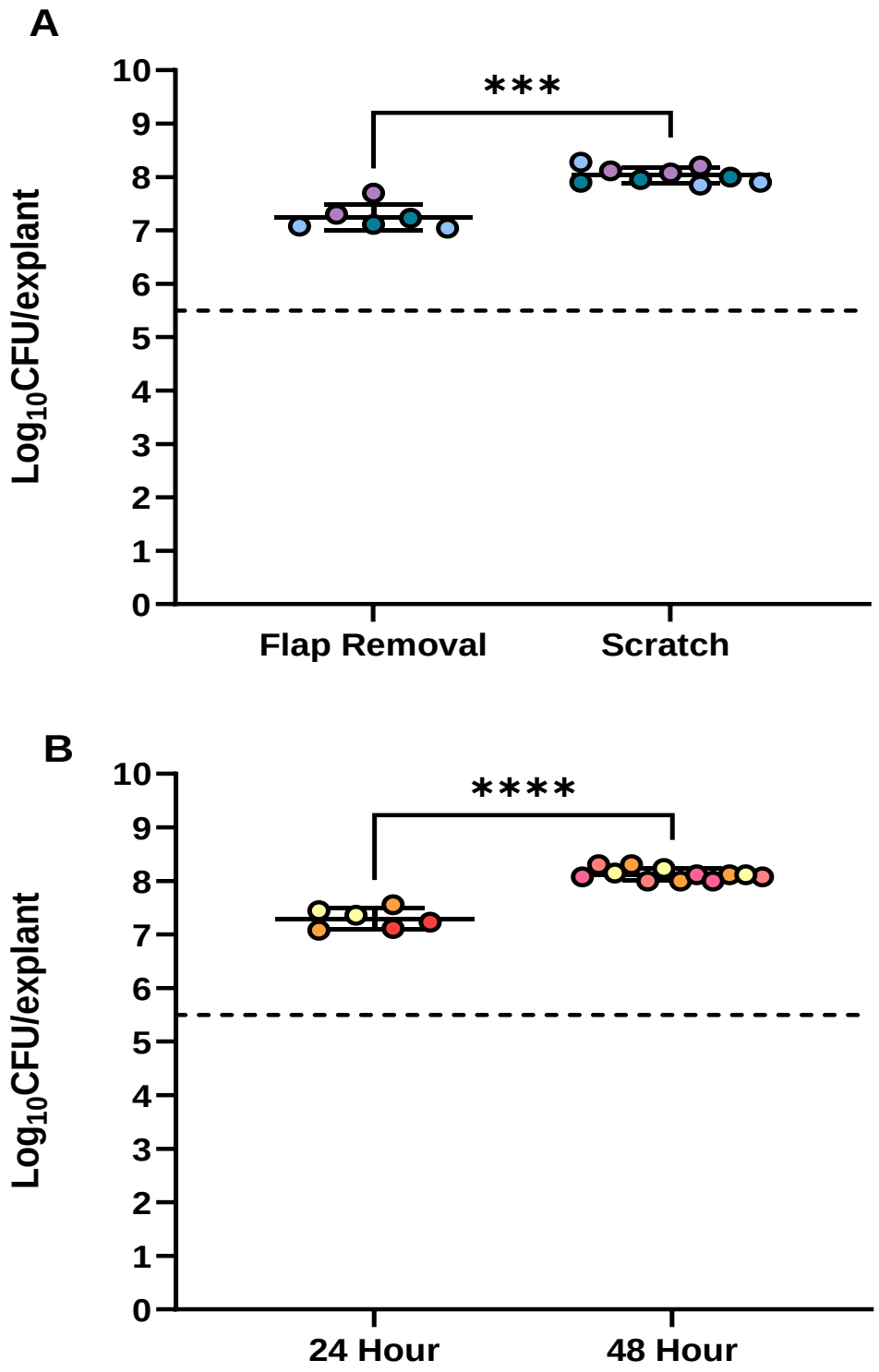


Figure 18 (A)Testing the effect of different wounding methods on the final colony forming unit (CFU) counts after homogenization. Flap removal (n = 6) and scratch (n = 9) infected with S. aureus for 24 h. Error bars indicate standard deviation. *** indicates a p-value < 0.0001. (B) Testing the effect of incubation times on CFU counts following homogenization. 24 h incubation (n = 6) and 48 h incubation (n = 12). Unpaired t-test, Error bars indicate standard deviation. **** indicates a p-value < 0.0001, Different colours indicate different donor heads. Results are from 3 repeated experiments.

An important consideration when setting up this model was whether there would be donor to donor variation in the explant CFUs. To observe any variation, samples from different donor heads were separated by colour as can be seen in Figure 18. In Figure 18, it can be observed that there is minimal clustering of data points from donor to donor. This suggests that there is minimal variation in the CFU counts from different donor skin, indicating that use of multiple donors is a feasible option going forward.

Gram-stained histology sections of infected tissue indicated the presence of *S. aureus* cells in the wound bed (Figure 19 A, B). This is evidenced by the presence of cocci aggregated on the surface of the explants 24 hours after inoculation with 1.5×10^5 CFU/explant of *S aureus*. The cocci are stained purple, which is representative of the presence of gram-positive *S aureus.* These areas are marked using the black arrows. Gram-stained histology sections of uninfected tissue are provided as a comparator (Figure 19 C, D).

These images show similarly clustered cells as observed in Figures 12 and 13 of the *in vitro* assay SEM micrographs. In the SEM micrographs it could be observed that there were surface aggregated microbial cells, adhering not only to the abiotic surface but also to other cells forming what may be called a biofilm. Interestingly, for the histological examination of the infected explants in this study it can be observed that there are Gram positive cocci adhered to the surface of the explant and also to other cocci. However, the resolution of these images are not of the same quality as the SEM micrographs. Therefore, the evidence of cocci on the surface of the explant suggests there may be the presence of a biofilm, yet further confirmation using SEM is required to confirm that the surface aggregated cocci have formed a biofilm.

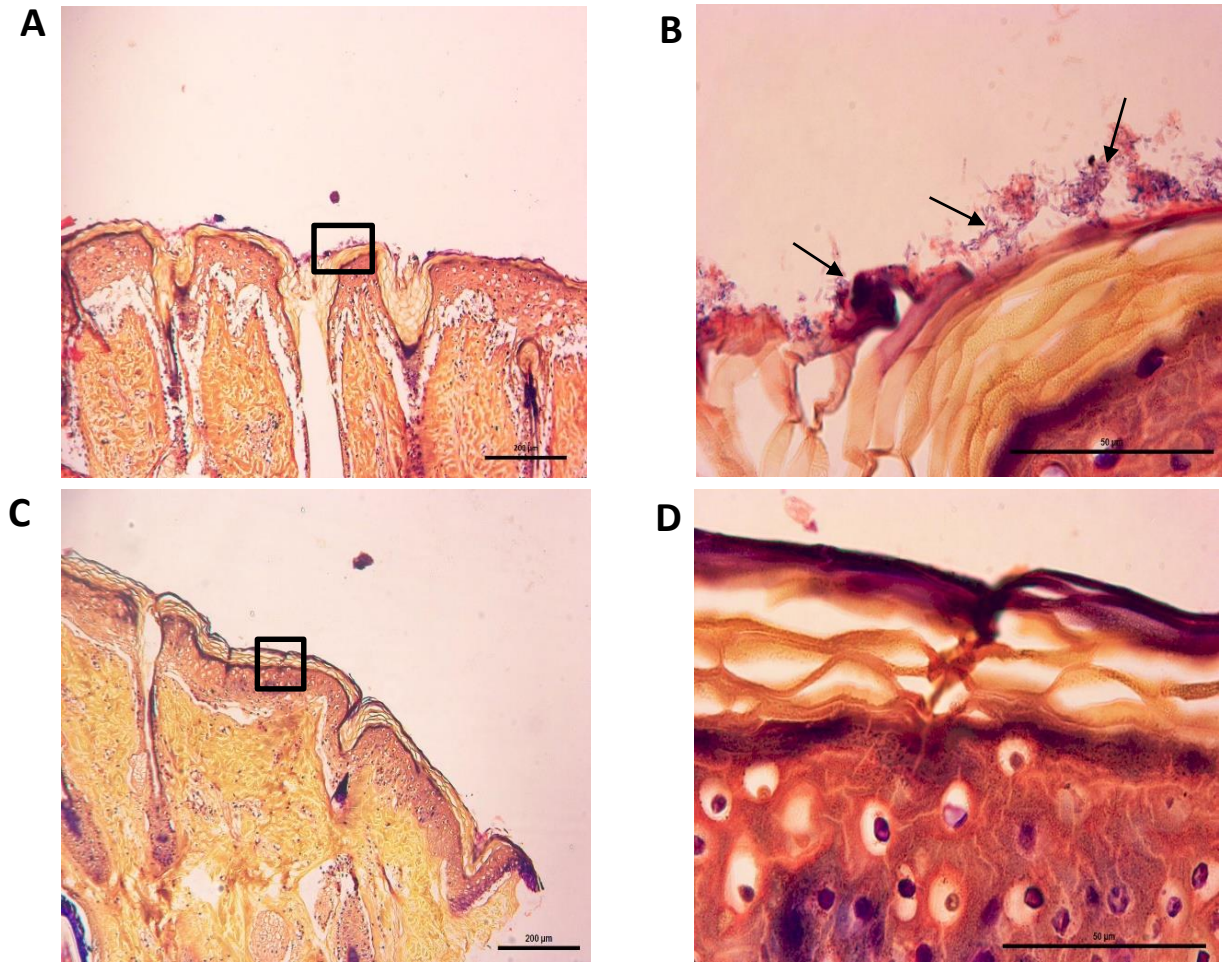


Figure 19 Representative images of histopathological analysis of infected ovine skin post 48 h infection with a modified gram stain (A) at 100x magnification (scale bar of 200 µm). The box indicates the area magnified in (B) at 1,000x magnification (scale bar of 50 µm). Black arrows indicate bacteria. (C,D) Representative images of histopathological analysis of uninfected ovine skin following a 48 h period (control) with a modified gram stain (C) at 100x magnification (scale bar of 200 µm). The box indicates the area magnified in (D) at 1,000x magnification (scale bar of 50 µm).

For the purposes of this model, it was essential that the method be scalable and high-throughput due to the necessity for drug development as previously discussed. Therefore, it was calculated that from one lamb's head, a 100 cm^2 section (10 cm x 10 cm) can be realistically accessed. Given each patch of skin is punched out of the forehead using a circular 8 mm diameter punch biopsy, approximately 100 skin patches can be obtained from one lamb's forehead per week. Procuring further lambs' heads proportionately increases the number of skin samples that can be obtained per week. Hence, it is claimed that the procedure is relatively high throughput. For this study, 24 skin samples were obtained routinely per week. Skin from various lambs' heads was processed as biological replicates to account for the potential donor to donor variability. During the preparation of the skin explants, the major time consumption is the 30 min incubation of the skin samples in the chlorine dioxide solution and the 2 x 35 min wait for the hair removal cream treatment. The use of punch biopsy to generate the 24 skin patches takes about 15 min. It is this time that would scale linearly if the number of skin patches were increased.

3.5 Discussion

The development of antimicrobials is an important but expensive venture that is estimated to cost around $1 billion and take around 15 years to complete. Over 90% of antimicrobial drug discovery and

preclinical studies of antimicrobial drug efficacy are carried out by academic researchers and small to medium companies with typically less than 50 employees (Theuretzbacher *et al.*, 2019). These teams are very financially constrained, which makes the failure of lead molecules in later stages of translational research calamitous. The rise in antimicrobial resistance is outpacing the development of novel antimicrobials, which further intensifies the need to steward the already limited investment in antimicrobial research responsibly (Miethke *et al.*, 2021).

The disparity in bacterial physiology between infections in *in vivo* and *in vitro* laboratory cultures tends to cause an overestimation of the antimicrobial efficacy of the hits during the lead identification stage. Such overestimation contributes to the high attrition rates seen during the subsequent stages of translation. The availability of *in vitro* antimicrobial efficacy testing platforms that incorporate bacteria with a physiology that better mimics that found during infections *in vivo* will enable a more accurate estimation of antimicrobial efficacy and provide researchers with greater control of lead optimization without reliance on expensive and tightly regulated animal trials. Such models will also decrease attrition rates during the subsequent stages of translation and may make antimicrobial drug discovery more attractive for investment.

This ovine skin infection model described here is a useful tool that will provide researchers with a physiologically relevant *in vitro* model of an infected wound to test the efficacy of emerging topical antimicrobial formulations at the preclinical stages. In contrast to the majority of *ex vivo* infection models described in the scientific literature (Yang *et al.*, 2013; Torres *et al.*, 2020; Guedes *et al.*, 2021; Johnson *et al.*, 2021; Horton *et al.*, 2020; Ashrafi *et al.*, 2018; Brackman and Coenye, 2016), in this model, the pathogens exposed to the *ex vivo* tissue are not supplemented with culture media during infection. Pathogen proliferation and subsequent infection are dependent on the ability of the organism to damage the tissue. Therefore, the pathogen physiology in this model may be more closely related to that during *in vivo* infections compared to conventional microbiological culture. A highly reproducible infection is obtained from this model, as judged by the number of CFUs retrieved from the tissue after incubation.

With the plethora of human skin equivalents and human and porcine *ex vivo* wound models, the use of ovine tissue in this model could be superficially argued to be a limitation. However, sheep are among the group of large animals used as models of infections *in vivo* (Rumbaugh and Carty, 2011; Boase *et al.*, 2011; Alharbi *et al.*, 2022). Moreover, sheep have been used as surrogates for humans in immunology research and vaccine development, indicating that their immune responses are similar to those of humans (Scheerlinck *et al.*, 2008). The tissue repair process during wound healing is similar in

large mammals, including sheep and humans (Metcalfe and Ferguson, 2007; Kazemi-Darabadi *et al.*, 2014), and interventions to improve wound healing that have been successfully demonstrated in sheep have been suggested for translation to humans (Martinello *et al.*, 2018; Roberts and Windsor, 2019; Mazzone *et al.*, 2020). Therefore, the use of *ex vivo* ovine skin in this wound model is not a limitation. Furthermore, this ovine model can be judged to be a reliable alternative to models incorporating human or porcine skin for the following reasons. The availability of human skin is limited and of variable quality when available (Olkowska and Gržinić, 2022). Tissue-engineered human epidermis and living skin equivalents require refinements in their culturing conditions to ensure reproducibility in tissue physiology (Couto *et al.*, 2021). Although porcine skin is more accessible than human skin, the widely available porcine skin is scalded as part of carcass processing in the abattoir, which removes the epidermis (Yang *et al.*, 2013). From experience, unscalded porcine skin is less readily available from abattoirs and from supplementary figure 4 the histological examination of the available local porcine skin, significant damage was observed.

Throughout the protocol described here, there are critical steps that ensure the success of the model. The most critical step involves the sterilisation of the tissue following harvesting. Chlorine dioxide solution must be mixed with the tissue samples, and a contact time of 30 min must be allowed to sufficiently disinfect the tissue and remove contaminants. Furthermore, when setting up this experiment, turbidity or fungal contamination may occur due to improper handling or ineffective treatment with disinfectants and antibiotic media. In such a case, samples that develop turbidity must be discarded. To reduce the development of turbidity and contamination, the laboratory environment should be kept clean, with frequent sterilisation of the incubator, the use of filter tips, and ensuring complete sterilisation of the tools and containers used with the samples. Another critical step in the protocol is when the 4 mm biopsy punch is used to wound the tissue. The use of this tool allows for similarly sized wound beds to ensure the repeatability and reproducibility of the protocol. Variability in the wound bed size impacts the endpoint CFU. When there is adequate removal of the tissue flap, the results are much more consistent. A further critical step associated with the integrity of the endpoint CFU count is the use of the fine-tipped homogenizer to liberate bacterial cells. The position of the homogenizer tip was seen to have an impact on the CFU count from sample to sample; when the tip was correctly placed directly on the wound bed, there was much less variability seen from sample to sample.

Nevertheless, the model proposed here is not without limitations. This model has limitations inherent to all *ex vivo* studies (i.e., the lack of active vascular flow, the absence of commensal microbiota, which

may modulate infection progress, and the absence of immune cells). It can be argued that, since the *ex vivo* wound model does not incorporate active immune cells, the infection progression *in vivo* in the presence of these cells could be different from that observed in *ex vivo* models. Regardless, *ex vivo* models provide a tissue surface for attachment and a nutrient source for bacteria and present a 3D diffusion barrier to formulations, which enables a more accurate assessment of antimicrobial efficacy than conventional microbiology culture techniques.

3.6 Conclusion

The main aims of this study were:

1. To investigate the suitability of ovine skin for use as an *ex vivo* wound infection model
2. To determine a suitable decontamination procedure for the ovine skin following collection and harvest

3. To investigate the ability of *ex vivo* ovine skin as a reproducible, high throughput infected wound model

A key advantage of the model is that the tissue is sourced from lambs that are grown for human consumption. More particularly, the model repurposes skin from lambs' heads, which are usually discarded. Moreover, the model has high throughput and enables cost-effective, rapid, and reproducible comparative studies. It can be contended that the availability of this model will reduce and refine the need for animals purpose-bred for translational research in line with the principles of the three Rs, which facilitate more humane research practices (Percie du Sert and Robinson, 2018). Although the model described here incorporates *S. aureus* as the exemplar pathogen, the model can be used with other microorganisms including other bacteria, fungi, and viruses, thus broadening the scope of drug development that the model enables. It can be envisioned that the use of this model will enable the rapid translation of much-needed antibiotics for skin infections by providing researchers with greater control over drug design and formulation at the preclinical stages.

When reflecting on the conlusions from this chapter, it was decided that the use of chlorine dioxide as a disinfectant was most suitable to be taken forward for further experimentation, as it provided the most replicable decontamination with minimal damage to the ovine skin. The removal of the full skin flap and subsequent 48 -hour infection was also determined to be the most suitable set up to bring forward as it yielded a less variable infection when compared with the scratch model.

Chapter 4: *Ex vivo* ovine skin as a biofilm model

4.1 Introduction

Chronic wounds can be seen as a loss of skin integrity, as absent or delayed healing, often requiring some form of medical or surgical intervention over an eight-week period (Renner & Erfurt-Berge, 2017;

Han & Ceilley, 2017; Kimmel, Grant, & Ditata, 2016; Sen *et al*., 2009; Parsek & Singh, 2003). Chronic wounds are rarely found in healthy patients, they are often seen in those with underlying diseases such as diabetes (diabetic foot ulcers), obesity, vascular disease, surgical site infections, pressure ulcers and traumatic ulcers. It is commonly reported that chronic wounds are non-healing due to the predominantly elderly patient population, this is likely as a result of the development of immunosenescence as a person ages. (Rahim *et al*., 2017; Ventura *et al*., 2017).

Delay in wound healing is costly for both the patient and national health authority with some international figures suggesting around 6.5 million patients being affected annually in the US, and almost 75,000 annual deaths in the UK (Rahim *et al*., 2017; Han & Ceilley, 2017; Sen *et al*., 2009; Sen, 2009). Significant humanistic and economic burdens are placed on these two demographics, with a high proportion of patients with chronic wounds reporting depression and anxiety as a result of their condition (Olsson *et al*., 2018; Renner & Erfurt-Berge, 2017). A commonly isolated pathogen from these life-altering wounds is *S. aureus* (Percival *et al.*, 2010).

Percival (2010) identified colonisation of *S. aureus* in 88% of presenting chronic leg ulcers, with Rahim (2017) isolating *S. aureus* in 37% of chronic wounds analysed with *Pseudomonas aeruginosa* being the second most commonly isolated at 17% of isolates. Evidence suggests that *S. aureus* colonises chronic wounds more readily, with around 48% of isolates from chronic wounds, there is additional evidence to suggest that these isolates can readily form biofilms (Neopane *et al.* 2018). Neopane *et al*. (2018) analysed the *in vitro* biofilm formation of clinical isolates of wounds and found over 60% of isolates analysed formed biofilms *in vitro*, thus underlining the prevalence of the biofilm forming phenotype in wound research. This work aims to investigate the biofilm phenotype within a wound environment, and to apply hypoxia as an external variable to investigate the impact low oxygen may have on wound based biofilms.

4.2 Aims

The main aims of this chapter were:

1. To determine if the model established in Chapter 2 would be suitable for antimicrobial treatment of *S aureus*, and if hypoxia could be applied to the model.
2. To determine if the infections present were biofilms formed in the wound bed.
3. To investigate the metabolic activity and healing capabilities of the *ex vivo* ovine tissues

4.3 Methodology

4.3.1 Bacteria and Growth Conditions

S. aureus ATCC 29213 was cultured on Tryptic Soy agar (Vegitone: 22091, Millipore, UK) at 37°C overnight. Several colonies were taken from the cultures and inoculated in 5 mL of Tryptic Soy broth (Vegiton: 14432, Millipore, UK) and incubated at 37°C in a shaking incubator overnight. Inoculum was prepared by centrifugation of overnight culture at 4000 × g for 3 minutes with the supernatant discarded and sterile phosphate buffered saline (PBS, P4417 Sigma-Aldrich) wash added. The wash was repeated 3 times then resuspended in sterile PBS, then was adjusted to 1 $\times 10^7$ CFU/mL using a spectrophotometer and sterile PBS to dilute, ready for inoculation onto the wounded skin samples

4.3.2 Media Preparation

Both antibiotic enriched and antibiotic free medium were used in this study. Antibiotic free medium was prepared by filter sterilising MK media (Medium 199) with Hanks' salts, L-glutamate, and 1.75 mg/mL sodium bicarbonate (M0393, Sigma-Aldrich) with added Ham's F12 in a 1:1 ratio (N4888, Sigma-Aldrich), with added FBS (10% v/v), (F9665, Sigma-Aldrich), EGF (10 ng/mL) (E5036, Sigma-Aldrich) , insulin (5 µg/mL), 91077C, SAFC), and for the antibiotic media penicillin-streptomycin (100 U/mL), (P0781, Sigma-Aldrich), and amphotericin B (2.5 µg/mL), (A2942, Sigma-Aldrich) was added.

4.3.3 Skin Preparation and Infection

4.3.3.1 Sample Collection and Preparation

Healthy ovine heads were collected from a local abattoir post-slaughter. Environmental contaminants were removed from the forehead section using a chlorine dioxide solution (Vimoba 200 ppm) before removal of all hair from the sample area using clippers and hair removal cream (Veet). Explants were removed using an 8 mm biopsy punch and submerged in chlorine dioxide solution for 30 minutes to ensure sterility of samples. Explants were subsequently washed in PBS and immersed in antibiotic medium and incubated at 37°C for 18 hours. The explants were washed and incubated in antibiotic-free medium for a further 18 hours.

4.3.3.2 Skin explant wounding

Explants were wounded and infected in the same method as reported in Chapter 3. Briefly, 4 mm biopsy punches were used to puncture the explant and a 15mm-blade scalpel was used to remove the top layer of skin from each explant. Wounded explants were then placed into transwell inserts with 200 µL of antibiotic free medium in the posterior chamber of each well. A schematic of the wounded explant set up is in figure 20.

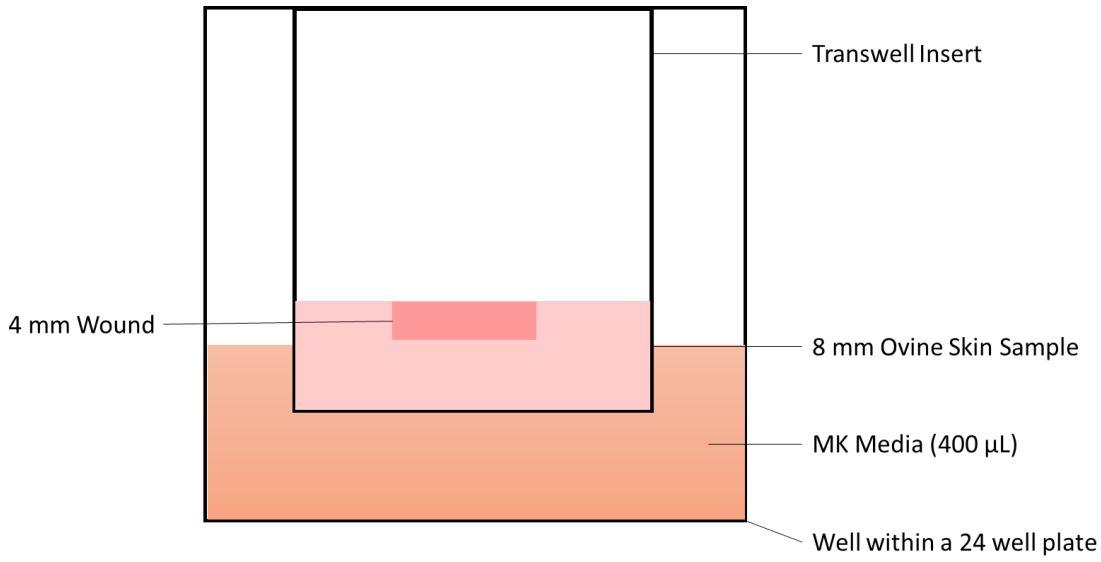


Figure 20 Schematic of the wounded ovine explant prior to inoculation.

4.3.3.3 Skin explant infection and Hypoxic Conditions

The infection of the explant with *S aureus* was performed by adding 15 µL of standardised inoculum (1.5×10^5 CFU/explant) (Section 4.3.1) to the apical wounded surface area of each explant. The 24 well plates were then incubated either normoxically in a Heracell Vios 160s with 5% CO_2 or hypoxically in a Heracell Vios 160s with 5% CO_2 and 1% O_2 at 37°C.

Antibiotic free media was changed at 24 hours post infection, taking care to not disturb the wound bed. Antibiotic treatment was added to the wounded surface of the sample in the apical chamber immediately following the media change.

4.3.3.4 Antimicrobial Treatment

The antimicrobials gentamicin (PHR1077, Sigma-Aldrich), clindamycin (PHR1159, Sigma-Aldrich) and vancomycin (SBR00001, Sigma-Aldrich) were selected for this study. Each antimicrobial was made up in sterile distilled water to form a 10 mg/mL Stock and filter sterilised using a 4 µm pore filter. The stock was kept frozen (-20°C) until use for a maximum period of 6 months. Working dilutions were made in sterile PBS for application to the infected samples.

Initial model validation experiments used gentamicin at a 0.1% and 1% solution at 20 µL and 100 µL to determine the most suitable volume and concentration of antimicrobial per sample. Following validation, 100 µL of 0.5% (5 mg/mL) was added to the apical wounded surface of each sample at 24 hours post infection and incubated at 37°C for 24 hours.

4.3.3.5 Recovery of CFU

After 48 hours of infection samples were placed into 1 mL sterile PBS and vortexed for 30 seconds continuously to disrupt the biofilm on the wound bed. Each sample was then vortexed for 10 seconds immediately prior to a 20 µL sample being removed. This step was to resuspend the bacterial cells to ensure a more homogenous solution. The 20 µL was subsequently placed into a 96 well plate with 180 µL of sterile PBS, each sample was serially diluted 10^{-1} to 10^{-8}, and plated onto tryptic soy agar plates and air dried. The plates were then incubated for 18 hours. Following incubation, the colonies were counted and colony forming units (CFU) per explant were recorded.

$$CFU\ per\ explant = average\ no. colonies\ \times \frac{1}{0.01} \times dilution\ factor$$

4.3.4 Wound Healing

4.3.4.1 Wound healing assay

Wounded sterile samples (as wounded in section 4.3.3.2) were incubated in the bottom of a 24 well plate in antibiotic media for 24 hours with care taken to maintain the air liquid interface (ALI). During a 10-day incubation the media was replaced every 24 hours. During media changeover samples were imaged using an iPhone camera. Each day, a single sample was removed from the assay and fixed by placing into 25 mL neutral-buffered formalin for histological analysis of the wound healing process over time. This process of sampling for histology was carried out for a 4-day period.

4.3.4.2 Image analysis

Pictures were taken of the wounded samples using an iPhone 12, these images were then analysed using ImageJ. The diameter of the wells was previously recorded in mm, and using ImageJ a pixel: mm ratio was set. Wounds were measured from the same reference points according to the sample (moles or skin discoloration were used to ensure the same diameter was being measured). The width was recorded and tabulated. Sample width was also recorded to ensure that the wound closure was not due to the shrinkage of the tissues over time.

4.3.5 XTT(2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-CARBOXANILIDE) tissue viability assay

Skin samples were assessed for their viability using an XTT assay as previously described in a paper by Elson *et al* (Elson *et al.*, 2015). Unwounded samples were incubated with antibiotic media for 24 hours

at 37 °C with 5% CO_2 under normoxia. Particular care was taken to maintain the ALI through careful movement of the plate, removal and addition of media.

Samples were removed from media using sterile forceps and then placed into a sterile 24-well flat bottom tissue culture plate, in the basal chamber. Then 1 mL of 1 mg/mL XTT solution with 1% of electron coupling reagent phenazine methosulfate (J61726-MD, Alfa Aesar) was added to the well, and the plates were incubated for 4 hours at 37°C in 5% (v/v) CO_2 in air with rocking. Following the incubation period, all XTT solution (1 mL) was removed and retained, then 500 µL of 100% DMSO (D/4120/PB08, Fisher Scientific) was added to extract the tetrazolium product from the tissues. Plates were incubated under rocking for 1 hour, then all of the DMSO extract (500 µL) was removed from each well in the plate. The DMSO extract and the XTT solution were separately added to a 96 well plate and absorbance was measured at 450 and 690 nm in a TECAN Spark Multimode Microplate reader. The OD was recorded, and samples discarded. The absorbances at 690 nm were subtracted from those at 450 nm. Samples were processed in triplicate.

$$XTT\ content\ per\ gram = average\ absorbance\ at\ 450nm - average\ absorbance\ at\ 690\ nm$$

4.3.6 Scanning electron microscopy (SEM).

4.3.6.1 Sample preparation for SEM.

Skin samples were wounded and infected as previously described and incubated for 12, 24 and 48 hours in either hypoxia or normoxia. The specimens were fixed by immersion in 2.5% (v/v) glutaraldehyde in PBS for a minimum of three hours at 4°C. The samples were then washed three times by submersion in PBS for 30-minute wash periods at 4°C. Secondary fixation was performed in 2% (v/v) aqueous osmium tetroxide for 1 hour at room temperature. Dehydration was accomplished through a graded series of ethanol, with 75%, 95%, 100% (v/v) ethanol for 15 minutes each, followed by drying over anhydrous copper sulphate for 15 minutes. All of the steps were performed at room temperature. The specimens were then placed in a 1:1 mixture of 100% (v/v) ethanol and 100% hexamethyldisilane (HEX, Merck) for 30 minutes, followed by 30 minutes in 100% hexamethyldisilane. The specimens were air-dried overnight, mounted on 12.5mm diameter aluminium pin stubs (G301P, Agar Scientific) with Leit Adhesive Carbon Tabs (AGG3347N, Agar Scientific).

4.3.6.2 Au sputter coating.

Edwards S150B Sputter Coater was used to coat the specimen with approximately a 25 nm thickness of gold (Au).

4.3.6.3 SEM microscopy.

Specimens were examined with the Tescan Vega3 LMU scanning electron microscope (EM Facility – The University of Sheffield) at a 10 kV operating voltage and micrographs were captured with the SE (Secondary Electron) detector.

4.3.7 Histology

4.3.7.1 Sample preparation

Samples were processed in the Department of Infection and Immunity in the Medical School. Samples were preserved in 10% (v/v) neutral buffered formalin for 24 hours prior to placement into cassettes and embedding in paraffin. Subsequently, sections were cut from the samples and mounted onto slides. The slides were dewaxed by washing twice with xylene for 5 minutes, followed by graded rehydration at ethanol concentrations of 100%, 95%, and 70% (v/v) for 5 minutes each, and then rehydration in running water for 5 minutes

4.3.7.2 Haematoxylin staining

Gill's haematoxylin was applied to the slides for 2 minutes, followed by a rinse in running water. Scott's tap water was added for 10-20 seconds, then the slides were rinsed again in running water. Eosin was applied for 5 minutes, followed by another rinse in water. The samples on the microscope slides were drained and dehydrated through three dips in increasing ethanol concentrations of 70%, 95%, and twice at 100% (v/v). Finally, the samples on the microscope slides were transferred into xylene and protected with coverslips.

4.3.7.3 Gram staining

Crystal violet solution was applied to the samples on the microscope slides for 1 minute, then thoroughly rinsed with deionized water. The samples on the microscope slides were treated with Gram's iodine mordant solution for 5 minutes, then rinsed and blotted. Differentiation was performed using absolute alcohol and rinsed with deionized water once more. The samples on the microscope slides were stained with Safranin O for 60 seconds, then rinsed and blotted again. The sections were treated with Tartrazine solution for 5 seconds, then blotted. The samples on the microscope slides were dehydrated through three dips in increasing ethanol concentrations of 70%, 95%, and 100% (twice), transferred into xylene, and protected with coverslips.

4.3.7.4 Imaging of Histology sections

Sections were imaged using an Olympus BX51 Fluorescence Microscope located in the Department of Chemical and Biological Engineering.

4.3.8 Statistical Analysis

Data was initially input into Excel spreadsheets to calculate the CFU/explant, closure of wound healing experiments, and the OD of the XTT assays. CFU/explant data was then transferred into GraphPad Prism for statistical analysis and graph synthesis. Ordinary two-way ANOVAs with multiple comparisons were performed to compare the antimicrobial treated biofilms to the untreated control. A t-test with Welch's correction was used to compare two data sets with each other.

4.4 Results

4.4.1 Biofilm confirmation

Electron microscopy provides an insight into the biofilm structure and can be used to determine if biofilms have formed *in vitro* (Vyas *et al.*, 2016; Relucenti *et al.*, 2021). Here, SEM was undertaken to visualise the wound bed for confirmation of biofilm formation.

The micrographs were taken of the *S aureus* inoculated wound bed as described in section 4.3.6 at 12, 24 and 48 hours in normoxia and hypoxia as seen in figure 21. In figures 21 A and B, long fibres can be seen which are indicative of the host fibres visible from the wounding process, amongst these fibres cocci can be observed attached to the fibres. The presence of attached cocci demonstrates the successful *S aureus* inoculation in the skin samples, indicating that an infection is established within the 12 hour period in both hypoxia and normoxia. Furthermore, the attachment of these aggregates of cocci onto the host fibres can suggest biofilm formation. There is a plethora of requirements for the confirmation of biofilm presence, here there is evidence of a multicellular agglomerate attached to a biotic surface, strongly suggesting biofilm formation in the wound bed at a 12 hour time point.

When comparing the 12 and 24 hour time points in normoxia (Figures 21A and 21C) there is an increased amount of the covering of the sample area by the attached cocci at the 24 hour time point compared with the 12 hour time point. This may demonstrate the further development of the biofilm, potentially indicating the maturation of the biofilm over time in this model. This increase in coverage from the surface attached cocci can also be observed in figures 21 B and D, representing similar processes under hypoxia as with normoxia. In Figure 18D, there is evidence of cocci attached to host fibres suspended across the wound bed, this further indicates the presence of biofilm, as *S aureus* can utilise host fibres to facilitate adhesion using methods such as the collagen hug and the dock, lock, latch methods (Foster, 2019; Zong *et al.*, 2005).

Over time, the development of biofilms covering the wound fibres and wound bed increased, as seen in the limited amount of visible wound fibres in figures 18E and F compared with figures 21 A and B.

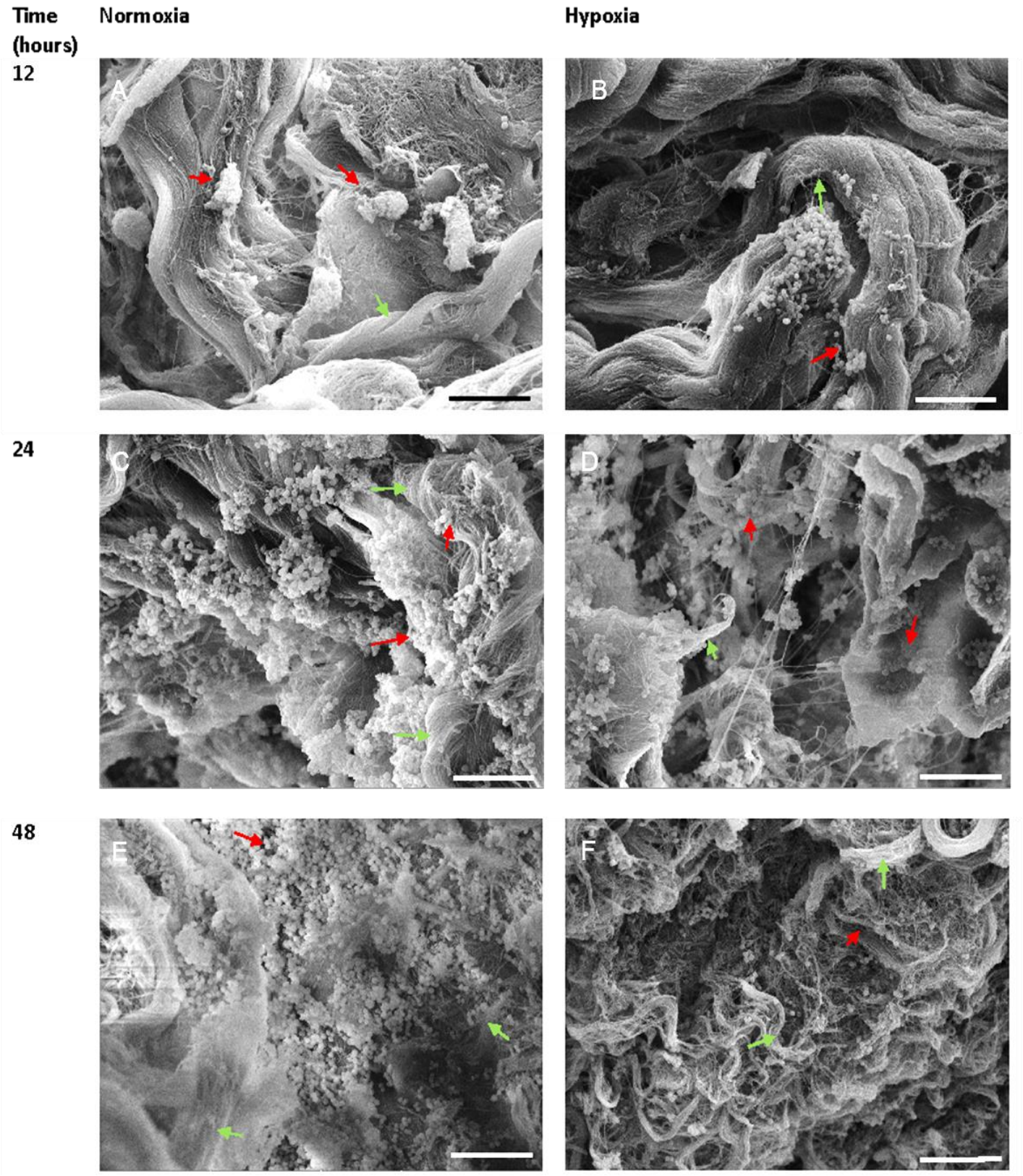


Figure 21 Scanning electron micrographs of wounded ovine skin infected with *S aureus* at 5000 x magnification. A) Normoxic incubation for 12 hours. B) Hypoxic incubation for 12 hours. C) Normoxic incubation for 24 hours. D) Hypoxic incubation for 24 hours. E) Normoxic incubation for 48 hours. F) Hypoxic incubation for 48 hours. Red arrows point to biofilm and green arrows indicate the wound bed. Scale bar 10 µm.

4.4.2 Validation of dosing method and hypoxic conditions

Initial validation that the *ex vivo* skin explants could support *S aureus* proliferation and subsequent investigation was conducted in Chapter 3, where the method was developed. Initially two different concentrations of high (1%) and low (0.1%) gentamicin at a higher volume (100 µL) and lower volumes (20 µL) were used to establish the ideal dosing strategy to study the effectiveness of topical application of antimicrobials in the wound bed. The results of this study are presented in figure 22.

Gentamicin was selected for the primary tests as it is commonly used in topical creams at a 0.1% concentration for infected wounds and 0.3% concentration for use in ophthalmological infections such as bacterial keratitis (Records, 1976; Chaves and Tadi, 2023). Additionally, as the MBEC values from chapter 2 were determined to be >512 µg/mL for all antimicrobials, it was then decided to investigate the efficacy in this model by using a concentration which was much above the reported MBEC (>512 µg/mL) and MIC (2 µg/mL) for the work previously conducted. As previously stated, the MBEC was above a concentration of 0.5 mg/mL (0.05%) therefore it was decided that an appropriate increase would be a 10 mg/mL concentration to be used for the high concentrated dose, as this was significantly higher than the MBEC, MIC and commercially available dosages.

Therefore, a range from the currently available topical concentration 1 mg/mL (0.1%) and a much higher concentration of 10 mg/mL (1%) were used in this initial investigation. There was greater variation in the results from the lower volume, with a much wider spread in the data this suggests that the lower volume has a more variable effect which may be as a result of less coverage of the wound surface. When regarding the low volume (20 µL) for both concentrations of gentamicin, there was a much lower reduction in CFU than that of the high volume (100 µL). This can be seen in table 3, where the results have been tabulated to show the log10 CFU/explant reduction compared with the untreated control.

As there is evidence of recalcitrance to antimicrobials from these results in figure 19 and table 3, it can be suggested that the bacteria *in situ* are utilising a tolerance response to the antimicrobial challenge. This can further suggest the presence of biofilm formation; as antimicrobial tolerance is a key marker of the biofilm phenotype (Uruén *et al.* 2021). This combined with the SEM micrographs strongly suggests that a bacterial biofilm had formed in the wounded ovine skin following 24 hours of incubation.

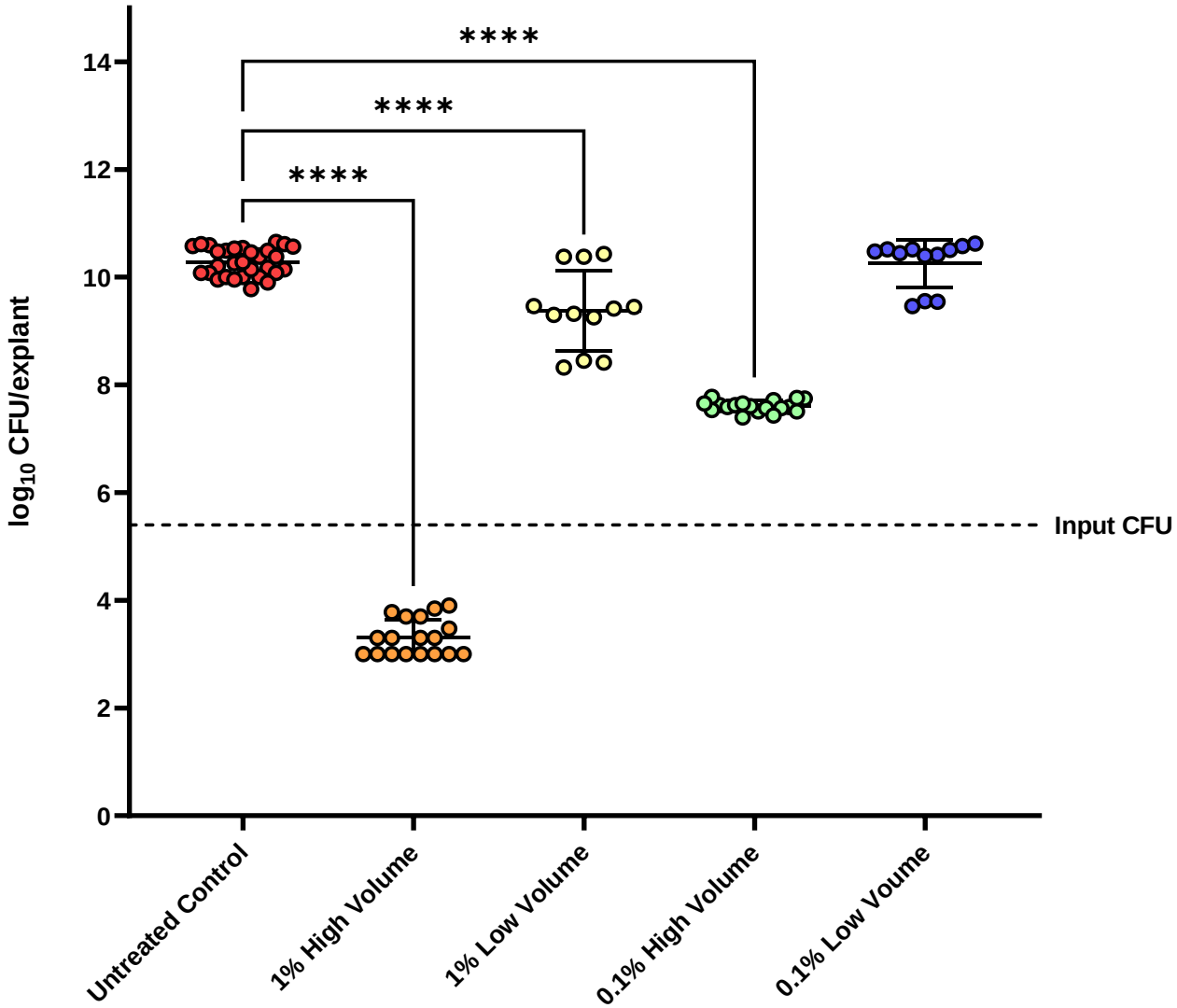


Figure 22 CFU comparison after 24 hours of treatment different volumes of 1% and 0.1% gentamicin. High volume=100 µL and Low volume=20 µL. One-way ANOVA with multiple comparisons ****=p value<0.0001. Error bars indicate standard deviation from the mean. (Untreated control n=36, High volume 1% and 0.1% n=18, Low volume 1% and 0.1% n=12). These results were from 3 repeated experiments.

Table 3 Mean log_{10} CFU/explant of wounded ovine skin infected with *S aureus* after 24 hours of treatment with gentamicin at 0.1% and 1% concentrations with either a high volume (100 µL) and low volume (20 µl). Results are from an ordinary one-way ANOVA with multiple comparisons. **Bold** indicates a statistically significant reduction (p<0.0001).

	1% High Volume	1% Low Volume	0.1% High Volume	0.1% Low Volume	Untreated Control
Log_{10} CFU/explant	3.3	9.4	7.6	10.3	10.3
log_{10} CFU difference from untreated control	**6.9**	**0.9**	**2.7**	0.02	

Additionally, as this study was looking to model an ischemic chronic wound it was important to validate the use of the model under hypoxic conditions. The hypoxic conditions were set as to be hypoxic incubation in a Heracell Vios 160s, with a 1% oxygen concentration and 5% CO_2 concentration. Figure 20 shows the comparison of hypoxic incubation CFU/explant to normoxic incubation. There was a significant reduction in the CFU/explant when incubated in a hypoxic environment (p<0.0001). As can be seen in Figure 23, there was a 2.7 log difference in the two environmental conditions indicating reduced growth within the wound environment under hypoxic conditions. This is further supported by the scanning electron micrographs at 24 and 48 hours (Figure 21 C, D, E and F), as coverage was reduced in hypoxic images compared to the normoxic images.

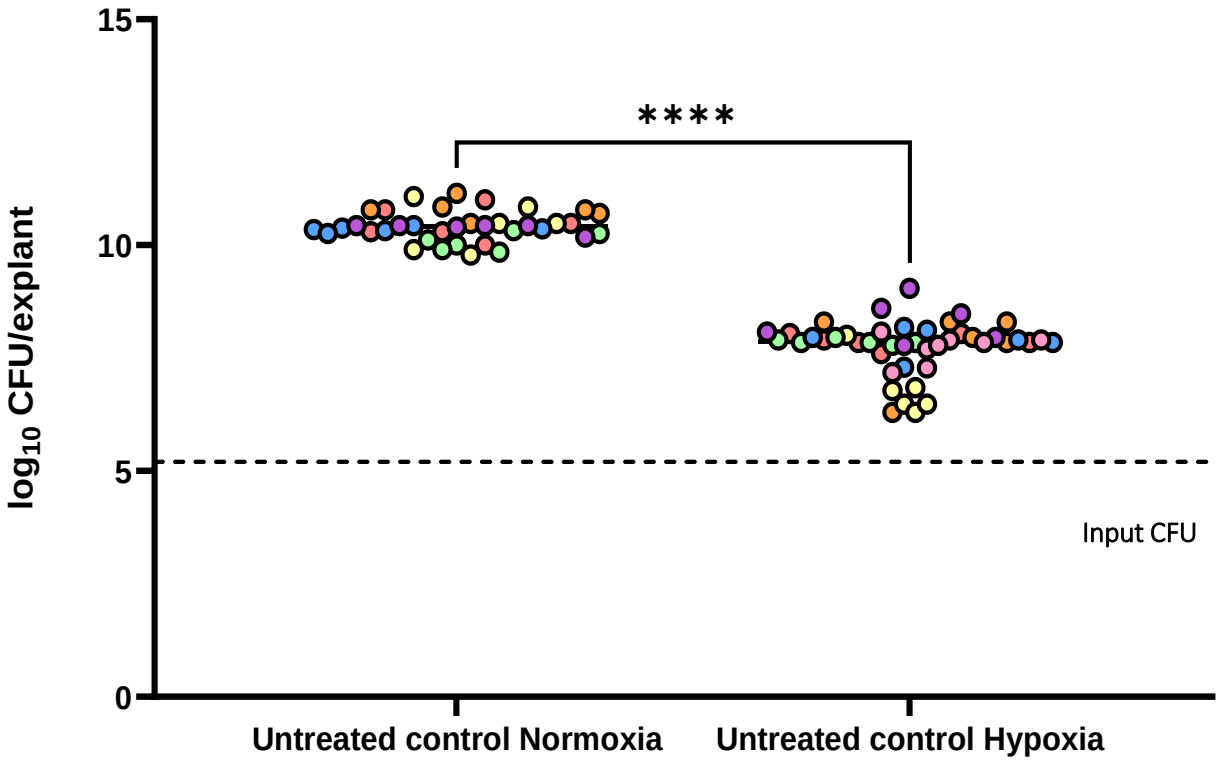


Figure 23 Comparison of wounded ovine skin infected with *S aureus* in normoxia and hypoxia. CFU counts from *S aureus* inoculated wounded skin after 48 hours incubation (Normoxia n=36, Hypoxia n=44). Different colours indicate different donor heads. Data sets were compared using a t-test with Welch's corrections for the comparison of means. Data with a p-value of <0.05 was considered statistically significant (****p<0.0001).

4.4.3 Antimicrobial challenge

Following on from the selection process for concentration and volume for antimicrobial treatment, the treatment with 0.5% gentamicin, clindamycin and vancomycin was undertaken. This concentration was chosen, as it represented a mid-point between the high concentration that was determined to be very effective at reducing the cell count of *S aureus*, and the low concentration that was determined to be ineffective in terms of eliciting the minimum of a 4 $\log_{10}$ CFU reduction that is required by the FDA for approval (Wilkinson *et al*, 2016).

S aureus infections were allowed to develop within the ovine wounds for 24 hours under normoxia or hypoxia before the application of antimicrobials. The effect of the antimicrobials was measured after 24 hours of antibiotic exposure under normoxia and hypoxia. The log reduction compared to the uncreated controls in normoxia and hypoxia were then calculated using viable counts, the results are presented in table 4.

When treated in normoxia, vancomycin and gentamicin were determined to be the most effective at reducing the CFU load of the explant at a 2.7 and 2.1 $\log_{10}$ reduction respectively ($p<0.0001$ for both). In hypoxia only gentamicin elicited a significant reduction in CFU of 1.7 $\log_{10}$.

Despite the same standardised inoculum, lower cell counts were observed in the antimicrobial treated wounds however, there was no significant difference from the untreated controls in hypoxic vancomycin (0.02 $\log_{10}$ reduction from control) in contrast to this, vancomycin does elicit a significant reduction in the normoxic CFU of 2.7 $\log_{10}$ comparatively to the control. When looking instead at the $\log_{10}$ reduction of cells (Figure 24), vancomycin in normoxia outperforms the reduction in CFU of vancomycin in hypoxia.

The reduction of biofilm was much more successful in normoxia than hypoxia, this can be seen in Figure 24 and table 4. When comparing the $\log_{10}$ reduction in biofilm, a 0.3 $\log_{10}$ higher reduction was seen in normoxic gentamicin, and a 2.7 $\log_{10}$ higher reduction in normoxic vancomycin when compared with hypoxic $\log_{10}$ reductions from the respective untreated control. This indicates potential impact of hypoxia on the ability for vancomycin to treat this strain of *S aureus*.

Table 4 Mean log_{10} CFU/explant of wounded ovine skin infected with *S aureus* after 24 hours of treatment with gentamicin, clindamycin and vancomycin. Statistical analysis undertaken on the antimicrobial treatment results. Results are from a one-way ANOVA with multiple comparisons. **Bold** indicates significant result ($p<0.0001$).

Condition	Normoxia log_{10} CFU/explant	log_{10} CFU difference from untreated	Hypoxia log_{10} CFU/explant	log_{10} CFU difference from untreated
Gentamicin	8.3	**2.1**	6.0	**1.7**
Clindamycin	10.7	-0.2	7.8	0.003
Vancomycin	7.7	**2.7**	7.7	0.02
Untreated Control	10.4	NA	7.8	NA

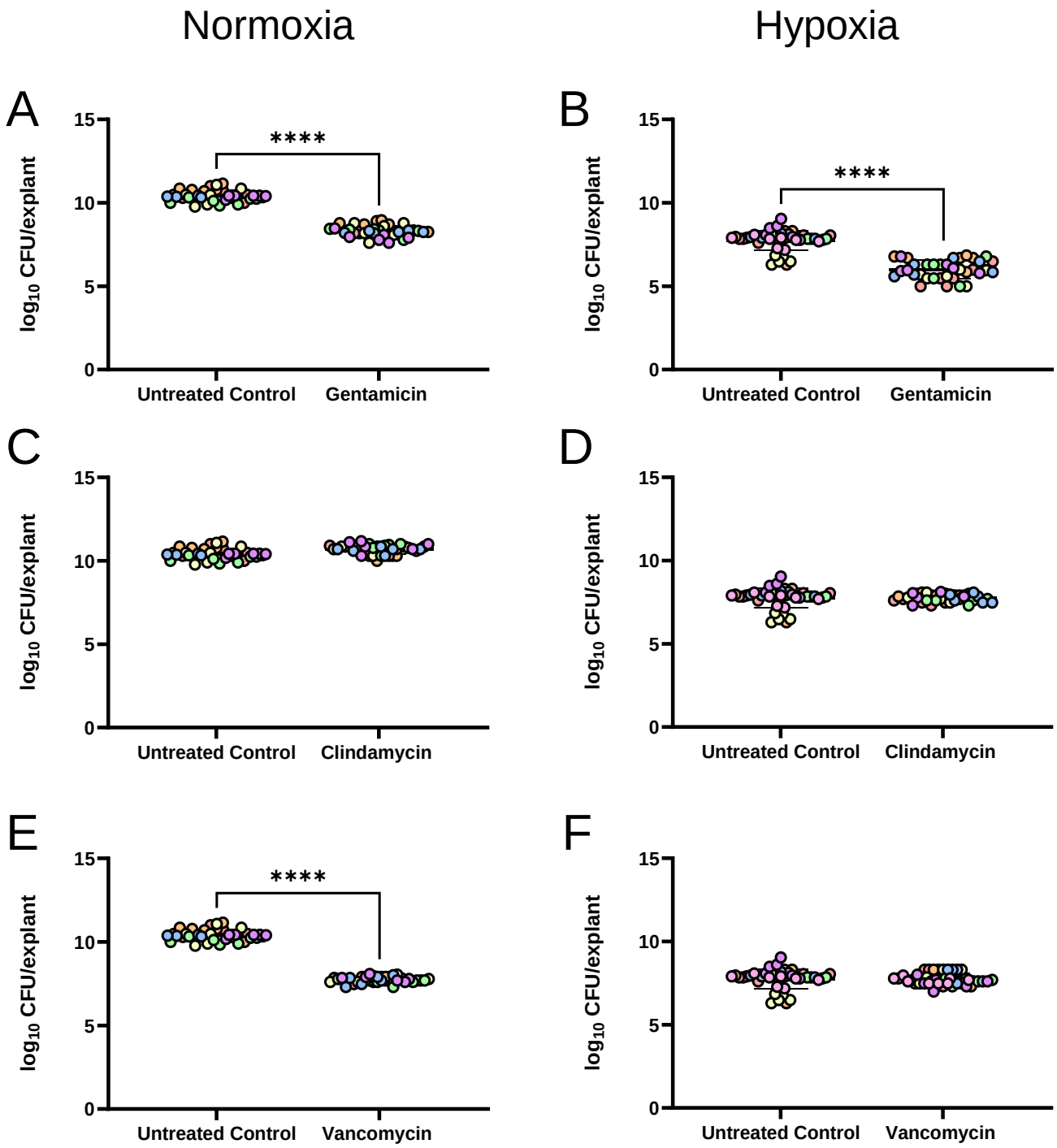


Figure 24 Graphs comparing the reduction in CFU to untreated control CFU following 24 hours of antimicrobial treatment in hypoxia or normoxia. A) CFU counts from gentamicin treated explant and the untreated controls in normoxia B) CFU counts from gentamicin treated explant and the untreated controls in hypoxia C) CFU counts from clindamycin treated explant and the untreated controls in normoxia. D) CFU counts from clindamycin treated explant and the untreated controls in hypoxia E) CFU counts from vancomycin treated explant and the untreated controls in normoxia F) CFU counts from vancomycin treated explant and the untreated controls in hypoxia. ****-p value<0.0001. Two-way ANOVA with multiple comparisons, different colours denote different donor heads. Untreated control was skin infected with inoculum and left untreated.

4.4.5 Wound Healing and Tissue Viability

The viability of the tissues used in this study was important, as it could determine the application of the model as either a substrate based model if the tissues were not viable, or as a biofilm infected wound model that may have the capacity to heal. Therefore, once harvested it was important to the scope of this study that the explants remained viable for the duration of infection. Preliminary experiments had confirmed the closure of scratch type wounds (Supplementary Figure 5); therefore, it was pertinent to determine if unwounded skin maintained viability over a 5-day period, and if wounded skin showed evidence of healing or wound closure over a 10-day period.

4.4.5.1 XTT Tissue viability assay

XTT is a colorimetric assay that can be used to determine cell and tissue viability, it can be converted into a coloured formazan product by being in the presence of metabolic activity. XTT can be converted through the reduction of the tetrazolium ring of XTT by mitochondrial dehydrogenases present in viable cells (Altman, 1976). Formazan is a water soluble product, and therefore can be measured in the supernatant after incubation with tissues. The 690 nm measurement is a reference wavelength and the subtraction of the 450 nm from the background wavelength of 690 nm is recommended to yield higher specificity for the results (Weislow *et al.*, 1989; Roehm *et al.*, 1991).

The data presented in Figure 25 shows the pooled XTT readings over a 5-day period, on day 1 viability will be at its highest as this step was conducted on tissues within a 6-hour time period of animal killing and harvest of explants. Therefore, with the results from Figure 25, it can be suggested that viability is 100% on day 1, and reduction in viability is in respect to day one. On day 2 viability reduced to 70.6%, day 3 56.5% and days 4 and 5 it was at 43.3% compared to the original value. The results from this assay indicate that tissue is metabolically active throughout the 5-day period, although decreased viability was observed over time, as seen in Figure 25. The decrease in tissue capability to convert XTT had a significant reduction at days 4 and 5 compared with day 1.

In addition to this, the data in Figure 25 shows that the viability of tissues does not seem to be donor dependent. Minimal clustering of data from the donor explants can be seen over the duration of the XTT viability assay, indicating that different donors have minimal influence over the viability of the explants. This further backs up the conculsions made from Chapter 3, wherein minimal donor to donor variability was observed in terms of CFU counts from the infected explants.

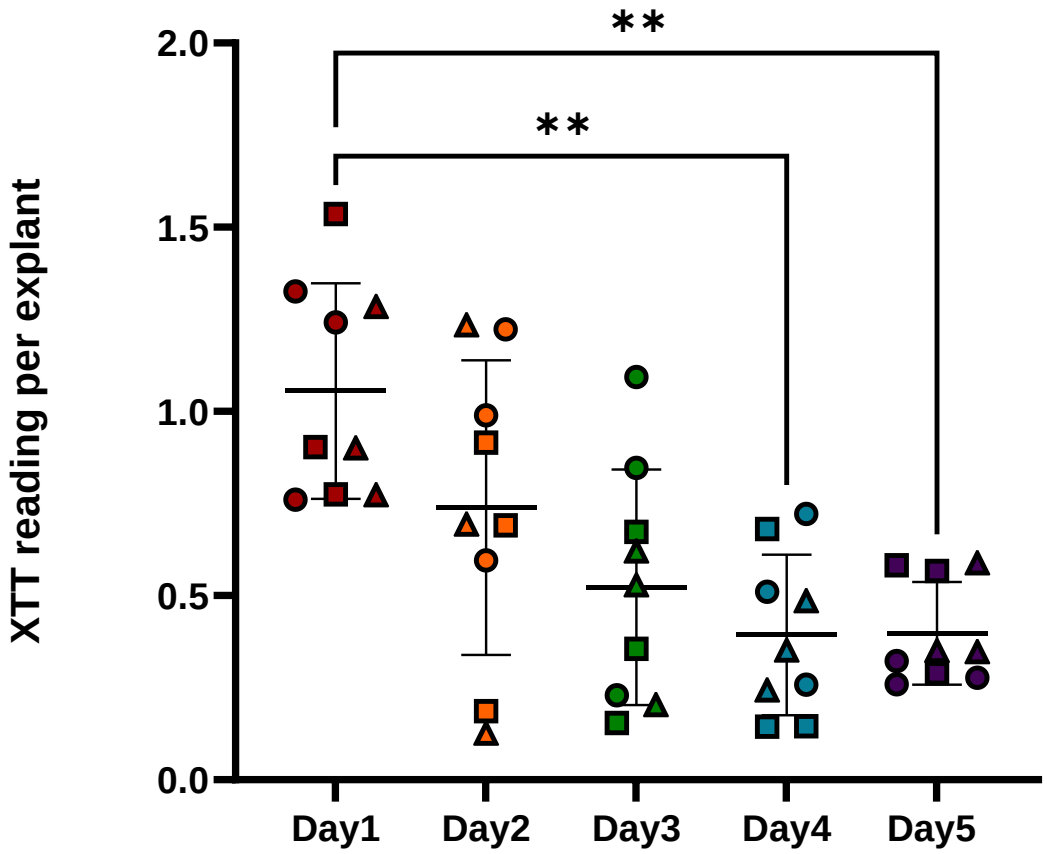


Figure 25 Viability assessment of ovine skin over 6 days in culture. XTT conversion by explants from 3 ovine donors. The data is presented as mean (n=9) ± standard error, with the shapes indicating different donor heads. Data were analysed by ordinary two-way ANOVA and significant differences are indicated by **p<0.005.

4.4.5.2 Wound healing observational study

After the tissues were observed to have viability, a preliminary observational study into the capacity of the tissues to heal was undertaken. Here, it was observed that tissues did reduce in the measurable wound over a period of 11 days, however this closure was very small and not visible to the naked eye. Once images were analysed using Fuji ImageJ the wound sizes were compared (Figure 26). The images were measured from one side of the wound bed to the other, with care being taken into the points taken as not all wounds were circular. To determine if any wound closure was due to healing not tissue shrinkage, measurements were taken of all samples using the same method of measuring each image in ImageJ at multiple points to determine an accurate diameter of the explants. Figure 26 A and B show the points of measurement on one sample taken on days 1 and day 5. Figure 26 C, D and E show the closure of a different sample on days 1, 5 and 11. These samples were from 3 ovine donors with 3 technical replicates.

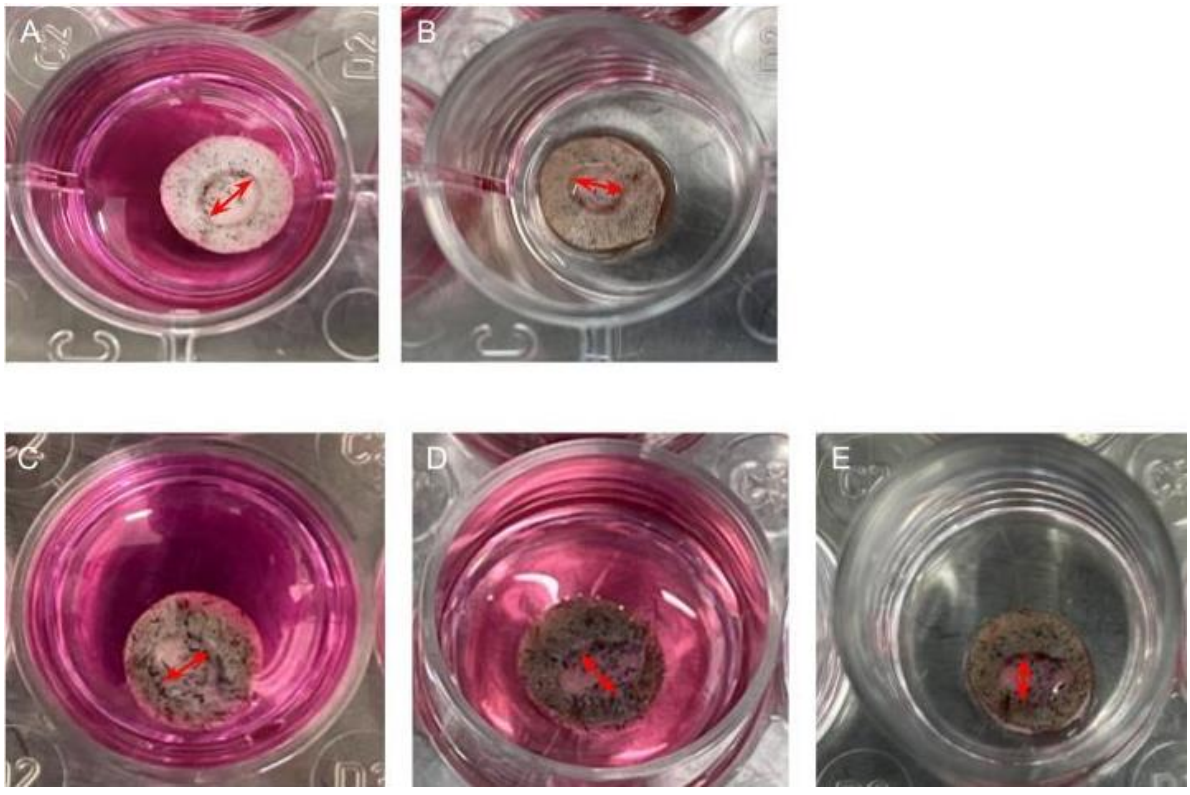


Figure 26 Images of wounded ovine skin. A and B show one sample at day 1 (A) and day 5(B). C, D and E show sample 2 at Days 1 (C), 5 (D) and 11 (E). Red arrows indicate the points measured to quantify wound closure.

Once the images were analysed in ImageJ, the measurements of the wound diameter were pooled and plotted as can be seen in figure 27. The wound diameter decreased over a 5-day period, with a significant decrease observed ($p<0.0001$) from day 1 compared with day 5 and 11. However, the measurements taken on day 11 indicated no significant decrease in wound diameter from day 5, although the diameter did decrease this was not found to be statistically significant.

Following on from this analysis, it was deemed important to investigate if explant shrinkage may be the cause of the decrease in wound size, and therefore the diameter of the explant was measured using the same images and technique. These results can be seen in figure 28, where a slight decrease in the diameter was observed and this was statistically significant after day 11 ($p<0.05$). Therefore, indicating that the decrease in wound diameter was as a result of wound closure.

These results suggest an ability for the explant wounds to decrease in size over a 5-day period and as a result, the ability for the explants to begin to heal. When considered with the context of the XTT assay results that demonstrate explant viability over a 6-day period, it can be suggested that the explants can heal. To further investigate evidence of explant healing, histological examination was undertaken (Figure 29).

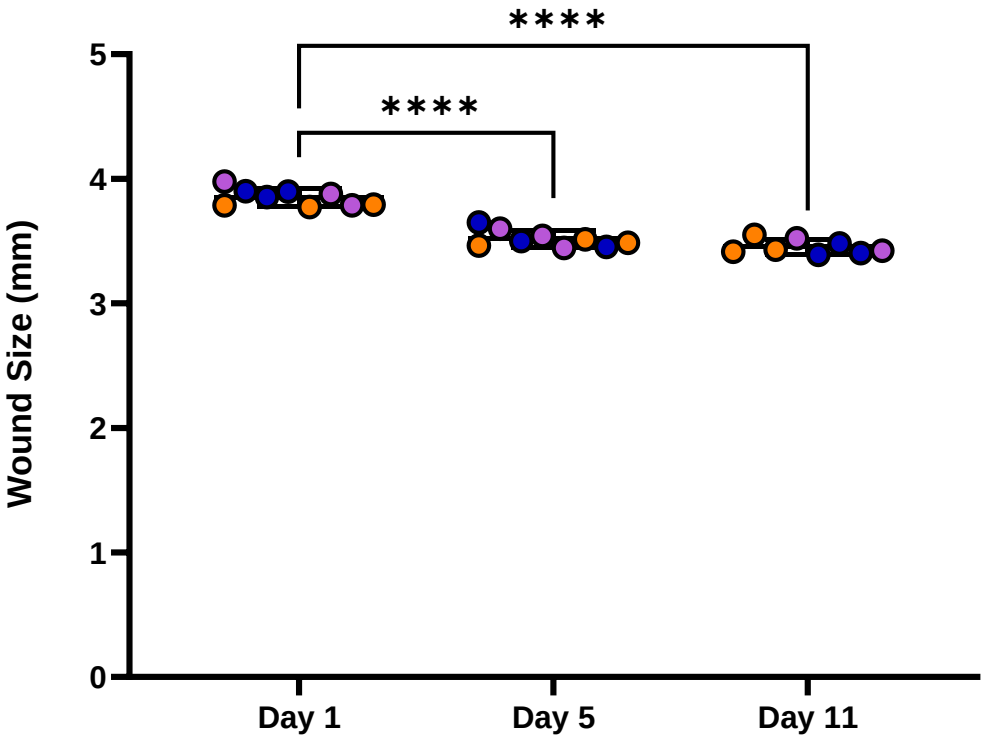


Figure 27 Comparison of the size of wounds made in ovine skin at Days 1, 5, and 11 post wounding. Ordinary two-way ANOVA statistical test used for comparison (**=p<0.0001), n=9, different coloured data points indicate different donor heads.**

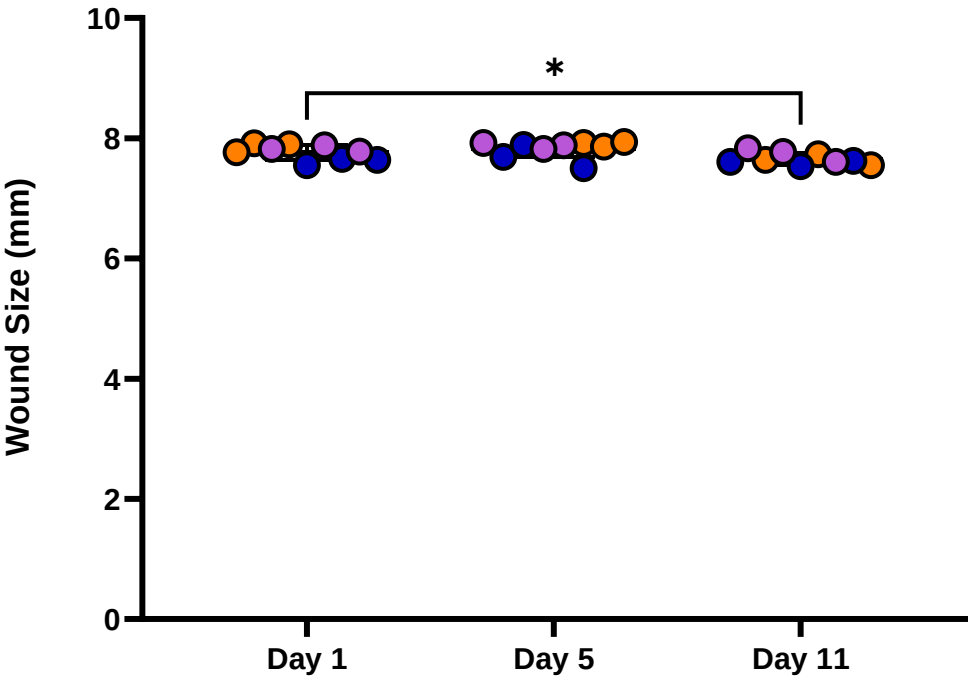


Figure 28 Comparison of the diameter of the wounded ovine explants at Days 1, 5 and 11 post wounding. Ordinary two-way ANOVA statistical test used for comparison (*= p<0.05) n=9, different coloured data points indicate different donor heads.

Histological examination was undertaken for wounded sterile explants to determine the healing capacity of the tissues over a 6-day period. These images indicate decreased tissue integrity over the 6-day period of histological examination as can be seen in figure 28. The explants on day one show standard staining and tissue organisation, with minimal keratin shedding and intact dermal and epidermal layers. The image from day three shows minimal signs of regranulation or tissue healing, as can be seen by the blue arrow only a small amount of granulomatous cells was evidenced in these examinations. There is also evidence of the dermis separating from the epidermal layer (marked D), and of subepidermal clefting which is when the epidermis begins to separate from surrounding structures due decreased tissue viability. The image from day 6 shows degradation of tissues surrounding the wound site, with debris visible in and around the wound bed. Collagen degradation is also seen in and around the wound bed, marked with the black arrows and further subepidermal clefting can be seen marked by the green arrows, this indicates that the explants are not able to heal the damage induced on day one. However, the stages of degradation are not significant, with the tissues on day 6 still remaining largely intact despite the quality decreasing. This indicates that although tissues are remaining metabolically active, and demonstrating signs of wound healing, the explants are degrading over a 6-day period in terms of viability (Figure 25), wound closure (Figure 26), and histopathological condition (Figure 29).

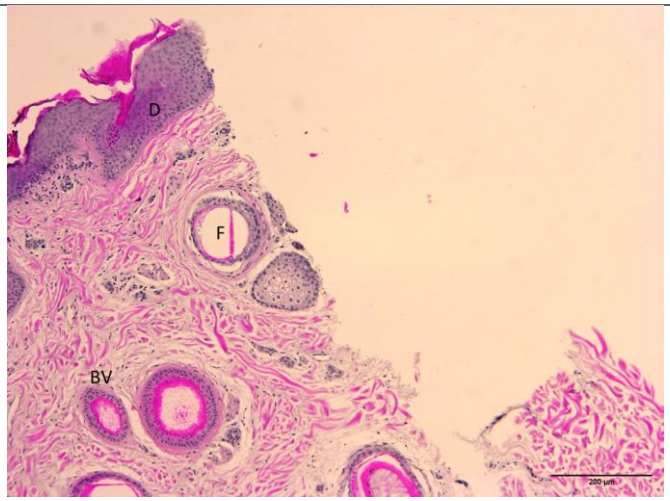
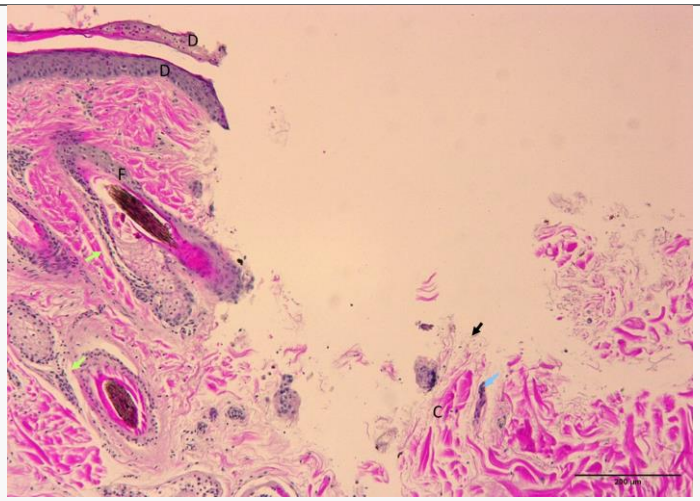
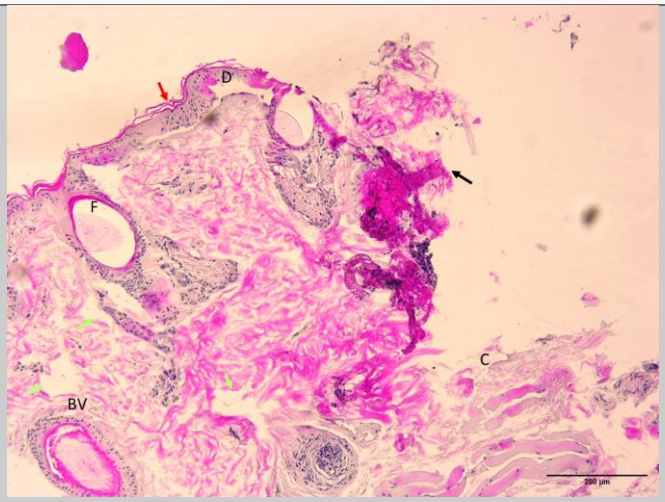
Day 1	
Day 3	
Day 6	

Figure 29 Histopathological images at 10 x magnification from wounded ovine explants at days 1, 3 and 6 post wounding.BV=Blood vessels, F=Hair follicle, D=Dermis, C=Collagen. Blue arrow indicates granulomatous cells. Black arrows indicate collagen degradation. Red arrow indicates keratin shedding. Green arrow indicates subepidermal clefting. Scale bars=200µm

4.5 Discussion

Recalcitrance to antimicrobials is a significant problem worldwide, and the impact of chronic non-healing wounds has a detrimental and loosely defined impact on patients due to the co-morbidity status of such wounds (Han and Ceilley, 2017). Therefore, better understanding of the recalcitrance to antimicrobials and novel wound treatments is essential for future treatment and resolution of wounds in patients. Tissue studies have indicated that more adequate *in vitro* models are needed for testing the clinical significance of novel antimicrobial treatments. This is mostly due to an increase in the amount of recalcitrance to antimicrobial treatment that has been evidenced from these studies (McMahon *et al.*, 2020; Seth *et al.*, 2014; Tran *et al.*, 2017). The opportunity for a model to indicate the interactions more closely between bacteria and host tissues is of clinical importance, as there are a multitude of host related factors that can impact the susceptibility profile of the bacteria that are not accounted for *in vitro,* from immune systems to interference in binding and uptake from host proteins. Therefore, an investigation into the ability of this model to form a biofilm within metabolically active tissues without the use of additives in the wound bed was pertinent. This would then enable the use of the model described within this chapter for antimicrobial susceptibility testing for biofilms in a wound environment.

It was then important to assess if the biofilms could be treated using topical application of antimicrobials. Subsequently, it was investigated if the application of hypoxia on the model would provide changes in the susceptibility of *S aureus* in the wound bed.

This *ex vivo* model used ovine skin as a substrate to investigate the role of environmental oxygen on the antimicrobial susceptibility of a *S aureus* biofilm. A supplement free wound bed was used to give insight on the antimicrobial susceptibility of bacterial biofilms developed within a clinically relevant wound bed. This consideration was determined to be essential as there is potential for some supplements that have the capacity to alter the bacterial load seen in these experiments and previous work elucidating the importance of media in bacterial growth (Chen *et al.*, 2020). These considerations are important as the other infection wound models available often use either pre-formed biofilms, add media to the surface of the wound bed, or wash the biofilms in high levels of antibiotics prior to the recovery of the viable cells (Andersson *et al.*, 2021; García *et al.*, 2018; Yang *et al.*, 2013, 2017; Phillips *et al.*, 2015; Schultz *et al.*, 2018; Roche *et al.*, 2019b). The implications of such are that the active modelling of an experimental wound may not closely mimic the clinical wound leading to failure at the clinical stages of drug development.

4.5.1 Biofilm Confirmation

Determining the presence of biofilm within the wound was an essential step in the validation and purpose of this method. Although there is some research into chronic wound infections and infection models within skin wounds there is significantly less focused on biofilms. Bacterial biofilms and the recalcitrance they present are a problem in terms of wound healing, and the establishment of a biofilm has been referred to as a hallmark of chronic wound infection (Calum *et al*., 2022). This impact has a profound effect on the lives of patients and also creates a significant cost and strain on the healthcare infrastructure that supports such patients.

The use of ovine skin and direct inoculation into the wound bed was undertaken to give a better clinical outlook of the establishment of a *S aureus* biofilm in a wound environment of a poorly perfused tissue. Other studies have used pre grown biofilms on membranes and achieved similar CFU/sample results (1 x 10^{11} of a 48 hour *in vitro* biofilm grown on porcine explant for 24 hours, 1 x 10^{8}) as found in our ovine model (1x 10^{10} CFU/explant after 48 hours) (McMahon *et al*., 2020; Wilkinson *et al*., 2016). However, in clinical infections it is likely that the infections establish through opportunistic ways as part of the natural microbiota, therefore biofilms establish through phenotypic switching to the biofilm phenotype and this switching to a more virulent and biofilm forming phenotype may be critical in the establishment of a chronic infected wound (Crabbé *et al.*, 2019; Luo *et al.*, 2021; Akers *et al.*, 2014). Additionally, there may be problems with using pre-grown bacterial biofilms on mesh or other substrate prior to placement in the wound as the amount of viable cells may be variable depending on the method of application. It is important to consider the bacterial load achieved within the wound environment as it has been reported that bacterial loads of greater than 10^{5} are linked to non-healing pathophysiology (McMahon *et al*., 2020).

Confirmation of biofilm within the wound can be undertaken in several different ways, such as direct visualisation using electron microscopy, confocal microscopy, histology for *ex vivo* and *in vivo* models; however, for this study SEM was used. The majority of literature concludes that the gold standard in biofilm detection is direct visualisation using high resolution microscopy and most commonly SEM (Lopez *et al*., 2021; Hurlow, Blanz, & Gaddy, 2016; Cryer *et al*., 2004). The ability to visualise the complex bacterial communities and presence of EPS is a very important factor. However, there are shortcomings with the use of SEM, it cannot be used for diagnostic purposes in clinical settings due to lengthy processes and additionally, there may be loss of biofilm biomass and EPS during the multi-step process to dehydrate the samples. Some research has been conducted into different methodologies that have less intensive dehydration processes such as variable pressure SEM (VPSEM), which yield much more

accurate EPS matrix morphology and hydrated 3D biofilm structure visualisation (Joubert *et al*., 2017; Weber *et al*., 2014). It can therefore be suggested that the use of SEM as a gold standard is appropriate, but the intensive dehydration steps will cause significant loss of matrix and associated biofilm characteristics needed for the confirmation of biofilm presence.

Here, biofilms were confirmed to have formed within the wound model in a 12-hour period following the application of a 1×10^7 CFU/mL inoculum (equivalent to 1×10^5 CFU per explant). These biofilms matured well over a 48-hour time period developing to form invasive biofilms that covered the entire wound bed and showed deposits of EPS interacting with the host dermal fibres at 48 hours untreated (Fig 18. E). These results indicate that at 24 hours post inoculum *S aureus* biofilms were mainly localised to the upper areas of the skin with greater coverage observed in the wound bed after 24 hours. The infection with a $1x10^7$ CFU/mL concurs with other skin infection models to form biofilm within a 24-hour period (Shepherd *et al*., 2009, 2011). The higher amount of bacterial inoculum is important as it corresponds more directly to bacterial densities that have been reported at the site of infection (Levison & Levison, 2009). Additionally, as the explants used in this model were on average 1 g/explant, the infections formed within this model correspond with the definition of a clinical skin wound infections, of a 1×10^5 CFU per explant is equivalent to the 1×10^5 CFU per gram of tissue (Alves *et al.*, 2021).

Wilkinson *et al* (2016) discussed the need for further investigation post *ex vivo* antimicrobial susceptibility testing and indicated that animal models were needed to investigate the impact of ischemic tissues on biofilm recalcitrance. This study was undertaken to see if a reproducible wound model could be developed; and used to study the impact of hypoxia. This initiates the further step to be taken in a much cheaper and more sustainable way compared with the animal models suggested by Wilkinson and colleagues. Here, it was found that the CFU in hypoxia was significantly less than in normoxia, this finding is supported by other demonstrations that *S aureus* biofilms in hypoxia had fewer CFUs (Lodge *et al*., 2017; Hess *et al*., 2013).

Once the presence of the bacterial biofilms in normoxia and hypoxia was confirmed the model was deemed fit for purpose and therefore antimicrobial susceptibility testing was essential to investigate the influence of the biofilm phenotype in a clinically relevant model and the impact of hypoxia.

4.5.2 Impact of antimicrobials on biofilms in an ovine wound model

Chronic wound treatment is a conflicting and limited area of research with few solutions available. A significant proportion of antimicrobial agents currently marketed at the treatment of infections have demonstrated limited treatment of biofilms (Vyas, Xia and Mai-Prochnow, 2022). Especially when

considering *in vitro* antimicrobial activity is measured using MICs which are based upon overnight aerobic incubation in low protein media at a controlled pH, when in clinical practice the infective environment is often acidic, anaerobic, and large amounts of tissue protein are present that can bind to the drugs and reduce their efficacy (Scheife, 1989). Therefore, the need for more clinically relevant models that account for some of these additional clinical factors along with being quick and simple to use is essential.

Treatment of chronic wounds with systemic antimicrobials is commonplace, however the efficacy against biofilms is reported to be as low as 25%, and this percentage will be significantly lower when considering chronic ischemic wounds in which appropriate levels of drug may not reach the wound as a result of inadequate perfusion and degradation of surrounding vasculature (Wolcott & Rhoads, 2008). However, the overall advice and guidelines for the treatment of skin infections is largely in favour of the use of systemic antimicrobials either orally or intravenously (Hernandez, 2006; National Institute for Health and Care Excellence, 2020). The use of topical antimicrobials is an area that has received much development over recent years, with the wide use of silver dressings and topical antimicrobials as these treatments may deliver a more targeted and concentrated antimicrobial delivery at the site of infection (Han and Ceilley, 2017b).

To determine the effect of antimicrobials on *S aureus* wound biofilms this *ex vivo* ovine model was infected and topically treated with 0.5% of gentamicin, clindamycin, and vancomycin for 24 hours. An untreated infected control was used as the positive control, and an uninfected wounded control was used as a negative control. The *S aureus* strain used here is a lab standard strain well documented and used for its susceptibility to antimicrobials.

A single application of gentamicin and vancomycin were the only antimicrobials that elicited a reduction in the CFU in normoxia. Limited success from single application of antimicrobials in a wound model has been reported and no significant reduction in CFU from the control compared was observed. The only sustained reduction seen was 1.40 $\log_{10}$ in comparison with the control (McMahon *et al.*, 2020). The CFU reduction is very similar to the CFU reduction seen in this study of 1.451 $\log_{10}$ following gentamicin treatment. Although the CFU reductions are comparable, all reductions observed from a single treatment in this study would not fit FDA threshold criteria of a 4 $\log_{10}$ reduction of CFU for an antimicrobial wound treatment (Wilkinson *et al*., 2016).

Although in vancomycin there was a reduction in the CFUs in normoxia the CBD, EUCAST, and MBC/MIC results indicate susceptibility at much lower concentrations than tested here, indicating that there should have been a higher reduction in CFU in this model. Despite being listed as susceptible at 2 μg/mL,

vancomycin treatment at this concentration carries the risk of therapy failure despite this concentration being an internationally accepted susceptibility breakpoint as determined by EUCAST (EUCAST, 2023). The impact of MIC values (high or low) on clinical therapy has been researched. The use of low MIC value antimicrobials may improve therapy efficacy; however, it has been reported that even in cases where these low MIC value antibiotics have been used that pathogen eradication is not achieved (Kowalska-Krochmal & Dudek-Wicher, 2021). This is due to many different reasons, one of which is heterogeneous resistance. This is when a small population of bacteria are resistant to the antibiotic and rapidly multiply in the presence of the antimicrobial whilst the susceptible population is killed (Band & Weiss, 2019). Detection of these cells is not possible due to the low population which may lead to incorrect estimation of susceptibility. A different theory as to the failure of antimicrobial treatment on susceptible cells is that of tolerance (Li *et al*., 2017). This is when the entire population survives treatment despite a previous susceptibility being given for that treatment. This can be associated with particular phenotypes or mutations that enable the bacteria to evade the killing mechanism of the chosen antibiotic, as often seen in bacterial biofilms. A third contributing factor to the recalcitrance to antibiotic treatment seen here could be a population of persister cells. The persister phenotype is a subpopulation of bacteria that are highly resistant to antibiotics and other antimicrobial agents. These bacteria are able to survive and persist in the presence of antibiotics, making them difficult to eradicate and contributing to the persistence of infections or antimicrobial susceptibility testing as seen in this work. In *S aureus*, the persister phenotype is associated with increased antibiotic tolerance and decreased susceptibility to killing by antibiotics. One of the mechanisms that contributes to the persistence of *S aureus* is dormancy in response to antibiotics. In this state, the bacteria are able to survive despite exposure to high levels of antibiotics and can re-emerge and resume proliferation freely once the antibiotic pressure is removed. Dormancy is particularly important as this phenomenon is often reported in biofilms. Finally, when treating in a clinic there are additional parameters that must be considered. This includes volume of distribution, clearance, max concentration, and other parameters that are associated with the patient's height weight and age, but also the immune response of patients is a massive advantage that means there will potentially be more resistance and failure of biofilm eradication in these skin models compared with clinical successes (Kowalska-Krochmal & Dudek-Wicher, 2021; Onufrak, Forrest, & Gonzalez, 2016). For example, in a study conducted by Onufrak *et al*, (2016) it was determined that the clinical AUC: MIC thresholds that predictively gave the best outcomes clinically had threshold upward of 600 µg/mL significantly higher than the 2 µg/mL MIC value, the reasons for these higher values can include the previously discussed factors involved in patient dosing specifically with soft tissue infections obesity and body composition is a critical factor. This *ex vivo*

model aims to provide more factors to interact with therefore giving a better picture of the susceptibility of an organism to a given antimicrobial within a clinical infection.

Interestingly, in the study by McMahon *et al.* (2020) antimicrobial susceptibility testing was conducted using the CBD and an *ex vivo* porcine dermal explant model, there they demonstrated a greater impact of antimicrobial treatment *in vitro* compared with *ex vivo*, as mirrored by the results seen from this thesis. McMahon demonstrated that antimicrobial treatment in the CBD elicited a higher CFU reduction than compared with the *ex vivo* porcine explant model. They suggested that there are model-dependent differences for the outcomes of antimicrobial efficacy studies, and that model selection was of utmost importance during product screening stages.

Change in efficacy of antimicrobials is also demonstrated by other studies that have investigated biofilm susceptibility and determined it is highly dependent on the environment in which the biofilm is grown, some studies cite an inability of biofilm formation *in vitro* and not *ex vivo* (Lorenz *et al*, 2023; Chen *et al*, 2021; McMahon *et al*, 2020). Lorenz *et al* demonstrated strains of bacteria that did not form biofilms in an *in vitro* method, when applied to their *ex vivo* model biofilms formed readily (Lorenz *et al*, 2023). Andersson *et al,* reported that the *in vitro* MIC of levofloxacin was not sufficient to eradicate the bacteria from their *ex vivo* infected wounds these results mirror the results from this *ex vivo* explant model and thesis (Andersson *et al,* 2021). The results from this model are additionally supported by the findings by Schaudinn *et al.* who report susceptibility of bacteria in biofilms in their *ex vivo* wound model decreased and were ineffective when treated with the MIC for the antimicrobial agents tested (Schaudinn *et al.* 2017). *In vitro* models have shown higher efficacy compared to *in vivo* models, this may be due to the inclusion of clinically relevant as there are more factors that may impede the antimicrobial actions against the biofilm such as inhibition through protein binding and differences in biofilm physiology (Flemming and Wingender, 2010). Biofilm maturity can have an impact on antimicrobial susceptibility, with immature biofilms often reported as being more susceptible to antimicrobial agents this may be due to lower cell density, a more homogenous population, or the EPS (Flemming and Wingender, 2010). The EPS plays a significant role in the antimicrobial resistance of biofilms, and the differences in efficacy may be attributed to the EPS maturity. Mature EPS can enhance antimicrobial tolerance or resistance in biofilms, as the hydrated matrix created gradients (oxygen, nutritional and density) that antimicrobial agents must penetrate through to allow eradication of the biofilm (Donlan and Costerton, 2002). The impeded diffusion of antimicrobials through several different processes; reduced diffusion, structural resistance to physical removal, chemical reactions, and interference with antimicrobials. All of these factors will be enhanced when in a mature, high CFU

biofilm as the components that cause the inactivation and slowing of the biofilms will be higher due to higher population (Ciofu *et al.*, 2022; Cao *et al.*, 2016; Flemming and Wingender, 2010).

Physiological differences in biofilms can also be attributed to the composition of the medium and culture conditions, in this *ex vivo* model a glucose rich media is used to sustain the tissue and may have an impact on the composition of the biofilm. Enriched media has already been observed to have an impact on biofilm composition, and therefore culture conditions in the *ex vivo* models must be taken into consideration. However, in a study by Lone *et al*. they determined that *S aureus* did not sustain growth in the culture medium for their study (DMEM) and therefore suggested that biofilm growth within their model was due to essential nutrients for the dermal explants (Lone *et al.*, 2015). Additionally, the differences between a full submerged static biofilm growing within a highly controlled environment may result in different phenotypic expressions when compared with a host biofilm interface with no media-based enrichment within the wound surface (McMahon *et al.*, 2020). Using the CBD as an example, the biofilms are grown on a large surface area in a nutrient rich environment before being exposed to antimicrobials, the antimicrobials when applied are applied to the whole biofilm in a standardised way and can penetrate the entirety of the surface of the biofilm. In some wound models the bacteria use the skin as the source of nutrients and therefore are applying potentially more virulence factors to penetrate and infiltrate the wound bed. The antimicrobials have to compete not only with the quenching of antimicrobial activity in the biofilm, but also the cross reaction of the skin components. However, there are many wound models that instead utilise additives such as agars and media to the wound bed which will impact the phenotype and growth of the bacteria.

Results from recent studies have investigated the impact on CFU following treatment with HOCl, the lowest efficacy was seen in the partial thickness *ex vivo* model, medium efficacy in the collagen model and highest efficacy reported in an abiotic model ((McMahon *et al*., 2020; Davis *et al*., 2017; Day *et al*., 2017; Robson, 2014). This further supports the findings of this thesis indicating that the more clinically relevant a model, the lower efficacy observed. Wolcott *et al*. (2010) demonstrated that immature biofilms are more susceptible to antimicrobial agents, and suggested that the difference in efficacy may be due to the maturity of the EPS (Wolcott & Rhoads, 2008).

Following the observation from this study, that the antimicrobials tested have limited effectiveness in normoxia when compared to an untreated control, the impact of hypoxia on the antimicrobial efficacy was undertaken to determine if hypoxic conditions have an impact on the bacterial biofilms in the ovine wound environment.

4.5.3 Impact of hypoxia on antimicrobial treatment of biofilms in an ovine wound model

Hypoxia can have a profound impact on the growth and survival of microorganisms, including bacteria. One way in which bacteria respond to hypoxia is by inducing changes in gene expression and metabolic processes. This allows bacteria to adapt to the low oxygen environment and maintain viability. However, these changes can also result in a fitness cost, which refers to a reduction in the overall ability of the organism to compete and survive in its environment. In the case of *S aureus*, the ability to form biofilms in hypoxic environments has been associated with a fitness cost Durhan *et al*., 2017). For example, some studies have shown that *S. aureus* biofilms formed under low oxygen conditions are less robust and less efficient in terms of metabolic processes compared to biofilms formed in a normal oxygen environment. This reduced fitness can lead to a reduced ability to compete with other microorganisms and to resist environmental stressors, such as antibiotics (Donlan & Costerton, 2002). It is important to note that the magnitude of the fitness cost associated with hypoxia can vary depending on the bacterium and the particular environmental conditions. Additionally, the fitness cost may be different for biofilm-associated bacteria compared to planktonic bacteria.

When comparing the effect of environmental oxygen concentrations on biofilms within the ovine wound environment, it can be observed that hypoxic biofilms were less dense with fewer CFUs than normoxic biofilms, additionally coverage of the wound bed was observed to be reduced, as evidenced in the SEM imaging at 48 hours. These results concur with previous work that reported control samples incubated under oxygen limited conditions were less dense with significantly fewer CFUs (Hess *et al*., 2013).

The antimicrobial effects of gentamicin, vancomycin, and clindamycin in hypoxic *S aureus* biofilms, it was observed that only gentamicin elicited a significant reduction in CFU compared with the untreated control. It has been demonstrated that gentamicin becomes less effective in oxygen limited conditions and this is in part, due to the oxygen dependent uptake of gentamicin (Hess *et al*., 2013). This study has demonstrated a reduction in the CFUs in hypoxia compared to the untreated control, however this reduction was significantly less than in normoxia. It can therefore be suggested that under a 1% oxygen concentration, gentamicin was able to have an effect on the biofilms as the 1% oxygen may still enable the oxygen dependent uptake of the drug. However, hypoxia may impair the efficacy of this antimicrobial potentially through a slower uptake in low oxygen conditions. The impact of hypoxia on

bacterial biofilms is far reaching and it is important to consider the wide- array of factors that may contribute to the changes observed in this study.

In contrast to this, under hypoxia vancomycin did not elicit a significant reduction in the CFU, and when comparing this result to normoxia, it is evident that hypoxia is inducing a change in the susceptibility of *S aureus* in the context of vancomycin treatment. Vancomycin, a glycopeptide antibiotic formerly designated as a last line of defence, prevents cell wall synthesis by forming hydrogen bonds with the D-alanyl-D-alanine C-terminal peptidoglycan precursor (Mühlberg *et al*., 2020). The lack of susceptibility under oxygen limited conditions to vancomycin specifically in this strain is corroborated by a study by Larsson *et al*, that observed a decrease in the effectiveness of vancomycin under anaerobic conditions (Larsson *et al*., 1996). The work from chapter 2 supports this, with findings of normoxic MBEC of 2μg/mL and hypoxic MBEC of 32μg/mL suggesting that hypoxia induces resistance to treatment with vancomycin. However, these differences were not seen in the MTA biofilms, where the recovered CFU counts where there was no difference seen in hypoxia compared to the normoxic MTA MBEC results. It poses an interesting finding associated with the resistance of *S aureus* to vancomycin under hypoxia within an *ex vivo* explant model, and using the CBD and MIC methods. Interestingly, resistance to vancomycin is reported to be increasing with new intermediate resistance and heterogeneous intermediate resistance being more prevalent in recent years (Stogios & Savchenko, 2020; Gomes, Ward, & Laplante, 2015). Resistance to vancomycin in hypoxia has not been widely reported in the literature, however there is significant evidence for mechanisms of resistance in *S aureus*.

S aureus has a number of virulence factors that enable its rapid growth and pathogenicity in a wide array of infections. It has been well reported that amongst the wealth of pathogenic virulence factors that *S aureus* possesses, *S aureus* often establishes a hypoxic environment (Lodge *et al*., 2017; Traci L Kinkel *et al*., 2013). Some virulence factors have been thoroughly reviewed and these factors include substances that impede phagocyte mobilisation, substances that prevent recognition and phagocytosis by host immune cells; toxins that destroy host immune cells, deterioration of host bactericidal peptides, immune modulators that enable escape from phagosomes, and elements to allow adaptation to adverse conditions such as hypoxia (Guerra *et al*., 2017). *S aureus* can sense and respond to environmental changes through a two-component regulatory system; a histidine kinase that senses a specific environmental stimulus and a corresponding regulator that controls the expression of target genes. The control of virulence factors that are linked to sensing hypoxia are *ssrAB* (staphylococcal respiratory response AB) and air*SR* (anaerobic iron-sulphur cluster-containing redox sensor regulator), these loci allow metabolic adaptation and enable the secretion of virulence factors to combat

environmental hypoxia. Hypoxia linked virulence factors have been identified as essential to the survival of *S aureus* in hypoxia (Hajdamowicz *et al*., 2019). SsrAB is essential in co-ordinating increases in quorum sensing dependent exotoxin production which in turn enhances the cytotoxicity to the surrounding wound environment (Wilde *et al*., 2015). It has also been shown to be essential in the growth of biofilms in hypoxic conditions, however the control of such virulence factors may be context specific dependant on the model and substrates used (Traci L. Kinkel *et al*., 2013; Pragman *et al*., 2004). Low levels of oxygen limits ATP synthesis and results in depletion of NADH/NAD+ pools, which subsequently restricts growth and promotes the emergence of SCVs (Christmas *et al*., 2019). SCVs are vital in a wound with respect to intracellular persistence, antimicrobial resistance, immune evasion, and biofilm formation, and have been shown to emerge in the setting of chronic staphylococcal infections (Kahl, Becker, & Löffler, 2016; Proctor *et al*., 2014).

There is literature that suggests that hypoxia has an impact on a variety of aspects of infection such as; decreased neutrophil mediated killing of *S aureus in vitro*, increased size of lesions in *S aureus* infected animal skin models, impaired clearance in *S aureus* infected lung models, and increased intracellular survival when *S aureus* was incubated with neutrophils *in vitro* (Christmas *et al*., 2019; Lodge *et al*., 2017; McGovern *et al*., 2011; Werth *et al*., 2010; Jonsson, Hunt, & Mathes, 1988; Mandell, 1974). Staphylococcal virulence mechanisms are intricately linked to neutrophil attack and evasion, leading to a significant diversity of *S aureus* clinical isolates and laboratory strains used in experimentation. It is important to note that these strains often carry mutations in regulatory genes, leading to variations in virulence factors and antibiotic sensitivity. For example, the laboratory strain RN1, isolated from a historical sepsis patient, is fully antibiotic sensitive but has defects in two regulatory genes, while USA300 is a virulent community-acquired MRSA clinical isolate with high haemolytic activity and leukotoxin secretion (Lodge *et al*., 2017)These variations between strains used in experimentation may contribute to conflicting results in research and give reasons as to the differences in susceptibility seen in this study compared to wider literature.

4.5.4 Tissue viability and Wound healing

XTT viability assays were used to determine the ability of the ovine explants to convert XTT to the tetrazolium product at a given time (Elson *et al.*, 2015; Berridge, Herst and Tan, 2005). XTT is a commonly used tissue viability assay that measures cellular metabolic activity. The assay is based on the reduction of XTT by cellular dehydrogenases to form a water-soluble formazan dye, which can be quantified spectrophotometrically. The XTT assay measures formation of formazan by the reduction of XTT from nicotinamide adenine dinucleotide (NAD) in its reduced form of NADH as an indicator of the

viability or proliferation of the target cells and tissues (Berridge, Herst and Tan, 2005). Oxidised NAD^+ is an electron carrier and is the main hydride acceptor used in intermediary metabolism. NAD cycles between the oxidised state of NAD^+ and the reduced state of NADH through reduction-oxidation reactions that are critical for multiple mammalian metabolic processes (Walker and Tian, 2018). Mammalian cells manage their energetic requirements by continuously adapting the intermediary metabolism in response to changes in the environment, therefore the breakdown of nutrients and subsequent transfer of electrons to NAD is an essential component of mammalian cell viability (Walker and Tian, 2018). NADH production will therefore decrease in tissues in which viability is decreasing, which will cause a reduction in the amount of formazan product and lower absorbance.

Despite its widespread use, there are several limitations to using XTT as a tissue viability assay. One of the main limitations is the inability to permeate cell membranes due to its net-negative charge, whereas other tetrazolium based assays such as MTT can permeate the cell membrane, equally the MTT assay does not require an intermediate electron acceptor for reduction (Stepanenko & Dmitrenko, 2015). However, both of these assays assess metabolic activity of cells and may not reflect the overall viability and function of the cells. Another limitation is that these assays are subject to interference from certain compounds, such as media components, that can interfere with the reduction of XTT and lead to false positive or negative results (Stepanenko & Dmitrenko, 2015; Funk, Schrenk, & Frei, 2007; Zhang & Cox, 1996). While XTT assays are a useful tool for assessing cellular metabolic activity, they have several limitations that should be taken into consideration when using the assay for tissue viability assessments.

Despite these limitations it is suggested that the tissue viability assay combined with the wound healing observational study and the histological examination of the wound closure over time indicates that the explants are still metabolically active despite decreasing formazan over 5 days of culture. These values are within other studies results for decreasing viability over a 6 day period from *ex vivo* animal tissue viability following slaughter and harvesting (Elson *et al.*, 2015). The histological examination also supports a decrease in tissue integrity over a 6-day period. The rate of degradation of the *ex vivo* ovine explants is supported by a study conducted by Maboni *et al.* in which ovine skin explants were utilised in an infection model, and following histological examination marked signs of tissue degradation were observed at after 52 hours of incubation (Maboni *et al.*, 2017). The study utilised similar parameters to this *ex vivo* model, wherein a heterogenous population of sheep were sampled from a local abattoir, however they utilised ovine feet, whereas this model utilised the ovine heads for the explants (Maboni *et al.*, 2017).

The importance of a metabolically active tissue is associated with a key aim of this work, which is to enable better translation of antimicrobial susceptibility testing for more relevant clinical applications. This aim also meant developing more accurate sustainable models that include the 3 R's in their ethos. It is important to develop a cheap, high-throughput model that could yield results for antimicrobial testing in a clinically relevant way. Current methods often include the use of expensive and highly regulated human tissues, difficult 3D models that require significant expertise and dedication, or animal models that are expensive and require significant investment and maintenance. However, it was important to investigate the scope of the model with respect to improved clinical modelling in terms of antimicrobial treatment, biofilm development and potential for an infected wound healing model. This study has given answers to each of those questions, and the scope of this model has been determined. This is a simple model that can yield biofilm and hypoxic wound relevant results within a week of experimentation.

4.6 Conclusion

The aims of this chapter were:

1. To determine if the model would be suitable for antimicrobial treatment of *S aureus*, and if hypoxia could be applied to the model.
2. To determine if the infections present in the explants were biofilms formed in the wound bed.
3. To investigate the metabolic activity and healing capabilities of the *ex vivo* ovine tissues

This study utilised *ex vivo* ovine explants to create a wound model that could host biofilm infection in a normoxic and hypoxic environment. The biofilm presence was confirmed through SEM examination, and antimicrobial susceptibility testing using gentamicin, vancomycin and clindamycin. These factors were considered suitable for the confirmation of biofilm, therefore the impact of hypoxia on these biofilms was investigated. Hypoxia was successfully applied to the model, and the impact of oxygen deprivation on the wound associated biofilms was evaluated. Biofilms grown in hypoxia were observed to be less robust (in terms of wound coverage and cell number) and the response to antimicrobials was markedly different for vancomycin.

The explants remained viable and metabolically active for the duration of the infection period, rendering this model a potential avenue to refine animal studies and expensive human tissue antimicrobial efficacy testing.

Chapter 5: Overall Conclusions and Future works

5.1 Overall Summary

Developing therapeutics for biofilm treatment is a difficult venture with significant barriers to overcome (Kranjec *et al.*, 2021). The overwhelming tolerance biofilms have to conventional antimicrobials give an added concern to the stalling of the antimicrobial pipeline (Theuretzbacher *et al.*, 2019; Donlan and Costerton, 2002). To give greater insight into the challenges of the translation of antimicrobials to clinical practice, better understanding is needed of the parameters and considerations for pre-clinical drug testing. Additionally, more information is needed about the resistance and tolerance of *in situ* biofilm infections to enable better disease management and outcomes for patients. The main findings of this thesis have begun to address some of those concerns.

This work has examined bacterial biofilm formation in several different models to determine the impact of model selection, substrate choice, and hypoxia on biofilm recalcitrance to antimicrobials. Experimentation was planned through a carefully considered escalation of complex susceptibility testing to determine if an effect was elicited by hypoxia and at which level of experimental complexity this recalcitrance occurred. Initially, the application of hypoxia elicited an effect on the first method tested- MIC broth micro dilutions, and these changes in susceptibility were seen across both the CBD and *ex vivo* models used in this thesis. After establishing that there were indeed effects being elicited on the bacteria in hypoxia a suitable method for chronic wound susceptibility testing was essential. This meant the development of a skin model that was easily accessible, and reproducible in terms of the infections formed. Additionally, it was of the utmost importance that this model fulfilled some elements of the NC3Rs principles to reduce the use of animals in research. As the aims were heavily associated around the reduction of animal use in drug testing and also the utilisation of clinically relevant factors, an *ex vivo* model was deemed the most suitable to fit these criteria. It was essential that the *ex vivo* tissues were from waste products of the food chain, as the utilisation of purpose bred animal tissues would have not fit the 3 R principles of reducing the need for animals bred for drug testing. Following discussion with abattoir staff, veterinary professionals and histological examination of porcine and ovine skin samples it was determined that ovine skin would be more suitable for the development of an *ex vivo* model. Then once a suitable decontamination process for the explants was determined, the model was validated for use as an ovine explant infection model.

As the basis for this thesis, and indeed the research group is biofilm infection it was essential to determine if the model formed biofilms within the wound bed. SEM, histology and antimicrobial challenge was undertaken to ensure that the model formed surface associated agglomerates that were tolerant of antimicrobial treatment. Hypoxia could then be applied to the model, to investigate the influence additional parameters may have on the wounded explant associated biofilms.

An additional important factor that was associated with this project was the significant impact of AMR and the associated devastating repercussions that are most felt in low- and middle-income countries (LMICS). These countries may not have appropriate access to human tissues for culture and testing or indeed access to animal models that can be developed and run in an appropriate way. Therefore, the development of a clinically relevant tissue model that relies on widely available waste tissues may aid the uptake of biofilm methods into these areas. The success of this model indicates a better access point for researchers and clinicians alike. It enables better control over testing in clinically relevant models such as biofilms and metabolically active tissues. The hope is that this work will provide an accessible platform for researchers to utilise readily available tissues for the purposes of antimicrobial development.

5.1.1 *In vitro* Susceptibility testing

Chapter 2 demonstrated the influence that hypoxia has on the susceptibility of *S aureus* through MIC, CBD, and MBEC models. The findings suggest the importance of hypoxia for inclusion in antimicrobial drug development. Particularly as a parameter for consideration when testing new formulations and classes of drugs. Using these parameters will enable researchers to have more control on the potential challenges that a clinical infection may pose on their novel drug. The findings suggest that mutations may occur to enhance the survival of *S aureus* in hypoxia. Previous reports of the involvement of a *gyrB* mutation can enhance survival of *S aureus* under hypoxia, as well as the two-component system *ssrAB* confers resistance to oxidative stress and enables metabolic adaptation to hypoxia (Hull *et al.*, 2022; Kinkel *et al.*, 2013).

Although hypoxia had an impact on the CBD and MIC methods, no differences were seen in the MTA, this was most likely due to the density of the biofilm. However, this is an area for further investigation as to the metabolic differences in cells in the CBD and MTA methodologies. The impact of model choice on the density of *S aureus* biofilms was found to be profound in the conditions used. This further underlined the importance of validation of the models being used, as significant discrepancies in the susceptibility were seen in between the two different biofilm methods.

The impact of additives within the experimental process must also be validated and understood that no differences in cell count or interaction between media or other additives within the models. The finding of a reduction in cell count from addition of tween 20 within the recovery media was an unexpected but crucial finding for this study. As the addition of tween is recommended by the original research paper for the CBD, it further highlighted the importance of thorough investigation into the impact of additives within the susceptibility testing process (Ceri *et al.*, 1999).

Overall, this study can suggest that the inclusion of clinical infection parameters-- such as hypoxia for ischemic wound infections- can yield a better picture as to the potential clinical success of a given antimicrobial. Additionally, the choice of model is critical for the development of a dense, mature biofilm. Therefore, cell motility and initial validation must be taken into account when selecting appropriate models for antimicrobial susceptibility testing.

5.1.2 *Ex vivo* Ovine infection and biofilm model

Chapter 3 demonstrated the successful transformation of waste ovine heads into an *ex vivo* high-throughput wounded skin model. The importance of appropriate selection of tissues and disinfection process was also demonstrated, with significant damage being caused to the dermis using standard disinfection practices. Although the use of ovine skin was preferable to the damaged porcine skin, there are less immunological similarities reported from ovine skin to human skin and this issue remains one of the main limitations of this study. Overall, this work enabled the utilisation of waste tissues into a reproducible infection model, suitable for antimicrobial treatment.

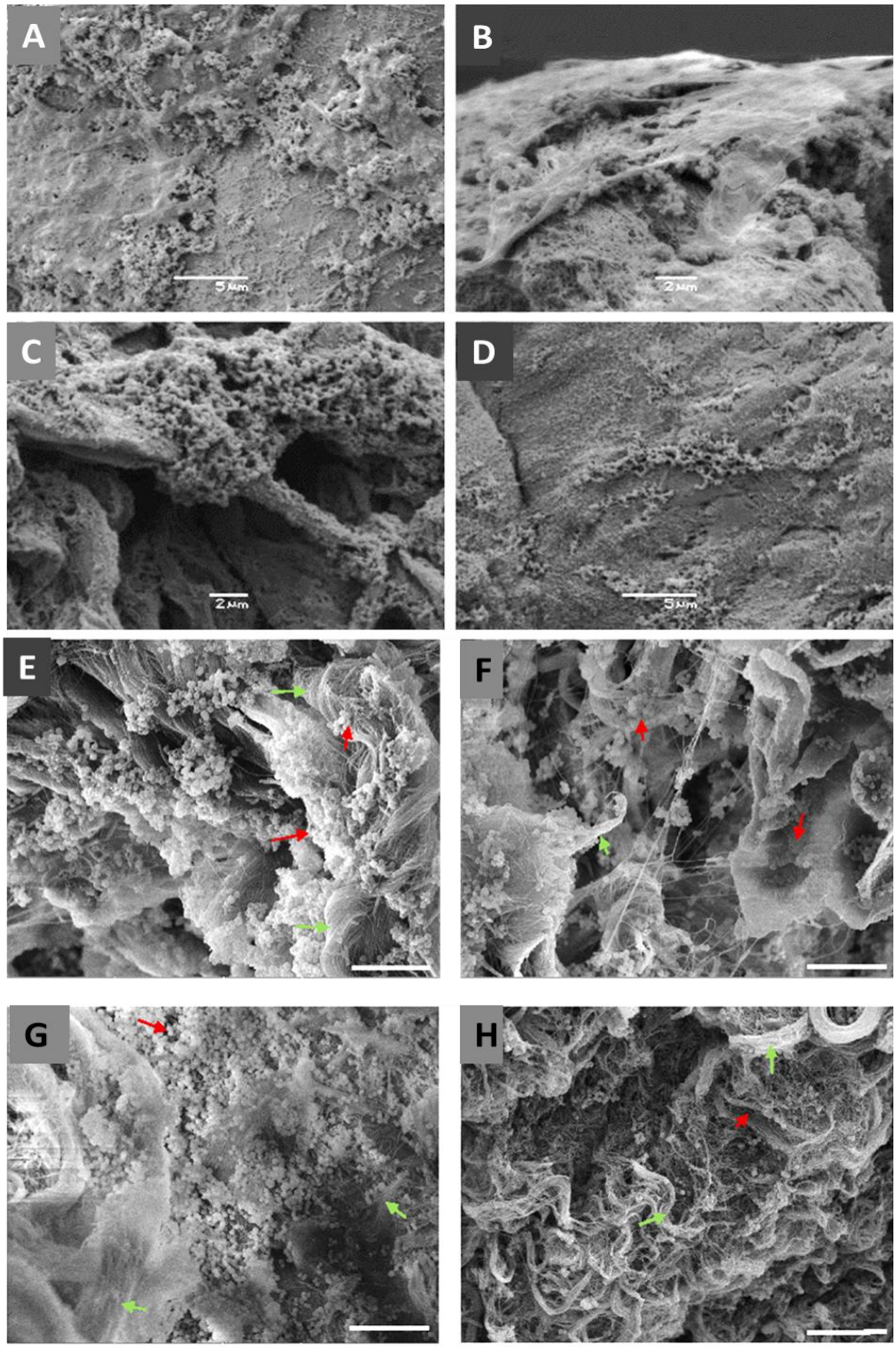


Figure 30 Scanning electron micrograph of DFU obtained from four patients demonstrating biofilm structure. A and B show large micro-colonies of coccoid microbial cells encased within an extracellular matrix anchored to collagen bundles within the wound. C shows large micro-colonies of coccoid microbial cells encased in thin layer of EPS. D shows large micro-colonies with eps, but far less than the first two samples. A, B, C and D are taken from Johani *et al.* 2017. E) Normoxic incubation for 24 hours. F) Hypoxic incubation for 24 hours. G) Normoxic incubation for 48 hours. H) Hypoxic incubation for 48 hours. Red arrows point to biofilm and green arrows indicate the wound bed. Scale bar 10 µm. E, F, G and H are taken from this study (Figure 21)

As part of the observation that the SEM imaging confirmed a chronic wound-like biofilm, the micrographs from this study, as can be seen in Figure 30 are compared with SEM micrographs of diabetic foot ulcers. Although the biofilms seen within this study (Fig 30, E, F, G, H) were not of the same density or coverage as seen in Figure 30 A, B, C and D, there are certainly some similarities in terms of the colonisation over the wound bed. For example, in Figure 30 A and C there are microbial cells adhered to the wound surface including the collagen bundles that were also seen in Figure 30 E and G, both indicate that there is an established infection within the sample. Both show an example of the previously mentioned multicellular agglomerate attached to a biotic surface which is often seen to strongly suggest the presence of biofilm formation in the wound. The differences between the micrographs are such that the biofilm in the clinical wounds (Fig 30 A, B, C, D) appears considerably more dense when directly contrasted with the micrographs from this study (Fig 30 E, F, G, H). This direct comparison indicates that the biofilms seen in this study are close in structure to biofilms seen in clinical wounds, however do not appear as dense as the clinical wounds.

Chapter 4 established that biofilms were forming within the ovine model at a 12-hour time point. Through SEM, histological analysis and antimicrobial challenge the biofilms were confirmed and the model was suitable for use as an ischemic biofilm infected wound model. Chronic ischemic wounds are a complex problem and given this complexity we can only provide interpretations based on the set of circumstances explored here. These suggest that *S aureus* is able to change its response to antimicrobials based on the environmental conditions it is present in. This includes a reduction in the susceptibility to gentamicin, clindamycin, and vancomycin in hypoxia comparatively to normoxia. There are a multitude of virulence factors and resistance mechanisms that may be affected by the exposure to a hypoxic environment which in turn has rendered the biofilms tested here, resistant. Although there is much more work that should be done within this model to investigate the causes of the recalcitrance to antimicrobials, this study has set up a reproducible, effective model in which these investigations would take place. Testing with multiple strains of *S aureus* to determine if the effect is as a result of global changes, or strain specific virulence factors-as previously discussed- would be pertinent for the overall picture of clinically relevant *S aureus* susceptibility testing.

5.1.3 Antimicrobial susceptibility observations

When considering the results from the different antimicrobial susceptibility tests utilised throughout this work, a few observations can be made. It would appear that as the complexity of the assay and the application of external variables to the antimicrobial susceptibility test there is a decrease in the susceptibility of the organism to the antimicrobials as seen in Table 5. This suggests that *S aureus* can adapt and respond to a variety of environmental triggers resulting in increased tolerance and resistance. This observation is an important part for further work into the applications of external variables in antimicrobial testing. As such variables may give a better picture of the success of failure of a novel agent prior to clinical testing.

It can also be observed that the application of hypoxia in different antimicrobial susceptibility tests can influence the tolerance and resistance of *S aureus* when challenged with antimicrobials. These observations are important to consider in the wider applications of these assays along with the main aim of this work which was to better understand why antimicrobials are not making it through to market.

Table 5 Antimicrobial susceptibility (MIC, MBC, and MBEC) values of the antibiotics tested (N=normoxia, H=hypoxia).

	EUCAST Breakpoint	MIC (μg/mL)		MBC (μg/mL)		CBD (μg/mL)		MTA (μg/mL)		Skin Model (μg/mL)	
		N	H	N	H	N	H	N	H	N	H
Gentamicin	2	2	>512	2	8	2	4	>512	>512	>5000	>5000
Clindamycin	0.25	<1	>512	16	256	16	>512	>512	>512	>5000	>5000
Vancomycin	2	2	64	16	64	2	32	>512	>512	>5000	>5000

5.2 Future Works

In the long term this PhD thesis provides a solid standing ground for future research to aid in the understanding of chronic wound biofilms and the influence hypoxia has on susceptibility. It has provided a model that is successful and is applicable to commercial antimicrobial testing to aid in the decision making process for new antimicrobial agents.

Future works include:

Investigation into the impact of hypoxia on other strains of clinically relevant bacteria would be pertinent. The further progression to a polymicrobial co-culture would be an interesting area to explore, as monoculture is not indicative of the infection processes clinically or otherwise, particularly when it comes to biofilms. Additionally, the influence of polymicrobial biofilms on resistance and tolerance to antimicrobials in normoxia and hypoxia will enable better understanding of the complex relationships that are formed within the biofilm environment.

As the gaps between the *in vitro* and *ex vivo* models are quite large in terms of variables and complexity, studies into co-culture with mammalian cells would be important to assess if there are similar increases in the susceptibility of *S aureus* in increasingly complex models. The BioFlux microfluidic system is a customisable system that enables the introduction of physiological shear flow into experimentation. This can add an additional clinically relevant parameter to the *in vitro* models used within this thesis. Use of the BioFlux would give excellent visibility of the dynamic process of adherence and organisation of the biofilm under hypoxia and normoxia.

Further examining the viability of the tissues, and potential for a healing model is important. This may be through cytokine panels, further histology such as DeadEnd Colorimetric TUNEL systems which can detect DNA fragmentation and yield a better assessment on the viability and degradation of the explants over time (Maboni *et al.*, 2017). Additionally, an investigation into the immune activity of the ovine skin would be an interesting avenue, as anecdotal healing had been observed. Cytokine panels can be used over time to measure the immune activity of the explants to better visualise viability and further scope for the model. If the tissues are immunologically active, then use in combination with infected samples could yield interesting results that may measure the impact of infection on the immune response within the ovine tissues. This could further be applied to antimicrobial drug development to give insight into the impact of a novel formulation with interplay from immune responses.

It would also be important to study a variety of species of bacteria within this model, as the current work is limited to only *S aureus*. *P aeruginosa* is a well-known pathogen that could be translated to this model, additionally the work could be moved forward to include *S epidermidis* and other commensal bacteria with the aim to form polymicrobial infections for susceptibility testing. Interactions between commensal organisms and opportunistic pathogens is an essential step for the wound infection process, as it has been reported that commensal organisms can prevent infection by pathogens during a skin breach (Tomic-Canic *et al.*, 2020).

The addition of other wound relevant parameters to be applied to the model is a recommendation of this study, as there are many factors associated with the impedance of wound healing. This may create a better clinical representation of the chronic wound environment, and in turn will give better insight into the parameters that are essential for consideration when tackling chronic wounds in a clinical setting. These parameters should include pH changes in the supplemental media, and the utilisation of an anoxic environment.

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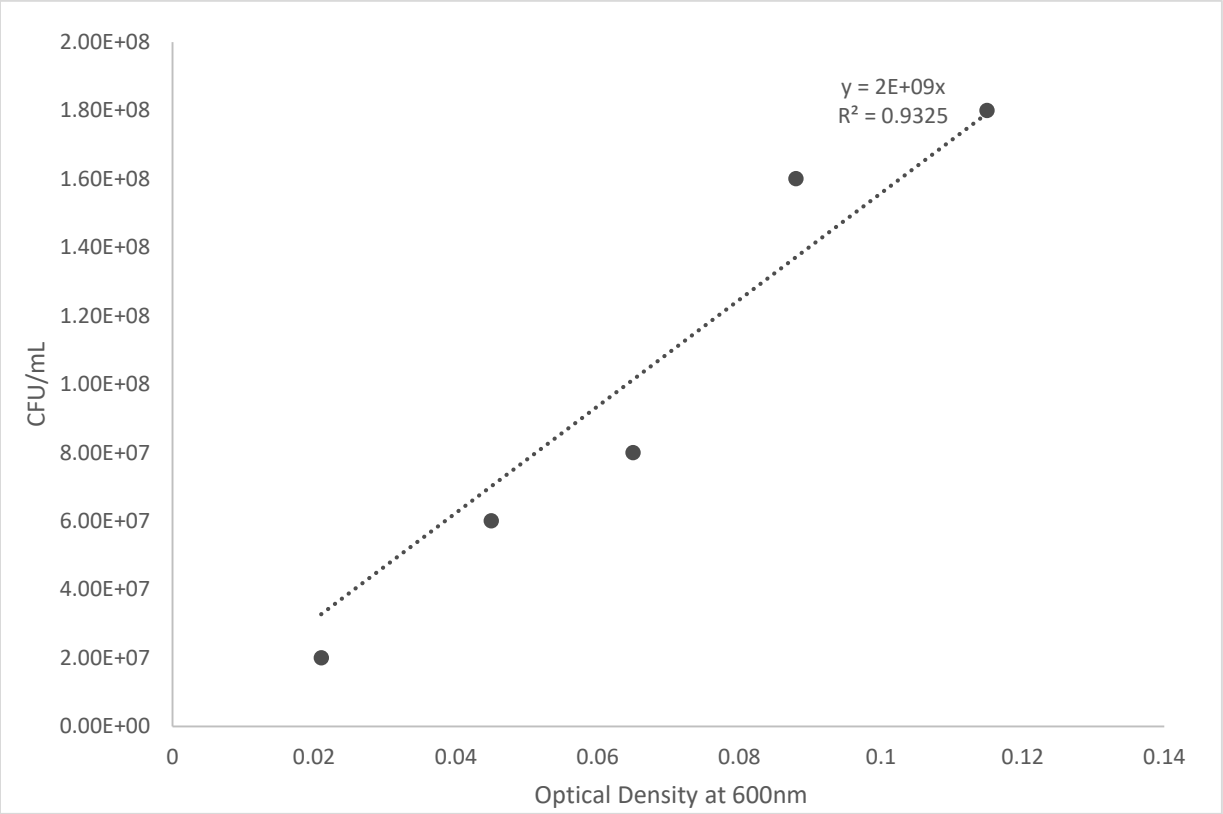
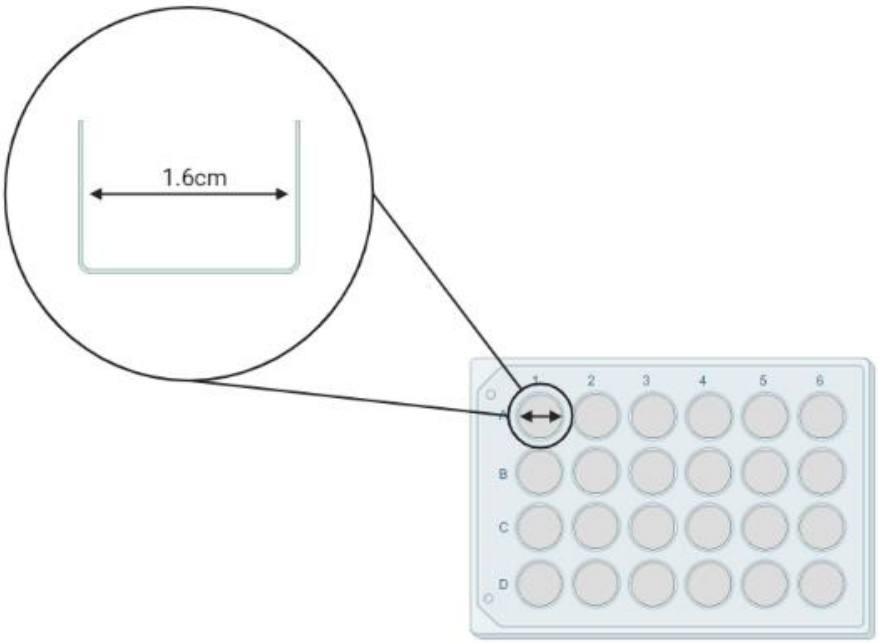
Supplementary Figures



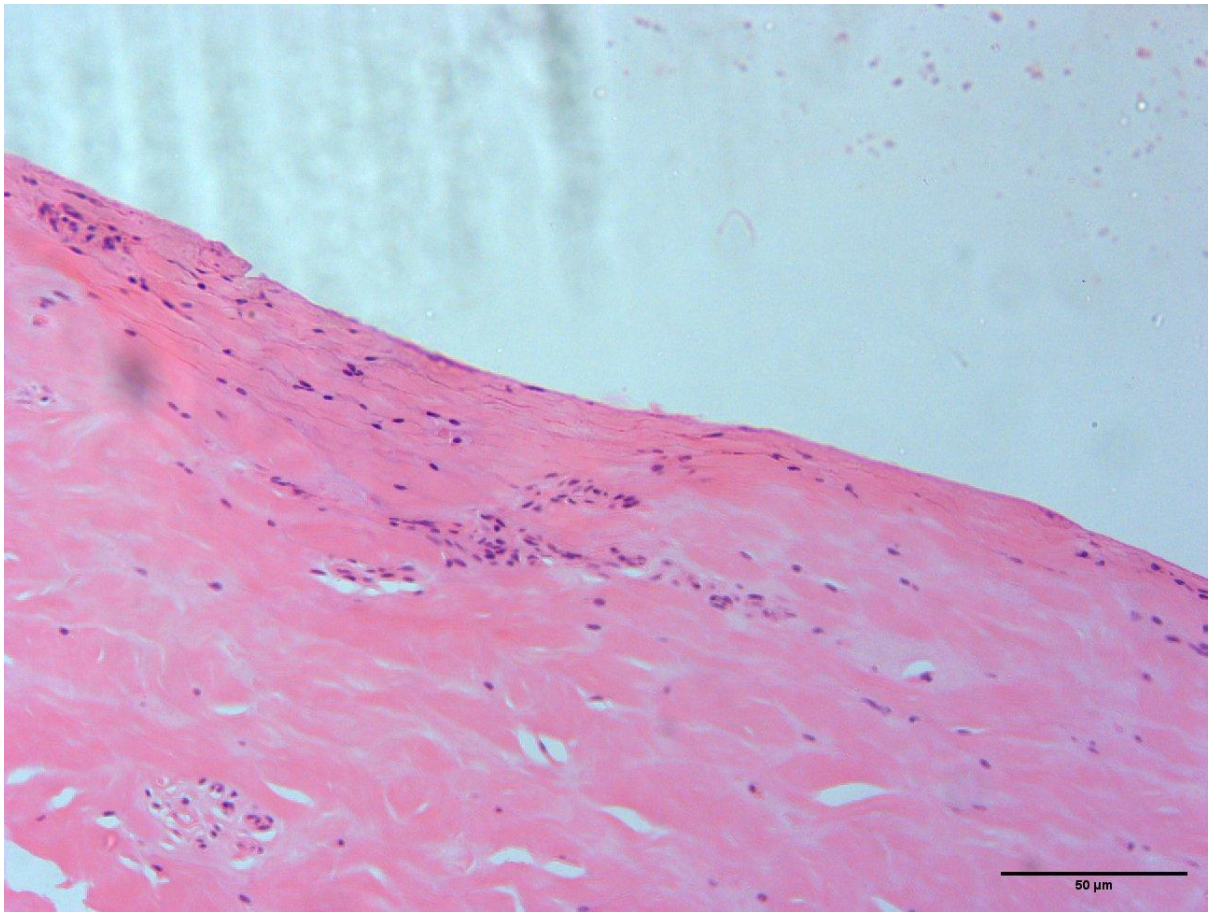
Supplementary Figure 1 Graph indicating the OD600 value and CFU of a S aureus inoculum at different OD600 values to validate the suitability of chosen inoculum for this thesis

Week 1 (g)	Week 2 (g)	Week 3 (g)	Total (g)
1.02	0.89	0.93	1.013
0.98	0.95	0.99	
0.88	1.21	1.09	
1.16	1.11	0.97	
0.97	0.96	1.06	

Supplementary Figure 2 Table showing the weight in grams (g) of individual 8 mm ovine explants weighed over a 3 week period. Explants were from 6 different ovine donors.

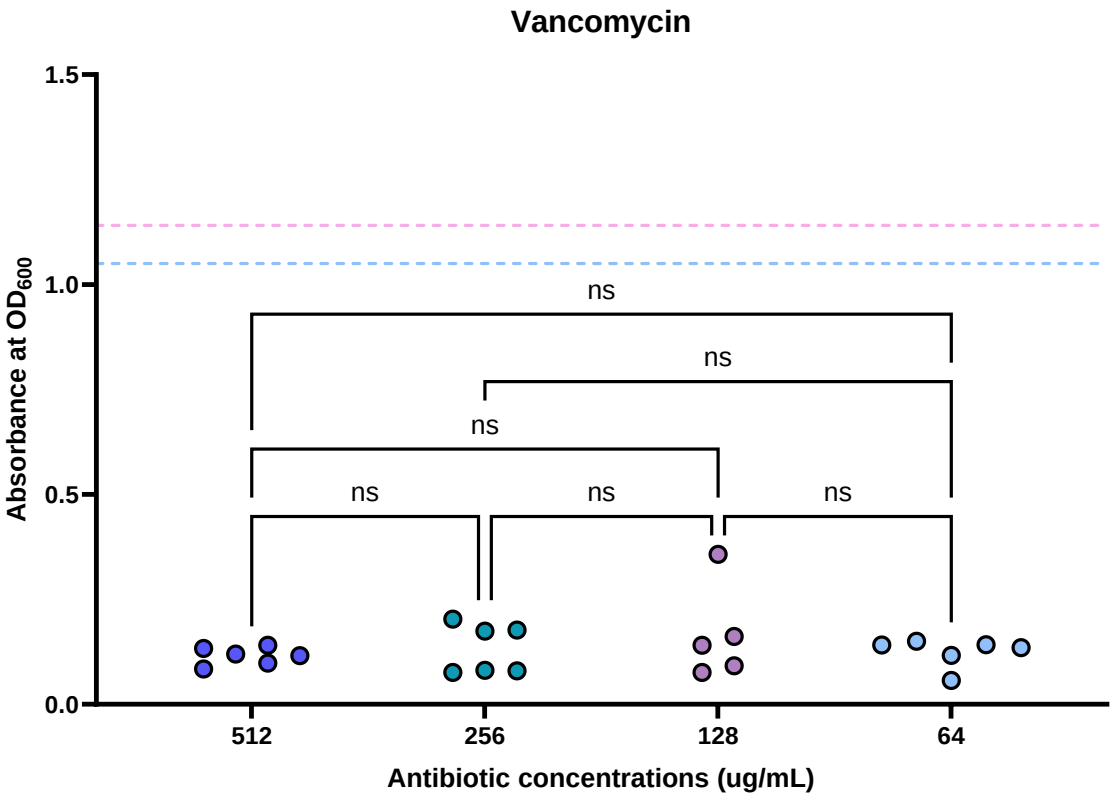


Supplementary Figure 3 Schematic of the diameter of the 24 well plate prior



Supplementary Figure 4 Histopathological image of porcine skin collected from the abattoir. Tissues were stained with an H&E stain. There is a lack of dermis and epidermis in these images indicating that porcine tissues from this supplier are burned or scalded prior to collection. Therefore, damaging the tissues. Magnification 40 X. Scale bar is 50 µm.

Supplementary Figure 5 Images of scratched wounded skin inoculated with *S aureus*. The wounded skin as can be seen on day 1, and it was incubated overnight. Following 24 hours of incubation (day 2) the sample showed no signs of wounding.



Supplementary Figure 6, Graph showing the MIC values of Staphylococcus aureus treated with Vancomycin in hypoxic conditions. A one-way ANOVA was conducted on these data sets to determine if the variance seen within the data was significantly different within this set concentration range. As no significant difference was seen in the analysis, 64ug/mL was determined to be the MIC value. (ns=not signficiant)

Supplementary Figure 7 Table with all reagents and equipment used throughout this thesis

Name of Material/ Equipment	Company	Catalog Number
24 Well Companion Plate	SLS	353504
4mm Biopsy Punch	Williams Medical	D7484
50ml centrifuge tubes	Fisher Scientific	10788561
8mm Biopsy Punch	Williams Medical	D7488
Amphotericin B solution, sterile	Sigma	A2942
Colour Pro Style Cordless Hair Clipper	Wahl	9639-2117X
Dual Oven Incubator	SLS	OVe1020
Ethanol	Honeywell	458600-2.5L
Epidermal growth factor	SLS	E5036-200UG
F12 HAM	Sigma	N4888
Foetal bovine serum	Labtech International	CA-115/500
Forceps	Fisher Scientific	15307805
Homogenizer 220, Handheld	Fisher Scientific	15575809
Homogenizer 220, plastic blending cones	Fisher Scientific	15585819
Heracell VIOS 160i	Thermo Scientific	15373212
Heraeus Megafuge 16R	VWR	521-2242
Hair Removal Cream	Veet	Not applicable
Insulin, recombinant Human	SLS	91077C-1G
Insert Individual 24 well 0.4um membrane	VWR International	353095
Microplate, cell culture Costar 96 well	Fisher Scientific	10687551

Multitron	Infors	Not applicable
Medium 199 (MK media)	Sigma	M0393
Plate seals	Fisher Scientific	ESI-B-100
PBS tablets	Sigma	P4417-100TAB
Penicillin-Streptomycin	SLS	P0781
Safe 2020	Fisher Scientific	1284804
Scalpel Swann Morton	Fisher Scientific	11849002
Scalpel blade number 15	Fisher Scientific	O305
Toothed Allis Tissue Forceps	Rocialle	RSPU500-322
Tryptic Soy Broth	Merck Life Science UK Limited	41298-500G-F
Tryptic Soy Agar	Merck Life Science UK Limited	14432-500G-F
Vimoba Tablets	Quip Labs	VMTAB75BX
Calgary Biofilm device	InnovoTech	19112
Mueller-Hinton Cation adjusted broth	Sigma-Aldrich	90922
Gentamicin	Sigma-Aldrich	PHR1077
Clindamycin	Sigma-Aldrich	PHR1159
Vancomycin	Sigma-Aldrich	SBR00001
XTT	Alfa Aesar	J61726-MD
Ultrasonic Bath	Bransonic	1800
Crystal Violet	Sigma-Aldrich	c3886
Glacial Acetic Acid	VWR	20C164022
Methanol	VWR	20847.3
DMSO	Alfa Aesar	D/4120/PB08
Plate Reader	Tecan	Spark

Spectrophotometer	Jenway	7310